COMPARISON OF TWO PROMOTERS DRIVING TRANSGENE EXPRESSION IN WATER-STRESSED SUGARCANE

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PREFACE

The experimental work described in this thesis was carried out in the Biotechnology Department of the South African Sugar Association Experiment Station (SASEX), Mt Edgecombe, from January 1997 to December 1998, under the supervision of Dr. B. I. Huckett and Prof. F. C. Botha.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

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I would also like to thank my supervisors Dr. Barbara Huckett and Prof. Frikkie Botha, as well as my co-supervisor Dr. Paula Watt, for their help and encouragement during this study.

To my wonderful family. As I write I am reminded of the abundance of love, support, encouragement and caring that exists in my life.

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A DEDICATION

'Whatever you can do, or dream you can,
Begin it.
Boldness has genius, power and magic
In it.'

Goethe

My thesis is dedicated to my parents, who have given me unconditional love. Thanks Dad and Mum, for guiding me through school and University, and for all your sacrifices. I also dedicate this thesis to those who, while no longer here in body, will always be with me in spirit.

ABSTRACT

For the expression of transgenes in plant cells, appropriate promoter sequences have to be introduced upstream of the gene to ensure efficient transcription. Tissue- or signal-responsive promoters are in high demand in practical plant biotechnology. The present study sought to characterise the activities of two promoters in sugarcane, namely the UBI (ubiquitin) promoter and the SUC-1 promoter (UBI linked in tandem to the cauliflower mosaic virus 35S promoter). It was hypothesised that the activity of UBI would be maintained or even increased under conditions of environmental stress, since it is well documented that ubiquitin is a stress-related protein. A further hypothesis was that SUC-1 might enhance overall gene expression since the CaMV 35S component is a constitutive promoter widely and successfully used in plant transformation. Plants of the sugarcane variety NCo310, containing the *cry1A(c)* (Bt) gene from *Bacillus thuringiensis*, were used as models in a system in which the plants were stressed by withholding water supply in a controlled manner.

Since large numbers of clones of both transgenic and wild-type plants were needed for the water stress and expression experiments, three micropropagation techniques, namely, shoot tip-, callus- and node culture, were optimised and compared. The objective was to propagate genetically stable plants rapidly. Compared to shoot tip culture, node and callus culture proved slow and inefficient. Shoot tip culture was thus chosen as the most suitable for the regeneration of experimental material.

Relative Water Content (RWC) determination, leaf elongation measurements and Infra Red Gas Analysis (IRGA) were compared in order to find the most appropriate method of measuring plant water status. In addition to being destructive, no observable differences were evident between the control (non-stressed) and water-stressed plants when using RWC as a measure. Results obtained from leaf elongation measurements compared favourably to the more sophisticated IRGA readings, showing that leaf elongation is as sensitive a measure of water stress. On the basis of preliminary studies with untransformed plants using the latter two

techniques, water regimes for stress-induction in the final experiments were designed. Leaf elongation measurements, which are simple and non-destructive, were ultimately chosen to measure plant water status.

In the final water stress experiment non-transgenic NCo310 and clonal populations of six transformants were used (three containing the UBI promoter; three the SUC-1 promoter). Exactly half of the plants of each type were stressed by withholding water supply, while the other half (controls) were watered manually twice a day. Leaf elongation measurements were made at the same time daily on the third youngest leaf of 6 plants from each population per treatment. At the same time, leaf samples were taken daily for molecular analysis. The stress regime led to marked differences in leaf elongation between control and water-stressed plants. In terms of physiological response (leaf rolling and senescing), plants containing the SUC-1 promoter appeared least affected.

The reverse transcription-polymerase chain reaction (RT-PCR) and Northern hybridisation were used to assay UBI and SUC-1 activity. RT-PCR revealed that both promoters drove Bt gene expression in controls and experimentals throughout the stress period, although differences in signal intensity were not observed. The extent of expression occurring in each type of plant was revealed in Northern blots probed with two genic sequences (1) the transgene and (2) sugarcane EST ME42, homologous to heat shock protein 82 in rice. Individual transformants showed overall levels of transgene expression that were variable, possibly due to insert position in the plant genome, as well as variations in relation to the application of stress. SUC-1 seemed superior to UBI in terms of driving transgene expression under stressful environmental conditions, since UBI promoter activity appeared to decrease under stress, while SUC-1 promoter activity remained constant. In addition to the expected 2.0 kb Bt transcript, transcripts of smaller than expected size were also obtained, leading to the suggestion of premature polyadenylation signals in the coding region of the wild-type Bt234 gene. Upon inspection of the transgene sequence, a number of motifs rarely present in plant genes were observed, namely A/T rich sequences, ATTTA motifs and numerous potential polyadenylation sites.

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ABBREVIATIONS

A	adenine
ABA	abscisic acid
ABRE	abscisic acid response element
Act-1	rice actin-1 promoter
Adh-1	maize alcohol dehydrogenase-1 promoter
AMV	Avian Myeloblastosis Virus
A. tumefaciens	*Agrobacterium tumefaciens*
AX	axillary bud
BAP	6-benzylaminopurine
bp	base pair(s)
Bt	*Bacillus thuringiensis*
C	cytosine
cab	chlorophyll a/b-binding protein
CaMV 35S	cauliflower mosaic virus 35S promoter
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
CSs	cleavage sites
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
dicot	dicotyledonous
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DRE	dehydration response element
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid

E. saccharina	*Eldana saccharina*
EST	expressed sequence tag
EtBr	ethidium bromide
FUEs	far-upstream elements
G	guanine
GUS	β-glucuronidase
HS	heat shock
HSEs	heat shock elements
HSFs	heat shock transcription factors
IBA	indole-3-butyric acid
ICPs	insecticidal crystal proteins
IRGA	infra red gas analysis
IPB	Institute for Plant Biotechnology
monocot	monocotyledonous
MOPS	morpholino-propane sulfonic acid buffer
mRNA	messenger ribonucleic acid
MS medium	Murashige and Skoog medium
MuLV	Murine Leukemia Virus
NIR	near infra red
nos	nopaline synthase
NS	nodal segment
NUEs	near-upstream elements
ocs	octopine synthase
PCR	polymerase chain reaction
PR	pathogenesis related
rbcs	ribulose-1,5-bisphosphate carboxylase-oxygenase
RF	root formation
RH	relative humidity
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute

rsp	root-specific promoter
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
RWC	relative water content
SASEX	South African Sugar Association Experiment Station
SDS	sodium dodecyl sulphate
SE	shoot elongation
SSC	sodium chloride sodium citrate
SUC-1	UBI and CaMV 35S promoters linked in tandem
T	thymine
TBE	tris-borate-EDTA
TCA	tricarboxylic acid
TE	tris-EDTA-buffer
tris	tris (hydroxymethyl) aminomethane
2,4-D	2,4-dichlorophenoxyacetic acid
U	units
UBI	maize ubiquitin promoter
UV	ultraviolate

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Stable expression of inserted genes is essential for the successful production of transgenic plants. Since promoters offer a fundamental control in gene expression, there is a great deal of interest in isolating and studying plant promoters. A number of constitutive promoters have been described. While these may be appropriate for the expression of certain types of transgenes, there is a growing need for promoters that confer unique patterns of transgene expression. This need is especially significant in monocotyledonous crop species as these have been more difficult to transform than dicotyledonous species (Torbert *et al.*, 1998). Since this has retarded the characterisation of promoters that are useful for transgene expression in monocots, investigation of expression patterns conferred by different promoters is warranted.

Expression cassettes commonly used in monocot transformation include promoter constructs of dicot origin, for example the 35S promoter from the cauliflower mosaic virus (CaMV 35S), as well as constitutive promoters of monocot origin, namely Emu (based on a truncated Adh1 promoter from maize), Act-1 (rice actin) and UBI (maize ubiquitin) promoters. The UBI promoter is of particular interest in sugarcane biotechnology as it appears to have a higher activity in these plants compared to other commonly used promoters. In separate studies carried out by Gallo-Meagher and Irvine (1993), as well as the South African Sugar Association Experiment Station (Snyman, personal communication), in which the efficiencies of CaMV 35S, Emu, Act-1 and UBI promoter constructs were compared in sugarcane leaf tissue, the UBI promoter resulted in the highest transient gene expression levels. In addition to this high activity, the possibility also exists that the UBI promoter might be environmentally responsive, since ubiquitin is known to be functionally related to stress. A promoter of this kind would be useful in sugarcane for driving the expression of *cry* genes, from the bacterium *Bacillus thuringiensis*, encoding insecticidal crystal proteins targeted to the stalk borer *Eldana saccharina* Walker. Eldana infestations in sugarcane are known to be exacerbated by plant stress (Paxton, 1982).

The present study is an investigation of the performance under stress of two variations of the UBI promoter. Clonal transformants of the sugarcane variety NCo310, containing the *cry1A(c)* transgene, are used as a model system.

1.2 LITERATURE REVIEW

1.2.1 *Eldana saccharina*: insect pest of sugarcane

1.2.1.1 General

Eldana saccharina Walker is an endemic lepidopteran pest found throughout the South African sugar industry. The caterpillar stage attacks sugarcane by entering the stalks and boring out the internal tissues on which it feeds (Anon, 1981). Several wild host plants have been identified, and it is from these that migrating moths gave rise to the problem which is now so widespread in cane fields.

Since the outbreak of eldana in 1970, emphasis has been placed on the factors that contribute to a high infestation. Age of cane was one of the first factors recognised to have an influence on eldana numbers (Carnegie and Smaill, 1980). Field survey results showed that eldana numbers were much lower in 12-15 month old cane than in 20 month old cane. In order to control infestation, the cane has to be harvested before the sucrose yield has peaked and thus profitability in these areas is decreased (Nuss, 1995). The age of cutting has important implications on the cost of sugarcane production, as the expensive operations in the crop cycle are at the beginning and end of the cycle. Reducing the time between these operations results in higher production costs.

Water stress also has a marked effect on eldana numbers (Anon, 1985). In a trial where cane was grown in drums, greater numbers and heavier larvae were recovered from stressed plants than from well-watered plants. The larval biomass in stressed cane was 3-5 times greater than that in unstressed cane. In a separate trial, the life cycle of eldana (eggs to moths) was 54 days in stressed cane and 84 days in well-watered cane. Soil type also affects eldana because of differences in water holding capacity (Nuss *et al.*, 1986). Most eldana damage to cane has been found in soils derived from Middle Ecca, Lower Ecca and Dwyka (Paxton, 1982). These soils are shallow, so they have a low water holding capacity and the cane has a shallow rooting system.

Yet another factor influencing eldana numbers recovered from cane is nitrogen application (Anon, 1985; Paxton, 1982). With low applications of nitrogen, eldana numbers were fewer and their masses lower than those where higher rates of nitrogen had been applied. The effect of nitrogen-fertilisation on eldana survival and growth was shown to be increased if the cane was stressed and in some trials the larval biomass was increased up to 10-fold.

1.2.1.2 The relationship between *Eldana* and stress

It is generally thought that eldana thrives best in droughted or stressed cane and that irrigated cane should therefore be less affected (Paxton, 1982). The results of pot trials carried out at the South African Sugar Association Experiment Station (SASEX) in 1984 to assess larval performance in cane subjected to water stress are summarised in Table 1.1. Data are the means of 14 pots per treatment except for stalk nitrogen determinations, which are means of 3 pots per treatment. The number of survivors and the biomass of larvae increased with the degree of stress, despite a decrease in the amount of stalk in which to feed. The percentage of fibre in the stalk decreased and the percentage of nitrogen increased, either or both of which factors could account for improving the quality of the food for the larvae, so making the stressed cane stalk more nutritious (Anon, 1985).

Table 1.1: Results of pot trials to assess larval performance in moisture stressed sugarcane (from Annual Report 1984-85; SASEX).

Trial # and cane age (mths)	*Stress treatment*	*Eggs placed / pot*	*Survivors / pot*	*Biomass of survivors (mg)*	*Dry weight stalk / pot (g)*	*Fibre % cane*	*Pol % cane*	*N % DM*
1	Slight	100.3	1.43	34.75	1400.4	7.7	1.99	0.45
(4-5)	Moderate	100.4	2.14	96.67	821.4	7.0	2.86	0.91
	Severe	100.3	2.86	178.94	627.3	5.9	2.78	1.12
2	Slight	101.3	2.79	124.96	-	12.6	11.9	0.30
(8-9)	Moderate	103.4	2.50	168.92	-	12.5	10.4	0.42
	Severe	103.0	4.50	447.54	-	12.3	9.0	0.50

Several trials were also conducted at SASEX in an attempt to measure the preference of moths for plants treated in different ways (Anon, 1985). Pot trials to assess the preference of moths for water-stressed cane plants showed that there were no significant differences in choice. It was therefore suggested that the susceptibility of stressed cane to attack by eldana borer is the result of better performance of the larvae in a stressed stalk rather than a preference of the moths for such cane, though it carries more trash, which the moth prefers as a site for laying eggs.

Where the effects of water stress were tested in the field, it was found that the number of larvae increased, whereas rind hardness decreased, as did fibre content but this was not always consistent or statistically significant (Anon, 1985). Sucrose content decreased consistently but not always significantly so. The nitrogen content of the stalk always increased with stress and the differences were always significant. As can be seen, the results of field observations support those from pot plant trials, indicating that increased stalk nitrogen was one factor associated with the susceptibility of droughted cane to eldana borer.

Three hypotheses have been put forward to explain why drought promotes outbreaks of phytophagous insects (in Rutherford, 1993). Firstly, drought increases insect survival and growth through elevated plant nutrient levels, especially nitrogen (White, 1984). Secondly, the plant's ability to defend itself is reduced, and thirdly, a more suitable physical environment, especially increased plant temperature, is provided (Rhoades, 1983). For example, there is recent evidence that heat stress causes a decrease in the level of unsaturation of plant lipid fatty acids (Rikin, Dillwith and Bergman, 1993). This might reduce the quantity of lipoxygenase mediated unsaturated fatty acid breakdown products produced during a wound response. Some of these, including fatty acid hydroperoxides, have been shown to be inhibitory to various fungi, insects and mites (Lyr and Basaniak, 1983). Shukle and Murdoch (1983) have shown that lipoxygenase can inhibit the larval growth of *M. sexta* in artificial diet in the presence of unsaturated fatty acids.

Mattson and Haack (1987) suggested that since the amino acid proline and the

sugar alcohol inositol are accumulated by many water stressed plant species, they might assist insects in detecting stressed plants. Both are common feeding stimulants yet neither are essential for insect nutrition. Kimmerer and Kozlowski (1982) have detected the release of ethylene, ethanol and acetaldehyde during stress. These might serve to attract insects by providing olfactory cues.

1.2.1.3 Economic impact of *E. saccharina* in South Africa

Figure 1.1 shows the type of damage caused to sugarcane by *E. saccharina*. Thompson (1983) estimated a loss of around 50% of sucrose for severe infestations of around 100 eldana larvae per 100 stalks (100e $100st^{-1}$) i.e. for every 1 larva per 100 stalks throughout the season, approximately 0.5 tons $hectare^{-1}$ of sugarcane is lost. It has been estimated that there was an average annual loss of R6 million in the drought years 1980/81 (Table 1.2). Collectively, the negative effect of eldana is estimated to cost the industry US $ 40m per annum (O' Reilly, 1998).

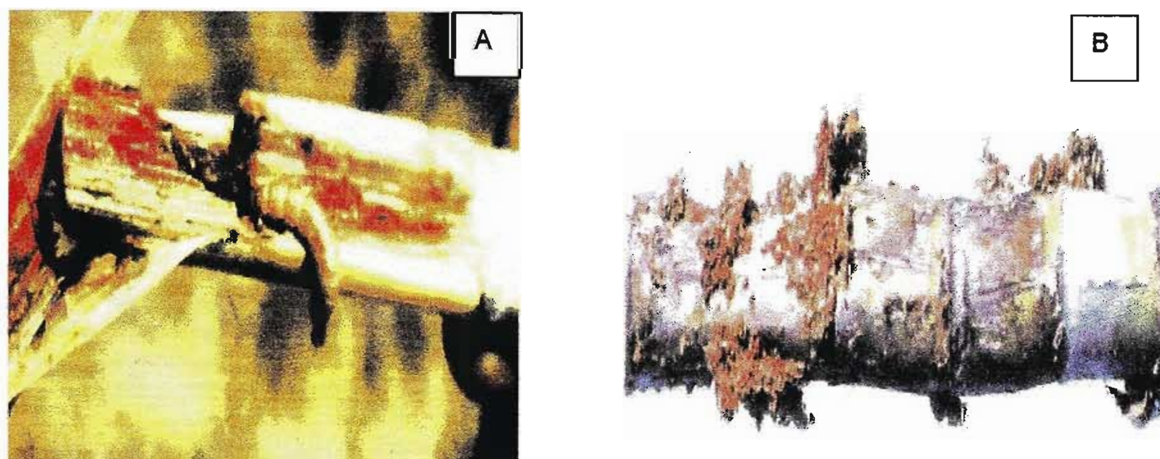


Figure 1.1: Damage to sugarcane caused by *Eldana saccharina* Walker.
(A) internal damage; (B) external symptoms of damage: feeding waste (frass) is pushed to the exterior (from Rutherford, 1993)

Table 1.2 Estimated annual loss of sugarcane (tons) and rand value in the South African sugar industry due to *E. saccharina* (1980 to 1986) (from Smith, 1990).

Season	*Average e 100st[1]*	*Tons cane lost (x 10^3)*	*Value in rands (x 10^6)*
1980/81	1.7	179	3.9
1981/82	2.7	351	7.0
1982/83	1.3	178	3.9
1983/84	2.9	334	10.1
1984/85	2.0	274	6.5
1985/86	1.8	247	6.2

1.2.1.4 Approaches by SASEX to the control of eldana

Past research programmes at SASEX have examined various approaches to the control of *Eldana saccharina* Walker (Carnegie, 1982; 1983). Arising from these were a number of recommendations based on reducing crop age at harvest, reducing use of nitrogen fertiliser, appropriate choice of varieties and a practice called pre-trashing. This comprises the manual loosening or removal of the dead leaf material attached to stalks (Carnegie and Smaill, 1982). The current research programme against eldana focuses on four areas: host plant resistance, the use of insecticides, biological control, and ecological and behavioural studies. These will now be discussed.

1.2.1.4.1 Host plant resistance

The basic approach, developed by Nuss and Atkinson (1983), comprises variety selection through bioassaying sugarcane in pots in a shade house. After about 9 months and after the plants are stressed by reduced watering, the stalks are inoculated with *E. saccharina* eggs. At sampling, which occurs 500 day-degrees after inoculation, assessments are made of damage and of recovered larvae. Each variety is then rated using these measures on a scale of 1 (resistant) to 9 (susceptible). An average rating is then obtained using all measures. Five such trials are conducted each year.

The use of biotechnology in the varietal resistance programme was also pursued

and comprised three approaches: the use of NIR to develop predictive models for assessing resistance to eldana, the development and testing of a genetically engineered bacterium (*Pseudomonas fluorescens*) and the use of a truncated gene, coding for a bacterial toxin, for insertion in the genome of sugarcane. Rutherford *et al.* (1993) developed 3 NIR models to predict varietal resistance or susceptibility to eldana. The first model was based on assessment of the tannin, lignin and fibre components of stalks; the second, on components of buds, and the third, surface wax components of stalks. Snyman *et al.* (1993) tested the efficacy of a sugarcane epiphyte, *Pseudomonas fluorescens*, genetically engineered to produce the toxin of the entomopathogen *Bacillus thuringiensis*. It was postulated that dispersing neonate larvae would come into contact with and ingest the engineered pseudomonad, leading to increased larval mortality. Controlled glasshouse studies (Black *et al.*, 1995) showed that while damage caused by eldana was reduced, the persistence of the bacterium was poor and this approach was abandoned. It was later envisaged that the insertion of the Bt gene into the sugarcane genome and its expression would confer resistance to eldana larvae feeding internally. This concept will be further discussed later in this chapter.

1.2.1.4.2 Insecticides

The current programme is based on the timing of insecticide application in relation to moth numbers. The rationale behind this approach stems from the finding that seasonal peaks occur in the number of *E. saccharina* moths caught in light traps, typically over the period September to November and again during April to May (Carnegie and Leslie, 1990). It is assumed that after a peak in moth numbers there is an oviposition peak, followed by a peak in neonate larvae. Because of the generally cryptic behaviour of eldana, only these dispersing larvae would be exposed to an insecticide. However, it is not possible to monitor neonate larval numbers directly, and so apply treatment at the correct time for maximum effect. Instead, treatments are applied at fortnightly intervals over the predicted period of a moth peak.

1.2.1.4.3 Biological control

A biological control programme against eldana was established in 1977 (Conlong, 1994), and comprised searching for parasitoids (both exotic and indigenous), the

identification of predators and screening feral material for pathogens. None of these approaches has so far produced a viable control option. A revised programme comprises the continued screening of local wild hosts for parasitoids; the search in other African countries for suitable parasitoids; the manipulation of indigenous predators and screening for a viral pathogen.

1.2.1.4.4 Ecology and behaviour

As noted previously, it is important that a monitoring method, other than light traps, be developed for eldana moths. The ecology and behaviour programme is aimed at developing a pheromone trap for moths. Initial work on pheromone trapping (Bennett *et al.*, 1991) was not encouraging, and this was attributed to the requirement for sound in the courtship system. Burger *et al.* (1993) list 31 compounds identified from wing and tail gland secretions of the male moths. The influence of temperature on eldana development is also being examined to obtain revised threshold temperature and thermal constants for different developmental stages and activities (for example, mating). The revised estimates obtained from laboratory studies are 6°C, 10°C and 10°C for egg, larval and pupal development respectively. The mating threshold is estimated to be about 15°C, which has implications for the survival of this pest in the cooler regions of the industry.

It is improbable that any of the above approaches will, alone, provide satisfactory long term control measures. Rather, appropriate combinations of varietal choice, timed insecticide applications and biological control strategies will be required. This illustrates the urgency to develop a cost effective control of this pest, preferably through the use of eldana resistant varieties. Genetic manipulation of sugarcane, to obtain insect resistant plants, is a suggested solution to this problem.

1.2.2 *Bacillus thuringiensis* (Bt) as a source of insecticidal protein

1.2.2.1 General

Bacillus thuringiensis (*Bt*) is a gram-positive spore-forming bacterium which synthesises a variety of insecticidal crystal proteins (ICPs) upon sporulation (Carozzi *et al.*, 1992). The structure of the ICPs results from the assembly of one or more types of protein subunits, the protoxins or delta-endotoxins. A number of different delta-endotoxins have been characterised, each having a relatively narrow

insecticidal spectrum (Mazier *et al.*, 1997). In order to exert their entomopathogenic activity, the delta-endotoxins have to be dissolved in the insect gut, then activated by gut proteases which specifically cleave the C-terminal half of the high molecular weight (130 kDa) protoxin as well as a few N-terminal amino acids, releasing smaller 60-70 kDa polypeptides which are the active toxins (Hofte and Whiteley, 1989). These smaller polypeptides comprise a toxic motif as well as the determinants responsible for specificity , and show blocks of sequences that are remarkably well conserved among the different toxins (Sanchis *et al.*, 1989).

1.2.2.2 Classes of Bt genes

Typically, 4 major classes of Bt crystal protein genes (*cry* genes) have been recognised:

I. Lepidopteran-specific
II. Lepidopteran- and Dipteran-specific
III. Coleopteran-specific and
IV. Dipteran-specific

These specify a family of related ICPs (Hofte and Whiteley, 1989). Table 1.3 gives a simplified classification of the Bt toxin genes, based on the nomenclature of Hofte and Whiteley (1989).

Table 1.3 Simplified classification of Bt toxin genes (Hofte and Whiteley, 1989)

Toxin gene	**Molecular wt. (kDa)**	**Insecticidal activity**
cryIA(a), (b), (c), B, C, D, E, F, G	130-138	Lepidoptera
cryIIA, B, C	69-71	Lepidoptera and Diptera
cryIIIA, B, B(b)	73-74	Coleoptera
cryIVA, B, C, D	72-134	Diptera
cryV - cryX	35-129	Various

a) *cryI* genes

Genes of the class *cryI* encode 130-160 kDa protoxins which combine to form bipyramidal protein crystals which typically contain more than one gene product (Bulla *et al.*, 1975). One half of the molecule (C-terminal end) is highly conserved (70% or more) and possibly involved in the packing of toxins within the crystalline

inclusion (Hofte and Whiteley, 1989), whereas the other half (N-terminal), which is the toxic domain involved in ICP formation, is a more variable region (Adang *et al.*, 1987). CryI-type proteins typically comprise approximately 1200 amino acids and range in size from 131.0 to 133.3 kDa (Gawron-Burke *et al.*, 1990). CryIA is the most common type of crystal protein found in cryI-producing Bt strains (Ceron *et al.*, 1994) and the *cryIA(b)* gene appears to be the most widely distributed gene amongst different Bt subspecies (Yamamoto and Powell, 1993). However, many strains produce several different types of ICPs simultaneously and the same (or very similar) crystal proteins occur in different strains (Hofte and Whiteley, 1989).

b) *cryII* genes

Genes of the class *cryII* encode 65 kDa proteins, which occur as distinct cuboidal inclusions in several Bt serovars (Yamamoto, 1983). The cryIIA protein is toxic to both lepidopteran and dipteran larvae, whereas the cryIIB toxin is only toxic to lepidopteran insects (Widner and Whiteley, 1990). These two toxins are 87% identical (Aronson, 1993). Another gene in this class (*cryIIC*) was cloned and sequenced by Wu *et al.* in 1991.

c) *cryIII* genes

Krieg *et al.* (1983) first described a Bt variety with coleopteran activity. Since then a number of other coleopteran-active strains have been identified, encoding cryIIIA, B, C, D and E type toxins (Ceron *et al.*, 1995). Herrnstadt *et al.* (1986) identified a Bt isolate which produced flat, rhomboidal ICPs of approximately 64 kDa. For most ICP genes, transcription is concomitant with sporulation (Adams, Brown and Whiteley, 1991). However, the cryIIIA protein appears to be unique in being expressed during vegetative growth of the Bt cell (Sekar *et al.*, 1987), i. e. independently of sporulation.

d) *cryIV* and *cyt* genes

The *cryIV* class of crystal protein genes (cryIVA, B, C, D) and the *cyt A* gene are located on the same plasmid and encode proteins with molecular weights in the range 28-140 kDa (Hurley *et al.*, 1985). These Bt crystals are composed of at least 5 polypeptides (Hofte and Whiteley, 1989). The proteins are all synthesised at different times during sporulation, and are added to the developing inclusion in a

step-wise manner (Lee *et al.*, 1985). The cryIV ICPs form an ovoid complex with the 27 kDa cyt A protein and is only active against dipteran larvae (Koni and Ellar, 1994).

e) *cryV* genes

Tailor *et al.* (1992) discovered a new class of ICP genes (designated *cryV*) which is reportedly most closely related to *cryIB*. In the original host, *cryV* genes are either cryptic or very weakly expressed.

1.2.2.3 Toxin specificity

Bt crystal proteins show a remarkable degree of insect specificity and the C-terminal part of the toxic fragment is considered to be responsible, via specific receptor binding (Honee *et al.*, 1991). Due to their ability to specifically bind Bt toxins, a number of proteins have being characterised as putative receptors (Mazier *et al.*, 1997). Different models have been described to explain the possible role of these receptors: the receptor itself could be a transmembrane channel which would be blocked in an open position by the toxin; the toxin and the receptor together could form a pore; or the receptor could catalyse the insertion of the toxin in the membrane, but would have no other role in pore formation. The subsequent steps in the intoxication process include the insertion of the toxin in the apical membrane of the epithelial columnar cells, including the formation of ionic channels or non-specific pores in the target membrane. As a consequence of pore formation, a series of events leads to lesions in the plasma membrane, and finally to the destruction of the integrity of the midgut. Histological observations suggest that osmotic lysis is a common phenomenon in intoxicated insects. The insects then stop feeding and die (Mazier *et al.*, 1997).

1.2.2.4 Bt234

A natural isolate of *Bacillus thuringiensis* identified at SASEX and named strain 234 was found to be at least twice as toxic to eldana larvae than the commercially available formulation Thuricide™ (Herrera, 1988). Crystals isolated from Bt234 are bipyramidal and the delta-endotoxin has an apparent molecular weight of 135, 000 (Herrera *et al.*, 1994). The *cry* gene of Bt234 is almost identical to that in *B. thuringiensis* subsp. *kurstaki* HD-73, indicating that this gene is widespread

(Herrera *et al.*, 1994). Southern hybridisation analysis of *Hind* III digests of the DNA of the two strains showed that whereas HD-73 carries only the 6.6 kb *cryIA(c)* gene, which is well known for its toxicity to eldana, isolate 234 carries, in addition to the *cryIA(c)* gene, the 4.5 kb *cryIA(a)* gene. It is not yet known whether the latter gene of Bt234 also contributes to its toxicity to eldana larvae.

1.2.3 Introduction of the Bt234 gene into sugarcane

1.2.3.1 General

Sugarcane plants expressing toxin genes have several advantages over classical chemical means used in plant protection. As previously mentioned, borers are difficult to reach by conventional means once they have penetrated plant tissues. Since toxin expression can be achieved throughout the whole plant during the entire life cycle, the deployment of transgenic sugarcane plants would allow the control of this major insect pest. Furthermore, eldana is exposed to the toxin at its most sensitive stage (the early larval instar) and conversely, the plants can be protected when they are most sensitive to insect attack. In addition, the system allows the toxin to be maintained in the plant tissues and has the potential of lowering costs by reducing the number of necessary chemical treatments (Mazier *et al.*, 1997). Finally, due to the relatively narrow host range of Bt toxins, which are active on target pests and not on beneficial insects, and to the particular mode of delivery of the toxin (the plant tissues), transgenic plants expressing Bt toxin genes can easily be integrated into an integrated pest management (IPM) scheme (Mazier *et al.*, 1997).

It was for the above mentioned reasons that the *cryIA(c)* gene from Bt234 was introduced into the sugarcane genome. A truncated version of the gene (Figure 5.8) was inserted into plasmid pLBR 19-1 and the resulting plasmid, referred to as pLBRtox (figure 3.2), was bombarded into sugarcane.

1.2.3.2 Transformation vectors

The ability to stably introduce foreign genes into plant cells is an essential component of genetic engineering (Gruber and Crosby, 1993). The ultimate objective is to regenerate normal, fertile plants which stably transmit the introduced gene to their progeny. Once a gene of interest has been isolated, it is introduced

into a plant transformation vector. The vector DNA facilitates manipulation of the gene in *Escherichia coli* prior to plant transformation, as well as transfer of the gene to the host plant (Greenberg and Glick, 1993). An idealised vector would contain a multiple cloning site, an antibiotic resistance gene allowing for selection in *E. coli* (e. g., a gene encoding ampicillin resistance), a broad-host bacterial origin of replication, and an antibiotic resistance gene for selection of the foreign DNA in transformed plants (e. g., a gene encoding kanamycin resistance).

There are many approaches for introducing DNA into plants, of which the most commonly used are the *Agrobacterium tumefaciens* system, electroporation, and the particle gun (Potrykus, 1991). Although most engineered plants have been generated through *A. tumefaciens*-mediated gene transfer, the system seems unsuccessful in the transformation of most monocotyledonous plants, especially cereals (Peferoen, 1992). Electroporation involves the electroporetic transfer of naked DNA into cells. Since any DNA fragment can be delivered to the cell, this technique has the advantage of allowing assimilation of a gene without having to clone the DNA into a vector. One disadvantage of this method, however, is that during incorporation into the host nuclear genome, DNA rearrangement sometimes occurs. Furthermore, only plants from which protoplasts can be isolated may be transformed by this technique and not all protoplast systems can be used to regenerate flowering plants (Greenberg and Glick, 1993).

For the present investigation, a technique known as microprojectile particle bombardment, which is the most common method used for monocot transformation, was used to deliver the Bt234 gene into sugarcane cells. With this technique, microprojectile particles (e. g. 1.2 μm tungsten beads) coated with DNA are accelerated at high speeds into plant tissue. This approach has the distinct advantage of applicability to any intact plant tissue or region of the plant (Wu, Kemmerer and McElroy, 1990) and any piece of DNA can be used for the transformation process (Greenberg and Glick, 1993). Unfortunately, the disadvantage exists that regions of DNA may be randomly lost during incorporation (Greenberg and Glick, 1993).

Two plasmid constructs, pEOT500 (Figure 1.2A) and pEOT510 (Figure 1.2B), were

used to transform the commercial cultivar NCo310 in the present study. Plasmid pEOT500 is 6650 bp in size and contains a maize ubiquitin promoter and a nos terminator, while pEOT510 is 7250 bp and contains the SUC-1 promoter (Groenewald, personal communication). Both plasmids carry the same truncated Bt gene (Figure 5.8) which is 1858 bp long. The plasmids were maintained and grown in *E. coli* (JM 83). White, embryogenic callus was co-bombarded with either pEOT500 and pEmuKN or pEOT510 and pEmuKN, using a particle inflow gun. pEmuKN is 5645 bp long and contains the npt II-gene cloned behind the Emu promoter and intron 1 of maize adh-1 (Meyer, personal communication).

1.2.4 The role of promoters in gene expression

The promoter of a gene contains the information required to direct when, where and to what extent this gene will be expressed (Datla, Anderson and Selvaraj, 1997). Gene expression is a complex, multi-step process which includes initiation of transcription, mRNA processing, transcript stability, mRNA transport to the cytoplasm, translation and finally protein modification. In general, gene expression can be regulated at any of the above steps. Of these, transcriptional regulation plays an important role for most genes, as it is the first committed step in the gene expression cascade.

1.2.4.1 Structural and functional organisation of promoters

Transcriptional activation of genes is basically controlled by *cis*-acting DNA sequences and *trans*-acting factors (Datla et al., 1997). The *cis*-acting sequences comprise the promoter region, representing the sequence immediately upstream to the transcriptional start site. *Trans*-acting factors (transcriptional factors), that include general and gene-specific DNA-binding proteins, recognise elements present in the promoter or other regulatory regions and modulate gene expression.

As shown in figure 1.3, a typical *cis*-acting sequence of a promoter contains two core elements, i. e. "TATA" box and "Inr" (initiator). The TATA box is an AT-rich sequence located 21 to 35 nucleotides upstream from the transcription start site (Breathnach and Chambon, 1981). This region is surrounded by GC-rich sequences which may play a role in its function (Lefebvre and Gellatly, 1997) and interacts with a TATA-binding protein necessary for accurate initiation of promoters

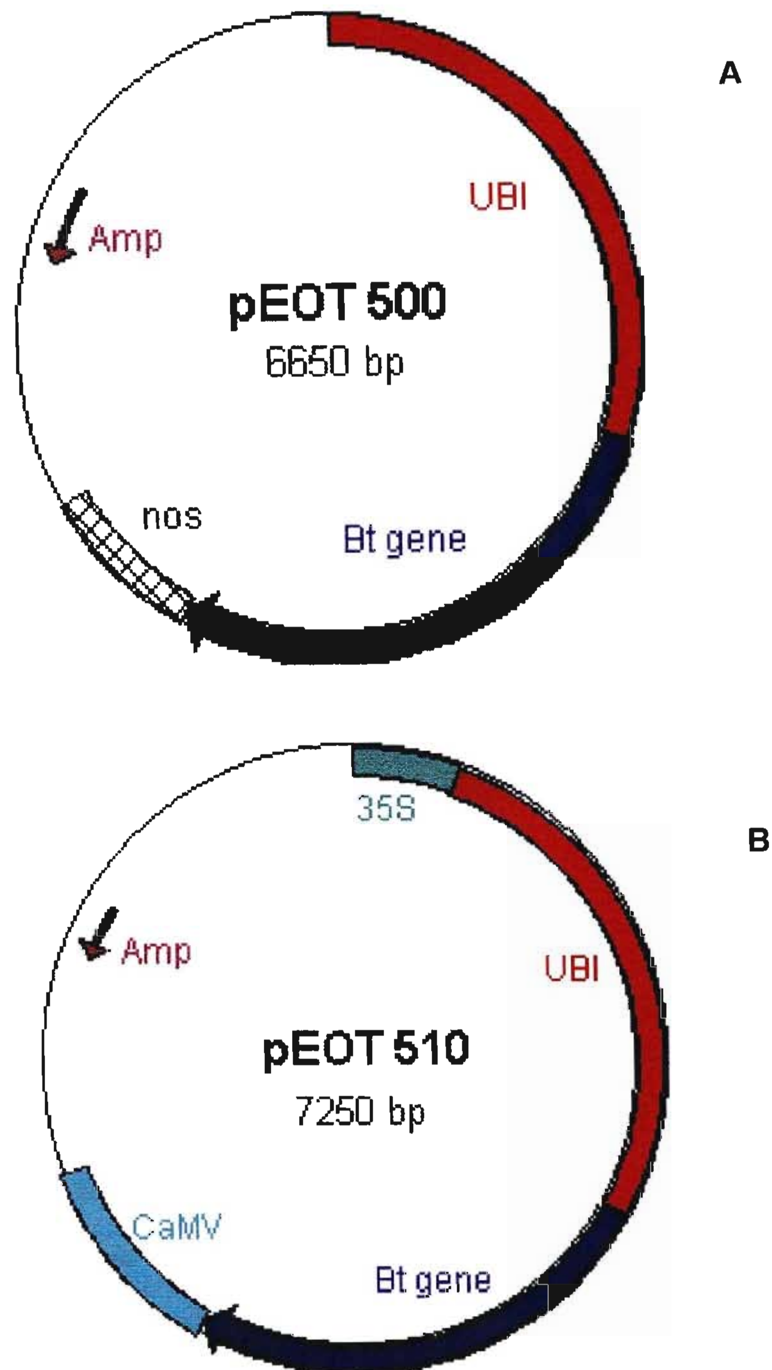


Figure 1.2: Schematic diagrams of plasmids pEOT 500 (A) and pEOT 510 (B). pEOT 500 contains the maize ubiquitin promoter (UBI), while pEOT 510 contains the chimaeric SUC-1 promoter (CaMV 35S + UBI).

transcribed by RNA polymerase II (Rosenthal, 1987; Mukumoto *et al.*, 1993; Czarnecka-Verna *et al.*, 1994). Deletion of the TATA box reduces promoter activity and results in multiple transcription start sites (An and Kim, 1993). In some promoters, such as those for the maize zein gene, two TATA boxes may be found as close as 10 nucleotides apart (Lefebvre and Gellatly, 1997). The TATA box also serves as an anchor for making contact with gene-specific transcriptional factors bound to upstream regulatory elements and thus to activate transcription. The second core element, Inr, determines the transcriptional start site for RNA polymerase II to initiate mRNA synthesis.

Another control motif, the CCAAT box sequence, is located close to the –80 region of several, but not all plant promoters and is functional in both orientations (Thangue and Rigby, 1988). It has been established that a family of transcription factors recognise this motif and that factors binding to this sequence can co-operate with those binding to surrounding motifs (Thangue and Rigby, 1988). A hexamer sequence, TGACGT, occurs in most constitutive promoters within a few hundred nucleotides from the transcription start site (An and Kim, 1993). These hexamer motifs are often found as repeats separated by six to eight nucleotides. Deletion analysis has indicated that these motifs are essential for transcription activity of the cauliflower mosaic virus (CaMV) *35S*, octopine synthase (*ocs*) and nopaline synthase (*nos*) promoters (An and Kim, 1993). Genes for the transcription factor that specifically interacts with the hexamer motifs have been isolated from both dicotyledonous and monocotyledonous plant species (Katagiri *et al.*, 1989; Singh *et al.*, 1990).

In addition to the core elements, the promoter also contains regulatory elements. The composition and organisation of these elements determine the regulatory properties and expression profiles of that particular gene. For example, as generalised in figure 1.3, the three boxes of positive regulatory elements, also referred to as enhancers, through interactions with transcriptional activators, impart developmental, hormonal, or environmental controls. Motifs that are found in promoters of genes that are environmentally inducible include the G-box sequence (CCACGTGG) in various photosynthesis gene promoters (An and Kim, 1993) and the H-box sequence (CCTACC) which, together with the G-box sequence, is

essential for the expression of the bean chalcone synthase gene (Arias *et al.*, 1993). AT-rich motifs appear to increase the amplitude of induction in heat-shock protein genes (Czarnecka-Verner *et al.*, 1994) and may play a regulatory role in several other higher plant genes (Forde, 1994). Examples of genes that contain AT-rich motifs are nodulin genes, seed-specific genes like lectin, trypsin inhibitor and β-phaseolin, photoregulated genes and some heat shock genes (Forde,1994). In contrast to enhancers, the negative regulatory elements, by interaction with transcriptional repressors, suppress gene expression (Datla *et al.*, 1997). The multilateral interactions of *trans*-acting factors, regulatory elements and basic transcription factors determine how a particular gene is regulated.

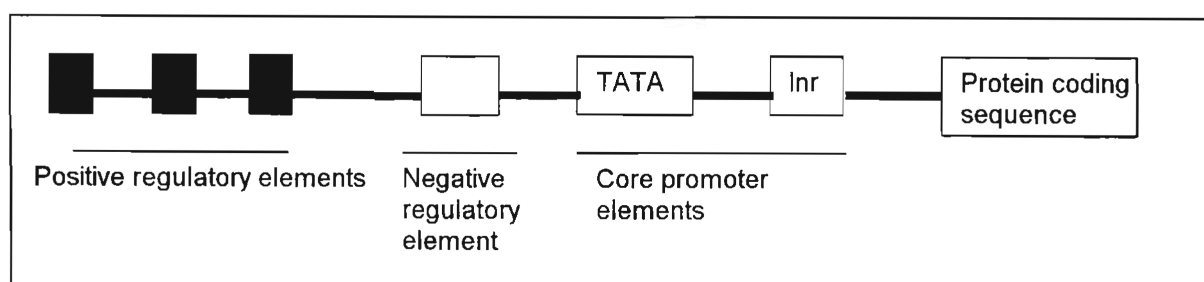


Figure 1.3 A generalised eukaryotic promoter (from Datla *et al.*, 1997).

1.2.4.2 Promoter classification

Promoters are classified into three groups, namely constitutive, inducible and tissue-specifically or developmentally regulated. The promoters that are chosen for transformation studies vary according to the expression pattern that is preferred (Gruber and Crosby, 1993).

a) Constitutive promoters

Constitutive promoters are active during most stages of development and in most plant tissues. There is little variation of mRNA abundance or translational efficiency during development, in different organs or upon application of various endogenous or environmental stimuli (Kuhlemeier *et al.*, 1987). Examples of constitutive promoters are the nuclear gene promoter for the β-subunit of the mitochondrial ATPase complex (Boutry and Chua, 1985), the rice actin promoter *Act I* (Zhang *et al.*, 1991) and the CaMV 35S promoter.

b) Inducible promoters

There is a wide variety of inducible promoters, a few of which include temperature-inducible, hormone-inducible, pathogen-inducible, light-inducible and water stress-inducible promoters (Gruber and Crosby, 1993).

Temperature-inducible promoters

i) Heat-shock promoters

The heat shock (HS) response has been documented in bacteria, as well as in lower and higher eukaryotes (Schlesinger *et al.*, 1982) where it occurs in almost every cell and tissue type. It is strongly induced by heat, but can also be induced by a number of other stress agents such as arsenite, ethanol, amino acid analogs, uncouplers of oxidative phosphorylation and transition metal ions (Ashburner and Bonner, 1979; Czarnecka *et al.*, 1984; Edelman *et al.*, 1988). The thermo-inducible expression of heat shock proteins is attributed to the presence of controlling DNA sequences located in their promoters (Scharf *et al.*, 1994). These conserved sequences were named heat shock elements (HSEs). Single, overlapping or multiple copies of these HSEs can occur in the promoter (Czarnecka-Verner *et al.*, 1994). The HSE confers both high selectivity of heat shock induction and high overall efficiency of transcription initiation (Scharf *et al.*, 1994). Transcriptional activation of heat shock genes in response to elevated temperatures or chemical levels is mediated by the interaction of the *trans*-acting heat shock transcription factors (HSFs) with HSEs of the promoter (Scharf *et al.*, 1994).

ii) Cold-induced promoters

Plant response to low temperature leads to alteration of the cellular metabolism and is correlated with significant changes in gene expression (Guy, 1990; Palva, 1994). Cold-regulated genes have been isolated and characterised in several plant species including alfalfa (Wolfraim *et al.*, 1993), *Arabidopsis* (Hajela *et al.*, 1990), spinach (Neven *et al.*, 1993), tomato (Schaffner and Fischer, 1990), barley (Cattivelli and Bartels, 1990) and wheat (Houde *et al.*, 1992). In the *Arabidopsis rd29a* promoter a dehydration-responsive element (DRE) was identified (Kirch *et al.*, 1997).

Hormone-inducible promoters

Plant hormones are considered to be an important class of internal signal compounds which are easily transported to target cells and have been shown to be directly involved in transcriptional and translational control of gene activity in a number of plant processes. Specific regulatory elements have been identified for hormone-responsiveness (Kuhlemeier *et al.*, 1987; Quatrano *et al.*, 1993; Giraudat *et al.*, 1994; Gatz, 1997).

The growth regulator abscisic acid (ABA) is known to induce the synthesis of specific proteins, among which are certain seed-storage proteins (Kuhlemeier *et al.*, 1987). Examples of genes that are expressed due to ABA influence include the beta conglycinin α-subunit, lectins and agglutinins. These may be involved in maintaining seed dormancy, in protecting seeds against desiccation and in preventing deleterious effects of hydrolytic enzymes (Kuhlemeier *et al.*, 1987). A protein that has been found to be ABA-inducible is the 10 kD soluble protein, *Em*, which accumulates in wheat embryos in the latter third of grain development (Williamson and Quatrano, 1988). The *Em* promoter contains an ABA response element (ABRE) which is responsible for the dramatic increase of expression in the presence of ABA.

Plants respond to pathogen infection or other stress by producing the stress hormone ethylene and this is the signal for plants to activate the defence mechanism against pathogens. Inducible defence responses are thus mediated by ethylene-induced gene expression (Shinshi *et al.*, 1995). Auxins and cytokinins also influence expression of genes including those that are involved in maintenance of apical dominance, cell elongation and xylem differentiation (Kuhlemeier *et al.*, 1987).

Pathogen-inducible promoters

In nearly all the systems studied to date, transcriptional activation of specific plant genes plays a key role in the defence of plants against pathogens (Dixon and Lamb, 1990). The proteins induced in this way are all involved in defending the plant against attack or preventing the spread of infection and include enzymes of the phenylpropanoid pathway, enzymes involved in phytoalexin production,

structural cell wall proteins, peroxidases, superoxide dismutase, proteinase inhibitors, thionins and the 'pathogenesis-related' (PR) proteins (Van de Rhee *et al.*, 1994). PR proteins have been identified as proteins coded for by the host plant but induced by various pathogens as well as under stress situations similar to those provoked by pathogens (Van Loon, 1990). These proteins may also play a key role in enhancing the levels of resistance against secondary challenges by pathogens since they not only accumulate locally around the sites of infection, but also appear in the uninfected parts of the plant (Van de Rhee *et al.*, 1994). Other stress conditions that induce specific subsets of PR proteins include wounding and ultraviolet light, chemical substances such as salicylic acid, the plant hormone ethylene and fungal elicitors (Van de Rhee *et al.*, 1994).

Light-inducible promoters

Light-induced responses require changes in both nuclear and chloroplast gene expression and provide the basis for much of plant development (Kuhlemeier *et al.*, 1987). Many of the regulatory effects of light on the growth and differentiation of plants are mediated by the control of gene expression for numerous proteins (Gilmartin *et al.*, 1990). The induction of gene expression as well as the degree to which the genes are expressed are not only determined by the quality and quantity of light, but also by the developmental state of the cells intercepting the light (Fluhr and Chua, 1986). The most extensively studied light-responsive genes are those encoding the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcs*) and the chlorophyll *a/b*-binding proteins (*cab*) (Gilmartin *et al.*, 1990). Numerous regulatory *cis*-acting sequences have been identified (Terzaghi and Cashmore, 1995).

Water stress-inducible promoters

Among the many different plant responses to water deficit, changes in gene expression have also been demonstrated. These changes are an important part of the ability of the plant to respond to the environment (Bray, 1994). Stress-related *in vitro* translation products have been observed indicating that a new population of mRNAs accumulates during stress imposition. For example, after wheat seedlings were subjected to an 80% relative water content, several different classes of *in vitro* translation products were observed: those that decreased,

increased and did not change with respect to nonstressed seedlings (King *et al.*, 1992). In dehydrated pea shoots, at least 13 mRNAs were increased in comparison to nonstressed shoots (Guerrero and Mullet, 1988). The accumulation of new mRNAs during stress has also been shown to occur rapidly. Ho and Mishkind (1991) identified a number of changes in *in vitro* translation products after 20 min of water deficit in tomato plants. These studies clearly demonstrate that there are many changes in mRNA accumulation during periods of water deficit and that the observed changes are highly regulated.

c) Tissue-specific expression

Development of a multicellular organism is critically dependant on the proper temporal and spatial cues that regulate gene expression. Thus, in any one cell at a particular time, only a subset of the total genetic information is expressed. The regulatory elements that are responsible for directing specific transcription initiation reside within the 5' promoter region of the gene (Lam, 1994) (figure 1.3). Numerous genes and associated promoters have been described which exhibit a wide range of tissue-specific or developmental expression patterns. Some include genes that are specifically expressed in pollen (Miyoshi *et al.*, 1995), flower (Faktor *et al.*, 1996), phloem (Yang and Russell, 1990; Rhode *et al.*, 1995), root (Depater and Schilperoort, 1992) and seed (Iida *et al.*, 1995; Nunberg *et al.*, 1995; Van der Geest and Hall, 1996).

1.2.4.3 Approaches to the isolation of promoters for transgene expression in sugarcane

At present, few regulatory sequences are available that specifically drive transgene expression in sugarcane. Patent limitations on established promoters and genes necessitate the isolation of novel regulatory elements. Options for isolating gene regulatory sequences with desired patterns of expression in sugarcane include the use of characterised promoters from other plant species. These promoters can be used directly to drive introduced gene expression in sugarcane, or indirectly as the basis for probes or primers used to isolate corresponding promoters from sugarcane (Birch *et al.*, 1996). This approach is, however, limited by the availability of suitable foreign promoters and sometimes by patent considerations.

Another approach concerns the introduction of promoterless reporter genes to tag sugarcane promoters revealed by interesting patterns of reporter gene expression (Koncz *et al.*, 1989). This approach is limited by the relatively low efficiency of promoter tagging by direct gene transfer into plants and the difficulties in recovering tagged promoters.

Hybridisation approaches may be used to isolate tissue specific cDNAs which can be used as probes to recover corresponding promoters from a sugarcane genomic library (Watson and Margulies, 1993). Constraints include the requirement for large amounts of RNA, technical difficulties in cDNA library construction, and a bias towards strongly expressed genes. The final approach to the isolation of promoters involves differential display of tissue-specific gene fragments following PCR amplification using random oligonucleotide primers on first strand cDNA (Liang *et al.*, 1993). These fragments are then used as a starting point for isolating corresponding promoters by PCR or hybridisation approaches.

Birch *et al.* (1996) used a sugarcane root-specific cDNA clone, isolated using a differential hybridisation approach, as a probe in detailed Northern analysis of sugarcane under various growing conditions. They found that the gene was strongly expressed in roots under a range of stress conditions, but not in leaves, stems or healthy field-grown roots. A corresponding 770 bp putative root-specific promoter (rsp) region, isolated from a sugarcane genomic library, was then transcriptionally and translationally fused to both the β-glucuronidase and firefly luciferase reporter genes (Birch *et al.*, 1996). Promoter analysis based on transient expression following particle bombardment into sugarcane and maize tissues was investigated. Expression levels in bombarded roots was, however, low. Insertion of an upstream enhancer (OCS) increased overall expression levels, but not root specificity. The translational fusions were then transferred into sugarcane by microprojectile bombardment of embryogenic calli, followed by selection for a co-transformed marker gene (*aphA*). Irregular patterns of reporter gene expression in transformed calli confirmed promoter activity of the cloned genomic sequence (Birch *et al.*, 1996).

A PFP-β promoter was isolated by PCR screening of a sugarcane genomic library

and characterised by sequence analysis (Groenewald, 1997). Known promoter motifs were identified and a fragment of 1072 bp of the 5' flanking area was tested for transient GUS reporter gene expression in sugarcane callus. Expression was observed and the results confirmed that the isolated promoter can drive transient transgene expression.

1.2.4.4 Activity of various promoters in monocot and dicot species

A promoter functional in plants that has been described in the most detail is the 35S promoter from the cauliflower mosaic virus (CaMV). This promoter has been used extensively in plant transformation (Gruber and Crosby, 1993). The CaMV 35S promoter is constitutive in its expression, although a certain degree of cell- and tissue-specificity has been observed. It has been studied intensively with respect to functional domains which define this sequence as a promoter, leading to the development of derivatives in which the intrinsic enhancer element has been duplicated. A construct containing such a tandem 35S promoter exhibited a 4-fold increase in steady-state expression of various marker genes relative to the unmodified 35S promoter in electroporation experiments involving plant species as diverse as maize, *Picea glauca* (White Spruce) and *Brassica napus* (Gruber and Crosby, 1993). Because of its early availability to plant molecular biologists, its broad host-range applicability and biological characterisation, the 35S promoter has found wide use in a variety of vector constructs, including chimeric constructs which combine regulatory elements from different promoters.

The 35S promoter does not usually function as well in monocots as in dicot species. Hence, other promoter elements which are efficiently expressed in monocots have been incorporated into vector constructs targeted to these species. In this regard, promoter Emu, which contains multiple copies of an anaerobic response element from the maize *Adh-1* gene and elements from the octopine synthase gene from *A. tumefaciens*, has been shown to exhibit 10- to 50-fold greater expression in five monocot species (Gruber and Crosby, 1993). During their studies on the effects of promoter, intron and enhancer elements on transient gene expression in sugarcane and carrot, Rathus *et al.* (1993) showed the artificial promoter Emu to be much more active than the CaMV 35S or maize Adh1 promoters. The Emu promoter, with multiple enhancers close to the core promoter

region, was even more strongly enhanced relative to the 35S promoter in sugarcane (400-fold) than in cereals (10- to 50-fold). The very strong expression from the Emu promoter has already been useful in optimising microprojectile bombardment conditions (Franks and Birch, 1991) and selecting transgenic sugarcane plants (Bower and Birch, 1992).

Other constitutive monocot promoters used in transformation studies are the rice actin promoter (Act-1) and the maize ubiquitin promoter (UBI), which achieved a far better expression than 35S in all monocots tested by Wilmink, van den Ven and Dons (1995). The increase in expression sometimes amounted to several tenfold. Dicot tissues showed a different response: the activities of monocot promoters were comparable to or less than the activity of the CaMV 35S promoter. In general, promoters isolated from monocots show a higher activity in monocot species (Wilmink, van den Ven and Dons, 1995).

The ubiquitin promoter has shown promise in sugarcane transformation. Gallo-Meagher and Irvine (1993) compared the strength of the UBI, recombinant Emu, Act-1 and 35S promoters to drive expression of the GUS gene in sugarcane leaves. GUS expression was histochemically measured 48h after microprojectile bombardment of the *uidA* gene into NCo310 leaves. The UBI promoter produced significantly more GUS foci and higher GUS activity levels compared to the recombinant Emu, Act-1 and CaMV 35S promoters. Leaf segments bombarded with 35S-GUS showed the weakest GUS histochemical staining. The authors concluded that UBI provides a strong constitutive promoter which will be advantageous for the development of an efficient sugarcane transformation system.

1.2.4.5 The ubiquitin promoter

Ubiquitin, a eukaryotic protein consisting of 76 highly conserved amino acid residues, is found in most cell types as either free monomers or conjugated to a variety of cytoplasmic, nuclear or membrane proteins (Hough *et al.*, 1988). It displays multiple functions in the cell, including selective degradation of proteins; control of chromatin structure and possibly gene expression; stress response; and participation in ribosome biogenesis. Ubiquitin is encoded by multigene families of 2 general types: i) genes encoding tandem repeats of ubiquitin monomers

(polyubiquitins) and ii) genes encoding ubiquitin fusions with other proteins (ubiquitin fusion proteins). Two polyubiquitin genes from maize, *ubi 1* and *ubi 2*, have been isolated and sequenced (Christensen *et al.*, 1992). Both genes are expressed constitutively at 25°C in maize seedlings and are inducible to higher levels upon heat shock.

In a study carried out by Cornejo *et al.* (1993), aspects of the activity of maize promoter sequences that regulate expression of the *ubi 1* gene in transgenic rice were characterised. The *ubi 1* promoter, first exon and first intron (UBI) supported the highest levels of expression of any of the promoter/ intron combinations that had been previously used to select rice transformants. It was also revealed that UBI was most active in rapidly dividing cells. In addition, it was reported that UBI expression occurs in many, but not all, cell types and undergoes important changes in activity during the development of transgenic rice plants. Those authors found that during differentiation of some tissues there are high levels of ubiquitin, but these decrease drastically with time. The UBI pattern of expression suggests that this promoter is most active in cells with high metabolic activity. Furthermore, UBI expression in transgenic rice calli increased in response to thermal stress. These results were encouraging as they suggested that the Bt gene, directed by the UBI promoter, would be expressed at high levels under stressful conditions of plant growth in transgenic sugarcane, for example, high eldana infestation and high environmental temperatures.

1.2.4.6 The SUC-1 promoter

The SUC-1 promoter is a combination of both the UBI and CaMV 35S promoters linked in tandem (i. e. a chimeric promoter). It was hoped that by joining the UBI and 35S promoters, expression of the Bt gene in transgenic sugarcane would be increased, since it is well documented that these genes are expressed at low levels in plants, in contrast to the high expression found in their natural host, in *E. coli*, or in *Pseudomonas* (Mazier *et al.*, 1997). At the outset of this project, the reasons for this low level of Bt gene expression in plants had not yet been clearly determined as few studies have been undertaken to identify the mechanisms responsible for the lack of transcript accumulation. Bt genes have conventionally been placed under the control of strong constitutive promoters in order to achieve the high level

of expression needed for good insect control (Mazier *et al.*, 1997) and for this the CaMV 35S promoter is generally used. Identification of an enhancer sequence in the CaMV 35S promoter by Kay *et al.* (1987) led to the construction of a duplicated 35S promoter with 5- to 10-fold higher levels of expression. This promoter has been used in several studies where higher levels of Bt expression were required.

Christensen *et al.* (1992) compared the separate activities of UBI and CaMV 35S promoters on the expression of the reporter molecule chloramphenicol acetyl transferase (CAT) in both maize (monocot) and tobacco (dicot). They reported that expression from the UBI promoter of pUBI-CAT yielded more than a 10-fold higher level of CAT activity in maize protoplasts than expression from the widely used CaMV 35S promoter of a 35S-CAT construct. Conversely, in tobacco protoplasts CAT activity from transcription of pUBI-CAT was less than one tenth of the level from p35S-CAT. They suggested several possible explanations for this different relative activity in maize and tobacco protoplasts. The maize UBI promoter may be monocot-specific, i. e. it is highly expressed in maize and other monocots, but requires a *trans*-acting factor(s) not present in a dicot such as tobacco. Alternatively, the intron in the 5' untranslated region of the transcription unit of pUBI-CAT may have contrasting effects on expression in the monocot and dicot cells. It may increase CAT expression in electroporated maize protoplasts similar to results obtained with the Adh-1 intron (Callis *et al.*, 1987), whereas in tobacco protoplasts, it may not be effectively spliced from the chimeric ubiquitin-CAT transcript and may result in the lower levels of CAT activity. It is well known that intron- exon-splice junctions are different between monocots and dicots. Monocot junctions are predominantly pyrimidine rich in the 3' splice junction, while dicot 3' splice sequences are higher in purine bases (Hanley and Schuler, 1988). From the results obtained in their study, Christensen *et al.* (1992) concluded that the ubiquitin promoter might be beneficial for generating a high level of expression of selectable marker genes necessary for generation of transgenic monocots. Also, since ubiquitin has a role in many important cellular functions, it is likely to be expressed in all tissues.

For the present investigation, it was envisaged that a combination of both UBI and 35S promoters might provide higher levels of Bt gene expression than the use of

either promoter alone. Thus, the activity of the chimeric SUC-1 promoter was compared to the UBI promoter.

1.3 PROJECT AIMS AND APPROACH

This project concerns the characterisation of the activities of two promoters, UBI and SUC-1, in driving transgene expression in sugarcane plants under stress. The SUC-1 promoter consists of UBI and CaMV 35S promoters linked in tandem. Since it is well documented that ubiquitin is a stress related protein, it was hypothesised that the activity of UBI would be maintained or even increased under conditions of environmental stress. A second hypothesis was that SUC-1 might enhance overall gene expression since the CaMV 35S component is a promoter widely and successfully used in transformation studies and commercially released transgenic plants. To test these hypotheses, transgenic sugarcane plants of variety NCo310 carrying the Bt transgene driven by the two respective promoters were used as experimental models, and the activities of the promoters analysed in both stressed and non-stressed (control) plants. Plants were stressed by withholding water supply in a controlled manner. The use of the Bt transgene in conjunction with the UBI and SUC-1 promoters was seen to be industrially significant in that eldana infestations (and thus the requirement for Bt expression) occur particularly in mature sugarcane that is physiologically stressed by drought.

In chapter 2 the micropropagation of NCo310 plants is described. The reason for utilising this technique is that large numbers of clones of both transgenic and non-transgenic genotypes were needed for the water stress and expression experiments. Micropropagation is well known for being much more rapid than conventional methods of vegetative multiplication (George, 1993). Three micropropagation techniques were compared in an attempt to find the most suitable for the present study in terms of the time required to successfully regenerate plants. These techniques were node culture, shoot tip culture (both of which follow direct organogenesis) and callus culture (representing indirect organogenesis). Node culture involves the cutting of sugarcane stalks into single-budded setts that are planted into polystyrene trays (Goodall, 1998). Each bud is capable of producing a new plant that can be transferred to the field. Shoot tip cultures are started from explants bearing an intact shoot meristem, whose purpose is shoot

multiplication by the repeated formation of axillary branches (George, 1993). Newly formed shoots serve as explants for repeated proliferation. Callus cultures include the initiation and continued proliferation of undifferentiated parenchyma cells (callus) from parent tissue (Brown, 1990). During indirect organogenesis, plants develop from specific regions of the callus via shoot morphogenesis followed by root development (Bhaskaran and Smith, 1990). Shoot tip culture was eventually chosen as the technique of choice to bulk up the transgenic and nontransformed plant material needed in the present study. Transgenic material consisted of three genotypes containing the UBI promoter (namely, #6.8, #6.12 and #18.1) and three containing the SUC-1 promoter (namely, #7.20, #7.47 and #7.33).

Chapter 3 focuses on the optimisation of molecular techniques which were to be later used for assaying UBI and SUC-1 promoter activity. These included RNA isolation, nucleic acid amplification (reverse transcription-polymerase chain reaction or RT-PCR) and Northern hybridisation. Care had to be taken with all three techniques not to contaminate samples with ribonuclease, a ubiquitous enzyme that destroys RNA. DNA contamination of RNA samples was a major problem encountered during RNA isolation and had to be overcome before RT-PCR could be successfully applied to amplify the Bt transgene. Optimisation of Northern analysis, which was required for the determination of size and abundance of the expressed Bt gene, made use of four protocols. The initial protocol failed to produce the Bt fragment, while the second and third protocols showed non-specific hybridisation of the probe to the 25S and 16S ribosomal proteins. The final protocol was successful, producing the expected 2 kb Bt fragment.

The aim of the investigation reported in chapter 4 was to develop successful watering regimes for the induction of plant stress. This included the development of suitable techniques to physically measure the stress response of these plants. Water stress was applied by completely withholding water supply to the plants to be stressed, while control plants were watered twice a day. Techniques employed for stress measurements were Relative Water Content (RWC) determination, leaf elongation measurements and Infra Red Gas Analysis (IRGA). Preliminary studies revealed that RWC determination was not a suitable measure of water stress since no trend was evident to indicate how stressed the plants were with time. It was

also shown that the simple method of measuring the elongation rate of the third youngest leaf was superior to the more sophisticated IRGA measurements which give information about the photosynthetic and transpiration rates, as well as stomatal conductance of the plants.

The effect of water stress on UBI and SUC-1 promoter activity is discussed in chapter 5. Transgenic as well as nontransformed sugarcane plants were subjected to periods of water deficit by complete withholding of their water supply. Leaf elongation was used as a physiological measure of plant stress, while RT-PCR and Northern hybridisation were employed to evaluate the effect of stress on the expression of Bt mRNA in transgenic sugarcane. In this way the activities of UBI and SUC-1 could be compared. A heat shock protein gene was also used as a probe during Northern analysis in order to provide a means of comparison of an endogenous stress gene with the behaviour of the UBI and SUC-1 promoters. RT-PCR revealed that Bt mRNA was expressed in water-stressed as well as non-stressed plants, suggesting that both UBI and SUC-1 promoters were active at all times. However, the extent of mRNA expression could not be determined from these results since the Bt bands were of similar intensity for most plants tested. Northern hybridisation revealed a multiple Bt banding pattern in all transgenic plants tested, as well as variations in signal intensity. SUC-1 promoter activity remained constant under stressful conditions whereas UBI promoter activity appeared to decline. The occurrence of smaller than expected Bt transcripts could be possibly due to the presence of polyadenylation signals in the coding region of the Bt gene (Adang *et al.*, 1987; Murray *et al.*, 1991; Diehn *et al.*, 1998) leading to premature termination of the transcript. The variable expression of the Bt gene when comparing expression levels in individual transformants could be due to position effects of the transgene in the plant genome (Broer, 1996).

A final discussion concerning the general conclusions which can be drawn from this study, as well applications for the future, are also given. Results obtained in the present study suggest that SUC-1 would be the promoter of choice for driving Bt gene expression in transgenic sugarcane plants in the future. Future work will, however, require the use of a modified version of the Bt gene, making it more 'plant like'.

CHAPTER 2: MICROPROPAGATION OF EXPERIMENTAL PLANTS

2.1 INTRODUCTION

Micropropagation describes the clonal propagation of very small parts of plants using *in vitro* culture techniques (Liu, 1971). These involve growing cells in a medium that will support them, keep them viable and allow plantlet multiplication. It can be initiated at three different levels of biological organisation, namely plant organs, tissues, or single cells (Hicks, 1980).

In culture, a flexible adjustment of factors influencing vegetative regeneration is possible such as nutrient and growth regulator levels, light and temperature (George, 1993). The rate of propagation is therefore much greater than in macropropagation and many more plants can be produced in a given time. This may enable newly selected genotypes to be made available quickly. The technique is very suitable when high volume production is essential (Vasil, 1986; Evans, 1990; Gnanapragasam and Vasil, 1990). Sugarcane, which provides about 65% of the sugar produced in the world, has been studied extensively using tissue culture methods (Ho and Vasil, 1983; Maretzki, 1987). Plant regeneration from tissue cultures of sugarcane has also been successfully applied to breeding and propagation programmes by many workers (Heinz *et al.*, 1977; Koga and Kudo, 1977; Nadar and Heinz, 1977; Ploper and Mariotti, 1978).

Two types of growth, namely organised and unorganised, are possible *in vitro* (Hicks, 1980; George, 1993). Organised growth contributes towards the creation or maintenance of a defined structure. It occurs when plant organs such as the growing points of shoots or roots (apical meristems), leaf initials, young flower buds or small fruits are transferred to culture and continue to grow with their structure preserved. Unorganised growth, on the other hand, occurs when the tissues which are formed in culture lack any recognisable structure and contain only a limited number of the many kinds of specialised and differentiated cells found in an intact plant. The following kinds of unorganised cultures are generally recognised, namely callus-, suspension-, protoplast- and anther cultures. Types of organised cultures include organ-, meristem-, shoot tip-, node-, embryo- and isolated root

cultures (Hicks, 1980).

In the present study, three types of culture, considered to be the most successful for sugarcane, were compared in order to establish one that would allow the rapid and successful regeneration of transgenic and untransformed plants. These were node culture, shoot tip culture and callus culture. The efficacy of each technique when using different sugarcane varieties was also investigated. The varieties tested were NCo376, NCo310, N12 and N19.

2.1.1 Propagation via direct organogenesis

2.1.1.1 Node culture

The culturing of separate lateral buds, each carried on a small piece of stem tissue, is referred to as node culture (Julien *et al.*, 1989). Stem pieces carrying either single or multiple nodes may be cultured and each bud is grown to provide a single shoot. Commercially, sugarcane is vegetatively propagated using setts, which are often referred to as 'seedcane'. These are sections of stalk containing 3-6 lateral buds and are traditionally planted into furrows in the field. The buds on the stalk germinate to produce primary shoots that form stools after development of secondary shoots from basal buds (Julien *et al.*, 1989). A new planting technique, which involves the cutting of stalks into single-budded setts that are planted into polystyrene trays, was introduced into South Africa in the early 1980s (Goodall, 1998). Each bud and band of root initials is capable of producing a new plant that can be planted into the field.

2.1.1.2 Shoot tip culture

Apical dominance, which is defined as the limitation or complete inhibition of the development of lateral buds by a vigorous terminal bud, has long been recognised (Mitchell, 1953). If the terminal bud is removed, one or more of the lateral buds close to the top of the stem usually commence development or grow with increased vigour. Once growing vigorously, these in their turn limit the growth of lateral buds below them (Snow, 1925; Thimann and Skoog, 1934). Application of cytokinin to inhibited lateral shoots can partly substitute for decapitation and stimulate their outgrowth (Sachs and Thimann, 1964). This is the essence of shoot tip culture, during which cultures are started from explants bearing an intact shoot meristem

and whose purpose is shoot multiplication by the repeated formation of axillary branches (George, 1993). In this technique, newly formed shoots serve as explants for repeated proliferation. Since cytokinins generally inhibit root formation (Hussey, 1976), normal rooted plantlets can be obtained by removal of these growth regulators from the culture medium. The inclusion of auxin into the culture medium is normally avoided so as to reduce the potential for callus formation.

Organogenesis and plantlet formation via shoot tip culture is a propagative technique in widespread use (Murashige, 1974; Miller and Murashige, 1976), particularly for the rapid and large scale clonal multiplication of plants, including sugarcane (Vasil, 1986; Debergh and Zimmerman, 1990). This type of culture finds special application in the regeneration of virus-free stock of commercial value (Mellor and Stace-Smith, 1977; Quak, 1977).

2.1.2 Propagation via indirect organogenesis

2.1.2.1 Callus culture

Callus cultures are composed of dedifferentiated, unorganised and actively dividing cells (Bartkowiak, 1981; Brown, 1990). Two types of calli can be formed, namely a white compact nodular embryogenic callus and a soft friable non-embryogenic callus (Ho and Vasil, 1983). Such cultures may be maintained for extended periods by subculture at 2-4 weekly intervals, and therefore represent a convenient form for the long-term maintenance of cell lines. Plants develop from specific regions of the callus via either somatic embryogenesis or by indirect organogenesis (shoot morphogenesis followed by root development) (Bhaskaran and Smith, 1990).

In vivo, callus is frequently formed as a result of wounding at the cut edge of a root or stem, following invasion by microorganisms or damage resulting from insect feeding. Its formation is controlled by endogenous auxin and cytokinin. By incorporation of these growth regulators into the medium, callus can be induced to form *in vitro* on explants of parent tissue (George, 1993). In the present study, sugarcane plants were regenerated from non-embryogenic callus via indirect organogenesis.

2.2 MATERIALS AND METHODS

2.2.1 Source of explant material

Explants of all varieties used in the present study were obtained from various sources at the South African Sugar Association Experiment Station, Mt Edgecombe, Durban. Transgenic experimental material was variety NCo310 derived and was obtained from plants maintained in the glasshouse, while non-transformed material comprised field-grown plants of varieties N19, N12, NCo376 and NCo310. Six to eight month old cane was used.

2.2.2 Preparation of explants for *in vitro* culture

All plants to be micropropagated were surface sterilised as follows. The outer mature leaves were removed to approximately the tenth node. Stalks were sprayed with 70% (v/v) ethanol and a further six outer leaves removed. Stalks were then soaked in 70% (v/v) ethanol for 1 min. Thereafter, they were soaked in 30% (v/v) sodium hypochlorite for 15 min and rinsed three times in sterile distilled water. The final step was to soak them in distilled water for 5 min.

2.2.3 Node culture

Node culture was performed on transgenic NCo310 plants containing the Bt gene with the SUC-1 promoter (#7.20, #7.47 and #7.33), as well as those containing the Bt gene with the UBI promoter (#6.8, #6.12 and #18.1). Sugarcane stalks (internodes 2-7) were cut into single-budded setts and planted in polystyrene trays containing sterile potting soil. The setts were grown at 27 +/- 1°C and 85% relative humidity (RH) in the glasshouse under natural daylight conditions. After approximately 14 days, plantlets were transferred to 160 mm long x 175 mm diameter pots and maintained at 27 +/- 1°C, ambient RH in the glasshouse.

2.2.4 Shoot tip culture

Shoot tip culture was performed on untransformed NCo376, N12, N19 and NCo310 plants, as well as transgenic NCo310 plants. The shoot tip (approximately 4 mm long x 4 mm in diameter) was aseptically excised from the apical portion of healthy stalks by completely removing the leaf roll tissue in a laminar flow hood. Apical and lateral buds were also excised. Explants were placed into 100 ml conical flasks containing a 20 ml volume of shoot proliferation medium, which consisted of

the basal salt formulation of Murashige & Skoog (1962) (Sigma, USA) together with 2% (w/v) sucrose, 0.1% (v/v) B5G stock solution (1 $g.l^{-1}$ nicotinic acid, 10 $g.l^{-1}$ thiamine HCl, 1 $g.l^{-1}$ pyridoxine HCl, 2 $g.l^{-1}$ glycine, 100 $g.l^{-1}$ myo-inositol), 0.2 $mg.l^{-1}$ 6-benzylaminopurine (BAP) and 1 $mg.l^{-1}$ kinetin (pH 5.7). Cultures were agitated on an orbital shaker at 40 rpm with a 24 h light (130-200 $\mu E.m^{-2}.s^{-1}$) photoperiod at approximately 26 +/- 1°C and ambient RH. After shoot tips had reached 10-20 mm in length, they were transferred to Magenta vessels [7.6 x 7.6 x 10.2 cm (Sigma, USA)] containing 50 ml shoot proliferation medium solidified with 5 $g.l^{-1}$ agar gel (Sigma, USA). At intervals of 8-12 days, proliferating shoots were separated into clusters of 4-6 shoots and each was placed in fresh shoot proliferation medium. Cultures were maintained in the light (130-200 $\mu E.m^{-2}.s^{-1}$) at 26 +/- 1°C and ambient RH.

Multiplication from original explants, as well as from propagated shoots, was continued until the desired numbers of shoots were obtained. When shoots had reached 6-8 cm in height they were transferred to Magenta vessels containing rooting medium which consisted of 0.1x MS basal medium (Murashige and Skoog, 1962), 2% (w/v) sucrose, 0.1% (v/v) B5G stock solution, 2 $mg.l^{-1}$ indole-3-butyric acid (IBA) and 5 $g.l^{-1}$ agar gel (Sigma) at pH 5.7. Cultures were incubated at 26 +/- 1°C and ambient RH with a 24 h light (130-200 $\mu E.m^{-2}.s^{-1}$) photoperiod . Once roots had developed, plantlets were hardened off as described in section 2.2.6 and maintained in the glasshouse.

2.2.5 Callus culture

Organogenic calli were initiated from the leaf roll tissue of untransformed NCo310 and N19 plants, as well as transgenic NCo310 plants. All outermost leaves were aseptically removed and the young, innermost leaf sliced into rectangular sections approximately 3 mm x 3 mm in size. Leaf sections were placed on to Petri dishes containing callus induction medium which consisted of MS salts (Murashige and Skoog, 1962), together with 3% (w/v) sucrose, 1 $g.l^{-1}$ casein hydrolysate, 3 $mg.l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 $g.l^{-1}$ agar gel (Sigma, USA) at pH 5.8 and incubated at 26 +/- 1°C, ambient RH in the dark. After 4 weeks cultures were transferred from medium containing 2,4-D (callus induction) to one containing BAP and kinetin (shoot proliferation medium, as in section 2.2.4) and maintained under

continuous daylight fluorescent illumination (130-200 $\mu E.m^{-2}.s^{-1}$) at 26 +/- 1°C to induce regeneration. When shoots had reached 6-8 cm in height, they were transferred to Magenta vessels containing rooting medium (as in section 2.2.4). Once roots had developed, plantlets were hardened off (section 2.2.6) in the glasshouse and grown under natural daylight at 27 +/- 1°C.

2.2.6 Hardening off regenerated plants

Plantlets exhibiting well-developed root systems were transferred to 90 mm long x 75 mm diameter pots containing sterile potting soil. These were placed in the glasshouse in a high relative humidity chamber (approximately 85% RH). Automatic misting was applied at 15 min intervals in order to keep the RH high. After approximately 2 weeks, plants were transferred to a chamber with ambient RH to continue growth. The temperature in the glasshouse was maintained at 27 +/- 1°C.

2.3 RESULTS AND DISCUSSION

As mature sugarcane plants were needed in order to bulk up material for stress studies, transgenic plant material in the glasshouse was allowed to reach maturity (approximately 8 months). In the meantime, using available material, different micropropagation techniques were compared. Once an efficient method had been established, this was used to clone transgenic NCo310 sugarcane plants containing the Bt gene. Table 2.1 provides the total number of plants successfully micropropagated in the present study.

2.3.1 Node culture

Figure 2.1 illustrates the results obtained using node culture. Out of all the micropropagative techniques investigated in the present study, node culture produced the poorest results. Only plants containing the SUC-1 promoter were regenerated (table 2.1). A possible reason for the poor performance of this technique is that it was undertaken during winter, when sugarcane growth is very slow. Low temperatures delay germination and thus give more time for disease organisms to gain access to the cutting, resulting in high mortality of the buds (Barnes, 1974). Other possible reasons for the absence of bud break are inhibition by apical dominance, physical injury, drying of the stem, excess water and poor

Table 2.1: Plant regeneration by direct and indirect organogenesis

Sugarcane variety	**Node culture**		**Shoot tip culture**		**Callus culture**	
	# Explants (nodes)	*Plants Regenerated*	*# Explants (shoot tips)*	*Plants Regenerated*	*# Explants (leaf discs)*	*Plants Regenerated*
nontransgenic N19	not applied	not applied	20	84	39	7
nontransgenic N12	not applied	not applied	20	0	not applied	not applied
nontransgenic NCo376	not applied	not applied	20	0	not applied	not applied
nontransgenic NCo310	not applied	not applied	20	75	39	0
Transgenic NCo310 plants containing Bt gene						
#7.20 (SUC-1 promoter)	25	12	5	0	12	0
#7.47 (SUC-1 promoter)	25	2	5	0	12	0
#7.33 (SUC-1 promoter)	25	14	5	0	12	10
#6.8 (UBI promoter)	25	0	5	110	12	8
#6.12 (UBI promoter)	25	0	5	0	12	18
#18.1 (UBI promoter)	25	0	5	64	12	0
Total number of plants	**150**	**28**	**110**	**333**	**150**	**43**

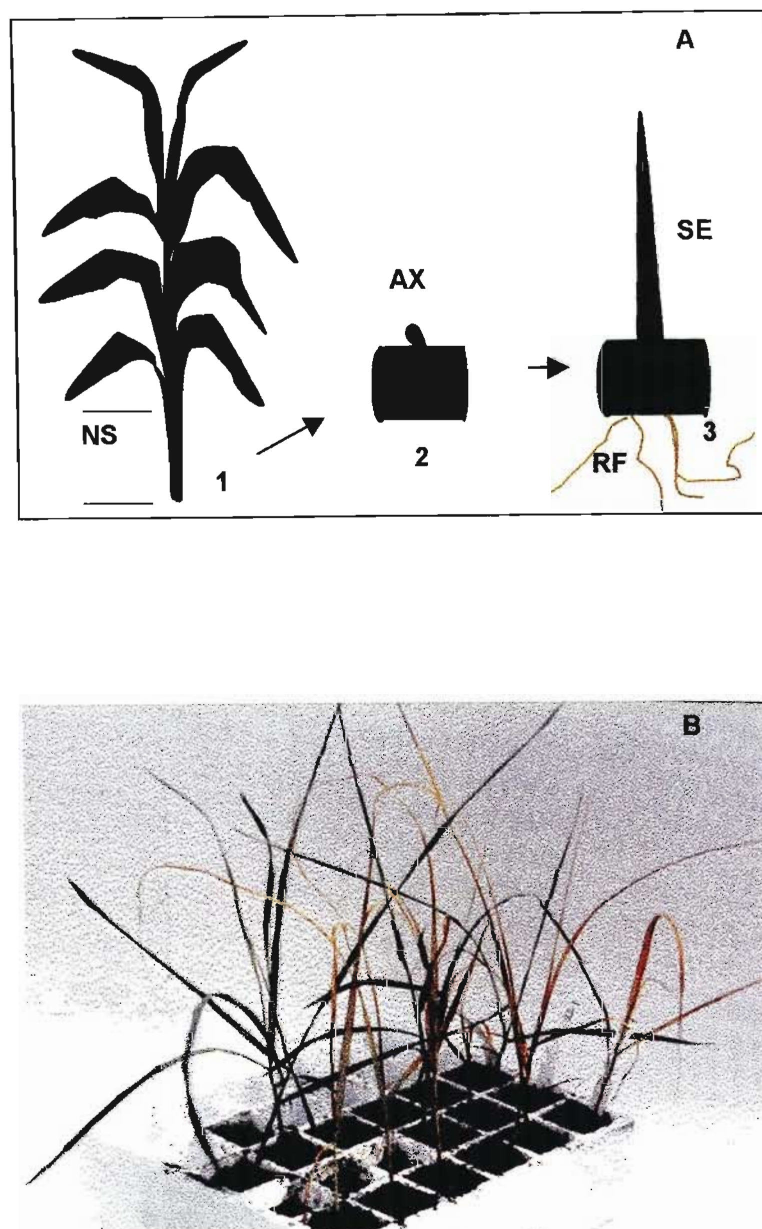


Figure 2.1 Micropropagation of sugarcane by node culture. (A) essence of node culture (adapted from Pollard and Walker, 1990): (1) plant showing nodal segment (NS) to be excised; (2) excised nodal segment showing axillary bud (AX) that will be responsible for subsequent growth; (3) pattern of development showing shoot elongation (SE) and root formation (RF); (B) regeneration of transgenic NCo310 sugarcane plants in speedling tray.

nutritional status of the seedcane. Infection of the setts by micro-organisms cause fermentation and the production of toxic materials, so depriving the buds of nutrients and inhibiting or retarding growth (Barnes, 1974).

2.3.2 Shoot tip culture

Shoot tips approximately 4 mm x 4 mm in size, as well as apical and lateral buds, were dissected out and grown aseptically on shoot proliferation medium as described in section 2.2.4. The cultures were continuously shaken to ensure aeration and diffusion of metabolic wastes. Initially, 0.1 $mg.l^{-1}$ kinetin was added to the medium to stimulate shoot proliferation. This concentration, however, proved insufficient for the production of multiple shoots and thus was increased to 1 $mg.l^{-1}$. Initial studies showed that although the liquid medium supported bud break, after approximately 1 month the explants died. Thus, as soon as the explants produced 1 mm shoots, they were transferred to semi-solid (5 $g.l^{-1}$ agar gel) shoot proliferation medium. Shoot tip and lateral bud explants were also placed directly on to semi-solid medium (instead of first into liquid medium) to investigate bud break rates. This treatment proved beneficial for shoot tips. In contrast, buds required an initial incubation in liquid medium before transference to semi-solid medium.

It was observed that explants produced shoots faster if placed with other explants in the medium than when alone. Large explants generally survived more frequently and grew more rapidly at the outset than very small ones. This phenomenon is described by George (1993) as the "feeder effect". That author claims that the loss of essential substances from a plant cell is of no consequence when the ratio of plant material to medium is high. However, when this ratio is low, the concentration of essential substances in the cells and in the medium can become inadequate for the survival of the culture. This means that there is a minimum amount of explant material per unit culture volume (inoculation density) for successful culture initiation.

An important observation during shoot tip culture was that excessive phenolic oxidation occurred. This phenomenon involves the oxidation of cells when they are wounded, following which the isolated tissue turns brown or black and fails to grow.

Substances known to be released into the medium by this means include alkaloids, amino acids, enzymes, growth substances and vitamins (Street, 1969). Explants from the variety N19 had the lowest phenolic oxidation and the highest regeneration rates (table 2.1). Variety NCo310 also had very little phenolic oxidation, while NCo376 exhibited the highest.

Explants from NCo376 and N12 were subcultured to fresh liquid medium every 2 days to prevent both exhaustion of nutrients in the medium and accumulation of toxic metabolites. N19 was subcultured less often (approximately every 9 days). In addition, lateral buds produced less phenolics than shoot tips and thus required subculturing less often.

When subcultured shoots had reached 6-8 cm in height, half were transferred to media in which kinetin and BAP had been omitted, since, as previously mentioned (section 2.1.1.2) high concentrations of cytokinin generally inhibit or delay root formation and also prevent root growth (Hussey, 1976; George, 1993). The other half were rooted in medium containing 1 $mg.l^{-1}$ IBA, with kinetin and BAP excluded, to see which rooting medium produced roots rapidly. Those shoots that were transferred to rooting medium which did not contain any growth regulators took 9 days to produce roots, compared to those that were placed on rooting medium containing IBA, which took approximately 13 days to root. Furthermore, roots were more abundant on those plants placed in rooting medium that did not contain IBA. Grisham and Bourg (1989) obtained similar results in their studies concerning shoot tip culture of sugarcane plants. Those authors found that increasing the sucrose content, reducing the concentration of MS salts and eliminating IBA resulted in the best root development.

Figure 2.2 is an illustration of a sugarcane plantlet developing roots during shoot tip culture. For both types of rooting medium used in the present study, it took approximately 18 days for roots to develop to an extent that allowed the plants to be hardened off in the glasshouse. It took approximately three months for plant regeneration, i.e. from the time cultures were initiated to the time plants were potted.

Figure 2.2 Development of roots in variety N19 during shoot tip culture.

Shoot tip culture proved to be the most successful of the micropropagation techniques used in the present study (table 2.1). Figure 2.3 shows some of the plants regenerated by this technique.

2.3.3 Callus culture

A variety of explants including young leaves, shoot apices and young inflorescences have been used for callus initiation in sugarcane (Heinz *et al.*, 1977; Liu, 1984). In the present study, young leaf tissue was the explant of choice. Small pieces of leaf tissue were placed on to Petri dishes containing callus induction medium (section 2.2.5) under sterile conditions in a lamina flow hood. Leaves formed callus within 2-4 weeks after explantation. Callus generally formed first on the cut edge, later spreading over the surface of the leaf (figure 2.4).

Histological studies have demonstrated that plant regeneration from sugarcane callus occurs either by organogenesis (Liu and Chen, 1974) or somatic embryogenesis (Ho and Vasil, 1983). In the present study, organogenesis was responsible for plant regeneration. A mucilaginous nodular callus was the first to develop. Subsequently, a white friable non-embryogenic callus developed over the mucilaginous tissue. Plants were able to regenerate only from the white friable callus. The formation of this type of callus was genotype dependent, with variation in the frequency of formation of this callus in the varieties N19 and NCo310. The variety which showed a low frequency of non-embryogenic callus formation, namely N19, also produced limited amounts of callus overall. However, white friable callus tissues from the two different varieties had similar growth characteristics.

Figure 2.3 Sugarcane plants regenerated by shoot tip culture. (A) untransformed plants; (B) transgenic NCo310 plants

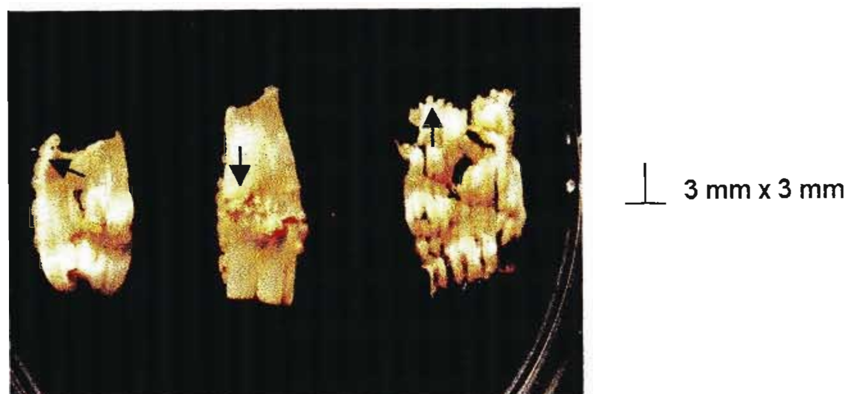


Figure 2.4 Pieces of leaf explant showing callus initiation (black arrows). Callus first appeared on the cut edges and then spread over the surface of the explant.

Proliferation of callus was stimulated by transferring the tissue to fresh callus induction medium (section 2.2.5). However, continued subculture caused callus production to decrease at each subculture with an increase in the proportion of nodular mucilaginous tissue. Careful visual selection at each transfer was effective in maximising the amount of white friable callus in the cultures.

Differentiation of plants from callus tissue was accomplished by transfer of callus to the basal medium without 2,4-D, but containing BAP and kinetin (shoot proliferation medium). Shoot formation via organogenesis occurred in the callus after approximately 5 weeks from callus initiation (figure 2.5A). After plants had been transferred to rooting medium, it took approximately 25 days for sufficient roots to form (figure 2.5B) to allow hardening off in the glasshouse.

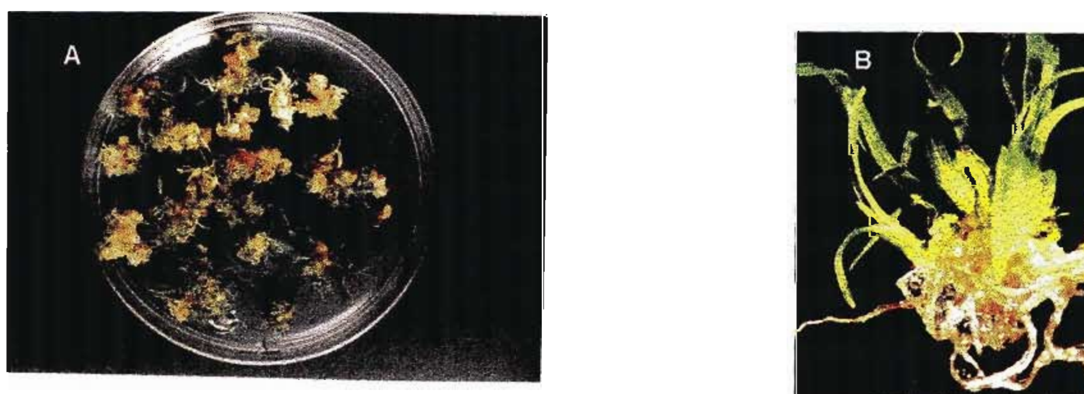


Figure 2.5 Plant regeneration during callus culture. (A) shoot formation on pieces of callus after transfer to shoot proliferation medium; (B) root formation

The production of plants from callus tissue has been described as a tool for plant breeders to create variability (Heinz and Mee, 1969). Murashige and Nakano (1966) found tetraploid plants in tobacco differentiated from callus, and Guha and Mahesewari (1964) have produced haploid plants from pollen callus. The formation of callus tissue and the subsequent differentiation of plants offered those authors the opportunity to change the chromosome complement and make-up without crossing. Heinz and Mee (1969) have also observed variation between plants differentiated from the same callus cultures. Among all the plants studied by those authors, morphological variants in relation to the parental clone were observed. In contrast, plants differentiated from callus in the present study were apparently derivatives from a single cell and remained morphologically uniform during growth.

2.3.4 Comparison of the techniques used to micropropagate sugarcane plants

Of all the techniques tested in the present study, shoot tip culture produced the most clones, especially in genotypes #6.8 and #18.1. However, for some genotypes no clones were produced by this method. The reasons for this are unknown. From 110 shoot tips (explants) used to initiate this culture, a total of 333 plants were regenerated (table 2.1), making shoot tip culture the technique of choice for future micropropagation of sugarcane. A total of 43 plants were regenerated from the 150 leaf discs used to initiate callus. Node culture was shown to be the least desirable with only 28 plants being regenerated from the 150 nodes used during initiation (table 2.1).

Regeneration of plants from organised meristems, as in node- and shoot tip culture, are usually associated with genetic uniformity (Walden *et al.*, 1989; George, 1993). In a study carried out by Walden *et al.* (1989) on maize meristem cultures, no instance of somaclonal variation was observed. Similarly, studies conducted by Hendre *et al.* (1983) on sugarcane showed that shoot tip culture produced plants very similar to their donor plants. Their plants exhibited very little deviation in the length of millable canes, number of internodes and the number of canes per clump compared to the mother plant.

Genetically altered plants are, however, occasionally obtained from shoot tip

cultures (Zimmerman, 1982; Vuylsteke *et al.*, 1988). This is associated with the formation of shoots from adventitious buds on basal callus rather than from axillary buds. Vuylsteke *et al.* (1988) found that about 6% of plants obtained from shoot tip cultures displayed phenotypic variation in inflorescence structure or in the shape, variegation or habit of leaves. The foliage malformations re-appeared when the plants were ratooned suggesting that they had a genetic basis. The occurrence of a significant proportion of deviant plants during banana shoot tip culture is very common and has been reported by Reuveni *et al.* (1984), Hwang and Ko (1984), Drew (1986), Hwang (1986) and Reuveni and Israeli (1990).

Although the rate of multiplication of node culture is generally less than that which can be brought about through shoot tip culture, there is less likelihood of associated callus formation and the formation of adventitious shoots (George, 1993). This type of culture thus carries very little risk of induced genetic irregularity. For this reason, node culture has been increasingly recommended by research workers as the micropropagation method least likely to induce mutation (Hussey *et al.*, 1980; Ammirato, 1976). However, node culture is not suited to all species and is still not as widely used as shoot tip culture. In the present study, node culture was not as successful in the rapid regeneration of plants as was shoot tip- or callus culture (table 2.1). Since the aim of the experiment was to produce a large number of plants in the shortest possible time, node culture proved undesirable.

On the other hand, shoot tip culture has proven to be an extremely rapid method of sugarcane propagation (Hendre *et al.*, 1983; Grisham and Bourg, 1989). Hendre *et al.* (1983) produced 15-20 shoots from a single shoot tip within 2-3 weeks. Within two weeks, 80% of the plants developed a healthy root system. This is in agreement with the results obtained in the present study in which shoots that were transferred to rooting medium containing IBA took approximately 13 days to root (section 2.3.2). Shoots placed onto medium that did not contain any growth regulators were rooted even faster (approximately 9 days). In contrast, root formation on calli took approximately 25 days (section 2.3.3).

In contrast to shoot tip- and node culture, callus cultures have generally proven

unsuitable for *in vitro* propagation because they tend to accumulate chromosomal abnormalities and lose their capacity to regenerate whole plants (Liu and Shih, 1933; Sheridan, 1975; Hussey, 1976; D'Amato, 1977; Evans *et al.*, 1984; Lee and Phillips, 1987; George, 1993). This was seen in the present investigation, where continued subculture caused callus production to decrease at each subculture (section 2.3.3). The presence of cytological variation in plants regenerated from callus was shown by Lee and Phillips (1987). Liu *et al.* (1984) found that one of their callus-derived lines was 38 cm higher in stalk height and 0.49 cm thicker in diameter than its donor. It also had a consistently higher sucrose content. In the present study, genetically identical plants were required and thus the above evidence of genetic instability induced by callus culture was particularly unsettling. Added to this was the low number of plants successfully regenerated by this technique (43 plants), compared to shoot tip culture (333 plants).

2.4 CONCLUSION

The aim of the present study was to clone transgenic and untransformed NCo310 sugarcane plants in order to bulk up experimental material for the stress studies described in chapters 4 and 5. Three culture systems were compared, namely node-, shoot tip- and callus culture, in order to establish the most successful in terms of rapidly regenerating genetically stable plants. Four sugarcane varieties, namely NCo310, NCo376, N12 and N19, were used to test the efficacy of these techniques in different varieties. Of the three, shoot tip culture proved the most successful, producing the largest number of plants (333 plants regenerated from 110 explants) in the shortest time (approximately 3 months). Percent efficiency of the three techniques were: 302.7% for shoot tip culture, 28.7% for callus culture and 18.7% for node culture. Shoot tip culture would therefore be the method of choice for future micropropagation of sugarcane plants.

CHAPTER 3: DEVELOPMENT OF MOLECULAR TECHNIQUES FOR ASSAYING PROMOTER ACTIVITY

3.1 INTRODUCTION

Since the expression of genes is driven by promoters, deductions about promoter activity can be made by studying the transcription product, mRNA (An and Kim, 1993; Glick and Thompson, 1993; Christou, 1996; Birch, 1997; Guilfoyle, 1997). Recent studies have made use of a variety of techniques to analyse mRNA expression, namely the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) (Chang *et al.*, 1993; Denis and Lustenberger, 1995; Ra and Saris, 1995; Richert *et al.*, 1996; Saric and Shain, 1997; Diehn *et al.*, 1998), Northern blot analysis (Chevet *et al.*, 1995; Triest *et al.*, 1995; Burnett, 1997; English and Baulcombe, 1997; Naitou *et al.*, 1997; De Rocher *et al.*, 1998; Diehn *et al.*, 1998), nuclear runoff transcription assays (Muellar *et al.*, 1995; English and Baulcombe, 1997; Kumpatla *et al.*, 1997; De Rocher *et al.*, 1998) and ribonuclease protection assays (Kumpatla *et al.*, 1997; Diehn *et al.*, 1998; Kumpatla and Hall, 1998). These will be described in brief.

RT-PCR is the technique of choice for analysing extremely low abundance mRNA derived from cells or tissues (Souaze *et al.*, 1996). The technique involves reverse transcription of target mRNA molecules to cDNA, followed by amplification of the cDNA by the polymerase chain reaction (PCR) (Dostal *et al.*, 1994). The exponential nature of PCR, however, makes it difficult to quantify transcript content because small variations in amplification efficiency often lead to dramatic changes in product yields (Sato *et al.*, 1994; Saric and Shain, 1997). This problem can be addressed by coamplifying a molecule of interest with an internal standard that has the same primer templates (Wang *et al.*, 1989).

Northern hybridisation, on the other hand, not only permits the qualitative analysis of a given mRNA but is also commonly used as a quantitative assay (Sambrook *et al.*, 1989; Triest *et al.*, 1995). This technique determines the size and abundance of specific mRNA molecules in preparations of total or poly(A)$^+$ RNA (Alwine *et al.*, 1977; Chevet *et al.*, 1995; Naitou *et al.*, 1997). However, Northern analysis is generally not very sensitive compared to RT-PCR (Ra and Saris, 1995). Ra and

Saris (1995) found that a specific transcription product could be detected by RT-PCR from as little as 2 μg of total RNA template, whereas 30 μg of RNA in a Northern blot failed to reveal the transcript. However, improvements can be achieved by using high specific activity antisense RNA probes and charged nylon transfer membranes (Ausubel *et al.*, 1988; Sambrook *et al.*, 1989).

Newly transcribed RNA can be identified using the nuclear runoff transcription assay (Groudine *et al.*, 1981; Greenberg and Ziff, 1984). This technique allows direct measurement and comparison of specific gene transcription in cells at various states of growth or differentiation. The runoff transcription assay is often used to assess whether changes in mRNA levels of a particular gene, that occur as a function of cell state, reflect a change in its synthesis as opposed to a change in mRNA degradation or transport from the nucleus to the cytoplasm (Marzluff, 1978). A point of controversy is whether some initiation of new RNA synthesis occurs in isolated nuclei during the runoff transcription reaction (Ausubel *et al.*, 1988).

In the final technique, namely ribonuclease protection assays, the steady-state levels of a particular mRNA can be detected (Kumpatla and Hall, 1998). This technique makes use of a single-stranded probe complementary to the sequence of mRNA to be measured (Ausubel *et al.*, 1988), with free probe being removed by ribonuclease treatment. Both the endpoint and the amount of a specific RNA can be determined. The most common problem encountered with this procedure is incomplete transcripts which may be caused by ribonuclease contamination or by pausing or termination of the RNA polymerase before completion of the transcript (Melton *et al.*, 1984).

The aim of the present investigation was to optimise two of the above mentioned techniques, namely RT-PCR and Northern blotting, to be used in later experiments concerning the characterisation of the UBI and SUC-1 promoters which both drive expression of the Bt gene. As mentioned, RT-PCR is an extremely sensitive technique and thus useful for the detection of rare transcripts, such as Bt mRNA, which might be from a single copy transgene in some transformants. Northern analysis is invaluable in terms of quantitating message abundance. These

techniques were chosen since they complement each other and thus a complete analysis of gene expression, with consequent deductions about promoter activity, could be obtained.

3.2 MATERIALS AND METHODS

3.2.1 Experimental plant material

Mature leaf tissue was obtained from 8-10 month old transgenic sugarcane plants (#7.20, #7.47, #7.33, #6.8, #6.12, #18.1) of the variety NCo310, maintained in the glasshouse at the South African Sugar Association Experiment Station. Fresh plant material was used for all experiments.

3.2.2 Precautions to prevent RNase contamination

To prevent RNase contamination from hands, gloves were worn at all times. Where possible, sterile disposable plasticware was used and nondisposable glassware and plasticware autoclaved. All buffers and reagents were sterilised and stored in small aliquots. Solutions were made up in diethyl pyrocarbonate (DEPC)-treated water prepared as follows: 1 ml DEPC was dissolved in 1 litre distilled water and incubated overnight at room temperature. The solution was then autoclaved for 30 min to remove any trace of DEPC.

3.2.3 RNA Isolation Protocols

3.2.3.1 SV Total RNA Isolation System (Promega)

Using a mortar and pestle, 100 mg leaf tissue was ground in liquid nitrogen. Ground tissue was transferred to a 1.5 ml microcentrifuge tube and 175 μl SV RNA lysis buffer plus 350 μl SV RNA dilution buffer added. After vortexing, the sample was placed in a 70°C waterbath for 3 min and then centrifuged at 12 000 rpm for 10 min.

The cleared lysate solution was carefully transferred to a new microcentrifuge tube by pipetting to avoid disturbing the pelleted debris. To the cleared lysate was added 200 μl 95% (v/v) ethanol and this was mixed by pipetting. The mixture was transferred to the spin column assembly and centrifuged at 12 000 rpm for 1 min. The liquid in the collection tube was discarded and 600 μl SV RNA wash solution added to the spin column. This was centrifuged at 12 000 rpm for 1 min. DNase

I (5 μl) and SV DNase incubation buffer (45 μl) were applied directly to the membrane inside the spin basket. The sample was then incubated for 15 min at room temperature (20-25°C). After the incubation, 200 μl SV DNase stop solution was added to the spin basket and the sample centrifuged at 12 000 rpm for 1 min. Thereafter, 600 μl SV RNA wash solution was added and the sample centrifuged at 12 000 rpm for 1 min. As a final wash, 250 μl SV RNA wash solution was added and the sample centrifuged for 2 min at 12 000 rpm. Finally, to elute the RNA, 100 μl RNase-free water was added directly to the membrane of the spin basket and the sample centrifuged for 1 min at 12 000 rpm.

The yield and concentration of total RNA obtained was determined spectrophotometrically at 260 nm, where 1 absorbance unit (A_{260}) equals 40 μg of single-stranded RNA/ml. The purity of the RNA was estimated from the A_{260}/A_{280} ratio.

The integrity of the purified RNA was determined by denaturing agarose gel electrophoresis with formaldehyde as the denaturing reagent (section 3.2.6).

3.2.3.2 RNeasy RNA Extraction Protocol (Quiagen)

Leaf samples were ground under liquid nitrogen to a fine powder using a mortar and pestle. Thereafter, 900 μl of RLT lysis buffer was added to 200 mg of tissue. After the reaction tube had been vortexed vigorously, the sample was incubated at 56°C for 3 min. The lysate was then applied to the QIAshredder spin column and centrifuged for 2 min at maximum speed. The flow-through fraction from the QIAshredder was transferred to a new 2 ml collection tube and approximately 225 μl 95% (v/v) ethanol added to the lysate and mixed by pipetting. The sample was applied onto an RNeasy mini spin column and thereafter centrifuged for 15 sec at 10 000 rpm. To wash the sample, 700 μl RW1 wash buffer was pipetted onto the RNeasy column and the sample centrifuged for 15 sec at 10 000 rpm. The RNeasy column was then transferred to a new 2 ml collection tube and 500 μl RPE wash buffer added. This was centrifuged for 15 sec at 10 000 rpm. A further 500 μl RPE wash buffer was added to the RNeasy column and centrifuged for 2 min in order to dry the membrane. The RNeasy column was finally transferred to a new 1.5 ml

collection tube and 50 µl RNase-free water added directly to the membrane. To elute the RNA, the sample was centrifuged for 1 min at 10 000 rpm.

Yield and purity of RNA obtained were determined as described in section 3.2.3.1.

3.2.4 Enzyme Treatments

3.2.4.1 DNase I treatment

The following were added to an RNase-free 0.5 ml microcentrifuge tube on ice: 1 µg RNA sample, 1 µl 10 x DNase I reaction buffer (200 mM Tris-HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl), 1 µl DNase I (AMP Grade, 1 U/µl; Gibco.BRL) and DEPC-treated water to a total volume of 10 µl. The reaction tube was incubated for 15 min at room temperature. To inactivate the DNase, 1 µl 25 mM EDTA was added and the reaction heated for 10 min at 65°C.

3.2.4.2 RNase A treatment

RNA samples were digested for 20 min with 2 µl RNase A (1 U/µl) at 42°C, as reported by Ra and Saris (1995).

3.2.5 RT-PCR Protocols

3.2.5.1 GeneAmp RNA PCR System (Perkin Elmer)

RT-PCR reactions were performed in a final volume of 50 µl. A master mix for reverse transcription was prepared for final concentrations of 5 mM $MgCl_2$, 1 x PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1 mM of each dNTP, 1 U/µl RNase inhibitor, 2.5 U/µl MuLV reverse transcriptase, 2.5 µM oligo $d(T)_{16}$ (5'-TTTTTTTTTTTTTTTT-3') and 1 µg RNA template. To control sample evaporation, 30 µl mineral oil was added to all tubes. Reaction tubes were allowed to incubate at room temperature for 10 min. Incubations in a Hybaid OmniGene Thermal Cycler were as follows: 15 min at 42°C; 5 min at 99°C and 5 min at 5°C (or on ice).

For PCR amplification, final concentrations of the following components were added to the above reaction tubes: 2 mM $MgCl_2$, 1 x PCR Buffer II, 2.5 U/100 µl *AmpliTaq* DNA polymerase and 0.15 µM each of the forward primer StoxF (5'-CGA TAT TTC CTT GTC GCT AAC G-3') and the reverse primer StoxR (5'-CGT TCT

AAT CCC GTA TTG TAC C-3'). Reaction tubes were spun for 30 sec in a microcentrifuge and the following temperature cycles carried out in a Hybaid OmniGene Thermal Cycler: 1 cycle of 2 min at 94°C; 40 cycles of 30 sec at 94°C, 45 sec at 50°C, 3 min at 72°C; 1 cycle of 8 min at 72°C. Samples were stored at 4°C until needed. Figure 3.1 provides an illustration of the binding sites for primers StoxF and StoxR.

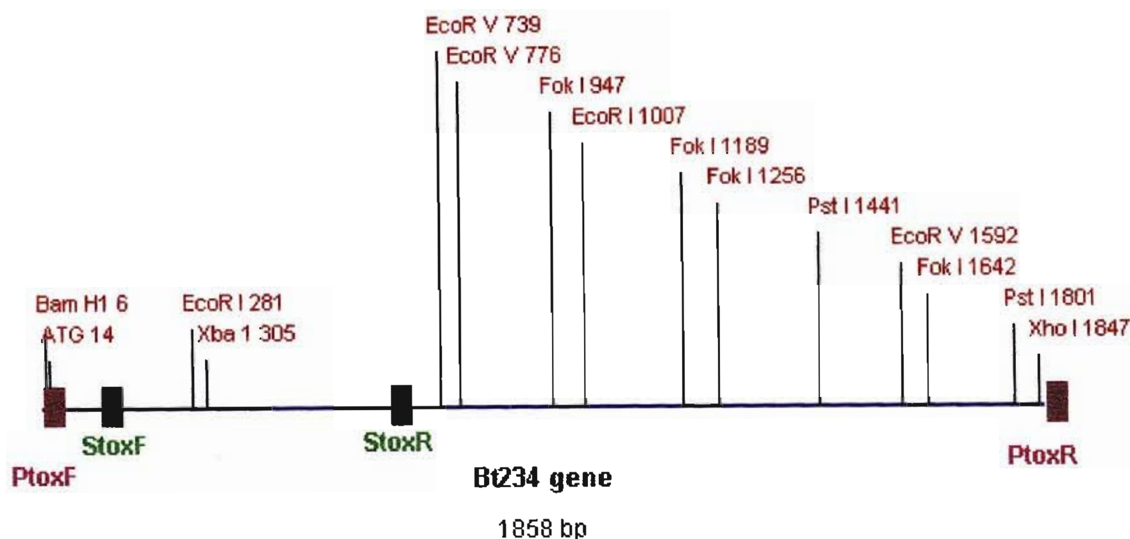


Figure 3.1: Schematic diagram of Bt234 gene showing the binding sites for primers StoxF and StoxR, as well as PtoxF and PtoxR. Primers StoxF and StoxR amplify a 543 bp fragment, while primers PtoxF and PtoxR (section 3.2.7), amplify the entire Bt gene.

3.2.5.2 Titan One Tube RT-PCR System (Boehringer Mannheim)

For the RT-PCR reaction, two separate master mixes were prepared in final volumes of 25 μl each. The first master mix consisted of final concentrations of 5 mM DTT, 0.2 mM of each dNTP, 10 U RNase inhibitor, 0.4 μM each of primers StoxF and StoxR and 1 μg RNA template. The second master mix contained 5 x RT-PCR Buffer (1.5 mM $MgCl_2$) and 1 μl enzyme mix (AMV and Expand^{TM} High Fidelity PCR System). Each master mix (25μl) was added to a labeled PCR tube and 30 μl mineral oil overlaid to prevent evaporation of the sample. The following temperature cycles were then carried out in a Hybaid OmniGene Thermal Cycler (programme 29 on card HB-TR3-MPC): 1 cycle of 30 min at 50°C; 1 cycle of 2 min at 94°C; 10 cycles of 30 sec at 94°C, 30 sec at 52°C, 2 min at 68°C; 25 cycles of 30 sec at 94°C, 30 sec at 52°C, 2 min 5 sec at 68°C; 1 cycle of 7 min at 68°C. Samples were stored at 4°C until needed.

3.2.5.3 Two Step Reverse Transcription and PCR

For reverse transcription, primer StoxR (30 pmoles) and 1 μg RNA sample were boiled together for 5 min and thereafter placed on ice. The following components were then added to each reaction tube in a final volume of 12 μl: 100 mM DTT, 1 x Expand™ Reverse Transcriptase Buffer (Boehringer Mannheim), 10 mM of each dNTP, 20 U/μl RNase inhibitor and 50 U/μl Expand™ Reverse Transcriptase. Tubes were incubated at 42°C for 60 min. They were then kept at 95°C for 2 min and thereafter immediately put on ice.

PCR was carried out in a final volume of 20 μl by adding the following reagents to 4 μl of the above reverse transcription reaction mixture: 1 x PCR Buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3), 0.2 mM of each dNTP, 1 mg/ml bovine serum albumen, 0.4 μM each of primers StoxF and StoxR and 1 U Taq DNA polymerase. The following temperature cycles were carried out in a Hybaid OmniGene Thermal Cycler: 1 cycle of 1 min at 94°C; 10 cycles of 25 sec at 94°C, 30 sec at 45°C, 4 min at 72°C; 30 cycles of 15 sec at 94°C, 30 sec at 40°C, 1 min at 72°C; 1 cycle of 2 min at 72°C.

3.2.6 Fractionation of RNA by Agarose-Formaldehyde Gel Electrophoresis

A 1.2% (w/v) agarose gel was prepared in 1 x TBE buffer. Each RNA sample was 15 μg, while 10 μl of a 0.24-9.5 kb RNA ladder (GibcoBRL) was used. The following denaturing reagents were then added to each sample: 10 μl formamide, 4.5 μl formaldehyde (12.3 M), 5 μl 5 x MOPS buffer [0.2 M 3-(N-morpholino)-propanesulfonic acid pH 7.0, 0.5 M sodium acetate, 0.01 M EDTA] and 4 μl loading buffer (50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue). Samples were heated for 10 min at 65°C. Thereafter, 30 μl of each sample was loaded onto the gel, in duplicate (on either end of gel), and run at 60 V. Half of the gel, containing each sample plus the RNA ladder, was stained for 10 min in ethidium bromide (5 μg/ml). The remaining half, containing the duplicated samples, was used for Northern hybridisation (section 3.2.10).

3.2.7 Probe generation by PCR and purification

Primers PtoxF (5'-ATG GAG GAT CCC CAT GGA TAA CAA TCC GAA CA-3') and

PtoxR (5'- TTA TCA CTC GAG TGT TGC AGT AAC TGG AAT AA-3'), which amplify a 2.0 kb Bt fragment, were used during PCR of plasmid pLBRtox (figure 3.2) according to the protocol described in section 3.2.5.3. Amplification products (500 ng - 1 μg) were run at 60 V on a 1.2% (w/v) agarose gel, containing 0.5 x TBE and 5 μg/ml EtBr. After visualisation under UV light, the Bt band was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen) as described by the manufacturer. Thereafter, either the Prime-It II Random Primer Labeling Kit (Stratagene) or the Megaprime DNA Labeling System (Amersham) was used for the radioactive labeling of DNA (section 3.2.8).

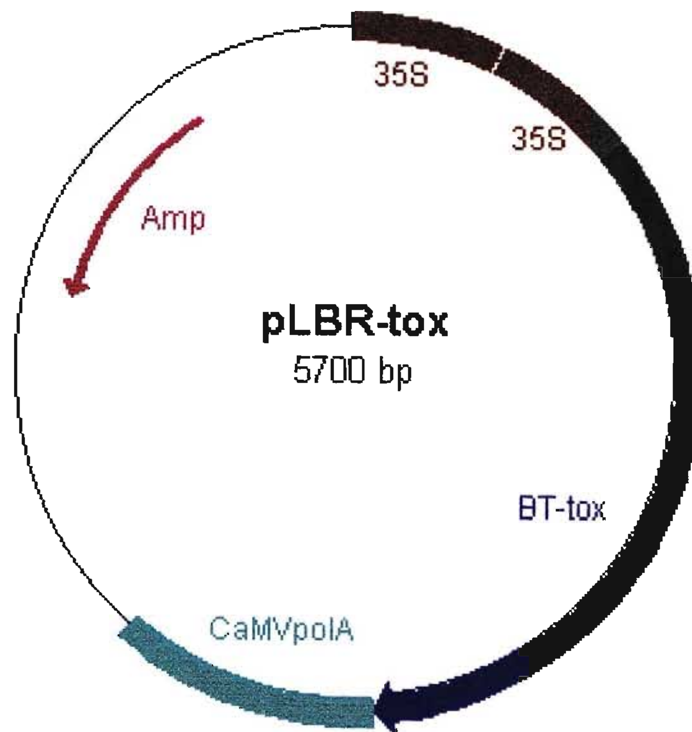


Figure 3.2: Schematic diagram of plasmid pLBRtox. The plasmid was constructed by inserting the 2 kb, Hpa I - Xho I, 5'-fragment of the Bt gene into the Sma I site of pLBR 19-1.

3.2.8 Probe Labeling

The following components were placed in a reaction tube: 20 ng DNA, 5 μl primer solution and DEPC-H_2O to a volume of 26 μl. The tube was then heated for 5 min in boiling water to denature the DNA. After a brief centrifugation (10 sec) the following components were added: 4 μl each of dGTP, dTTP and dCTP (10 mM each); 5 μl reaction buffer; 5 μl ^{32}P-labelled dATP and 2 μl Exo(-) Klenow enzyme (5 U/μl). Components were mixed thoroughly and the reaction incubated at 37°C

for 30 min. To stop the reaction, 5 µl 0.25 M EDTA was added.
Efficiency of the above probe-labeling reaction was checked by adding 200 µl TE buffer (pH 8.0) to the probe and dotting 1 µl of this onto a cellulose strip. The cellulose strip was then placed in a beaker containing 0.75 M NaH_2PO_4 and the liquid allowed to migrate approximately 10 cm up the strip. The cellulose strip was then placed in a Hypercassette (Amersham) and left at room temperature for 15 min to expose. Thereafter, the x-ray was developed as described in section 3.2.11

3.2.9 Measurement of Probe Specific Activity

To a reaction tube containing 100 µl of 500 µg/ml salmon sperm DNA, 2 µl probe was added and 10 µl of this spotted onto a glass microfibre filter (representing total radioactivity). To the remaining 90 µl, 1 ml ice-cold TCA was added and this incubated for 10 min on ice. The precipitate was collected by filtration through a second glass microfibre filter (representing incorporated radioactivity). The reaction tube was rinsed with 3 ml TCA and this poured through the filter. The filter was then washed four more times with 3 ml TCA, followed by 3 ml ice-cold ethanol. Both filters were then allowed to dry at room temperature and thereafter placed in separate vials containing 5 ml toluene-based scintillation fluid (Ultimate Gold). Radioactivity was measured in a liquid scintillation counter. Probe specific activity and percent incorporation of the radiolabel was calculated using the following formulae:

specific activity = (incorporated radioactivity - background)(volume labeled / volume counted)(1000 ng / ng labeled)

% incorporation = incorporated radioactivity / total radioactivity x 100%

3.2.10 Northern Hybridisation

A positively charged nylon membrane (Boehringer Mannheim or Amersham) and a 1.2% (w/v) denaturing agarose gel containing the RNA samples (section 3.2.6) were incubated in 10 x SSC for 20 min and thereafter downward capillary blotted (in 10 x SSC) by the method described by Chomczynski and Mackey (1994). RNA was bound to the nylon membrane by UV cross-linking (Hoefer Scientific Instruments, San Francisco) the RNA-containing side of the membrane at 120 000 microjoules per cm^2 for 2.5 min. The positively charged nylon membrane was then placed in a hybridisation bottle and 10 ml prehybridisation buffer added. Three

buffers were used, namely 1) Rapid-Hyb (Amersham), 2) a formulation containing 50% (v/v) deionised formamide, 5 x SSC, 5 x Denhart's solution, 200 µg denatured salmon sperm DNA, 0.1% (w/v) SDS and 3) a formulation containing 0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA, 7% (w/v) SDS. The membrane was prehybridised at either 42 or 65°C for 4, 5 or 24 h periods. Probes were boiled for 5 min and thereafter added to the membrane. Hybridisation occurred over 24 h at either 42 or 65°C.

To prevent non-specific hybridisation, wash solutions [from 2 x SSC (0.3 M NaCl and 30 mM Na_3citrate, pH 7.0) to 0.2 x SSC; 0.1% (w/v) SDS] were kept at 65°C before beginning, as well as during washes. After the incubation period, the hybridisation solution was discarded and the membrane washed briefly at room temperature. The membrane was then washed for 20 min at either 42 or 65°C at low stringency and thereafter, if necessary, for 20 min at either 42 or 65°C at high stringency. Radioactivity was monitored with a Geiger Counter.

3.2.11 Autoradiography

The membrane was sealed in a plastic bag and placed, together with x-ray film (Hyperfilm-MP; Amersham), in a Hypercassette (Amersham). This was left to expose by storage at -80°C from 2 to 10 days. Thereafter, the film was processed by incubation in developer solution for 5 min, 1 min in stop solution (acetic acid) and 1 min fix solution. The film was then washed with water and allowed to dry.

3.3 RESULTS AND DISCUSSION

3.3.1 Development of reliable RNA extraction and RT-PCR protocols

RNA extraction. At the outset of the study, it was essential to develop a reliable extraction protocol for the isolation of high quality RNA to be used in RT-PCR and Northern analysis. Initially, the RNeasy Plant Mini Kit (Quiagen) was used to extract RNA from mature leaves of variety NCo310 transgenic plants. Samples used were #7.20, #7.47 and #7.33, containing the Bt gene driven by the SUC-1 promoter and #6.12, #6.8 and #18.1, containing the Bt gene driven by the UBI promoter. The extracted RNA was quantified spectrophotometrically. Results showed that RNA yield was high. Figure 3.3 represents a typical RNA absorption

spectrum.

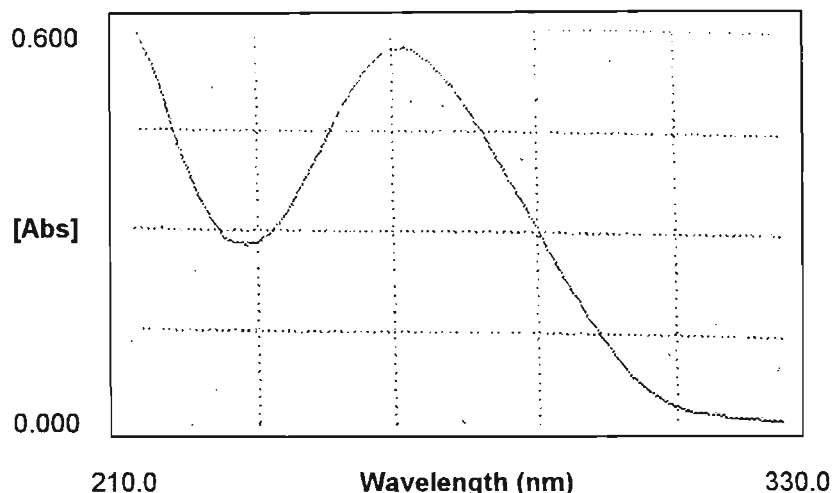


Figure 3.3: Typical absorbance profile for RNA extracted from sugarcane plants using the RNeasy Plant Mini Kit. A_{260} = 0.4972 and A_{280} = 0.2846.

The absorbance at 260 nm (A_{260}) was used to calculate the concentration of RNA. Since 1 absorbance unit equals 40 μg of single-stranded RNA/ml, and the absorbance of the sample in figure 3.3 at 260 nm was 0.4972, the concentration was calculated as follows:

Concentration of RNA = A_{260} x 40 x dilution factor

= 0.4972 x 40 μg/ml x 20

= 397.76 μg/ml

Purity of the RNA was calculated by taking the ratio of A_{260}/A_{280}

Purity = A_{260}/A_{280}

= 0.4972 / 0.2846

= 1.7

This indicates that the RNA was relatively pure, since the purity of uncontaminated RNA is approximately 2. Agarose gel electrophoresis of the isolated RNA confirmed the undegraded nature of the samples (figure 3.4).

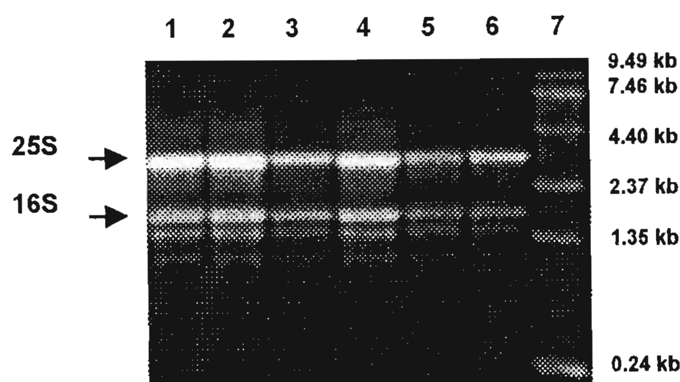


Figure 3.4 Agarose gel electrophoresis of total RNA from various transgenic plants. Lanes: (1) #7.20, (2) #7.47, (3) #7.33, (4) #6.8, (5) #6.12, (6) #18.1, (7) RNA molecular weight marker. Distinct 25S and 16S ribosomal bands (absence of smearing) indicate that the RNA was non-degraded.

Initial RT-PCR products. Using the Titan One Tube RT-PCR System, RT-PCR was performed on all samples. As a negative control, PCR without prior reverse transcription of samples was also performed. A band of approximately 543 bp was expected after RT-PCR (refer to figure 3.1). Since the polymerases used during PCR require a double-stranded template, no band was expected after PCR. Four samples showed an amplification product and thus appeared to be contaminated with DNA. The results showed a 543 bp band after PCR for samples #7.20, #7.47, #7.33 and #6.12 (figure 3.5).

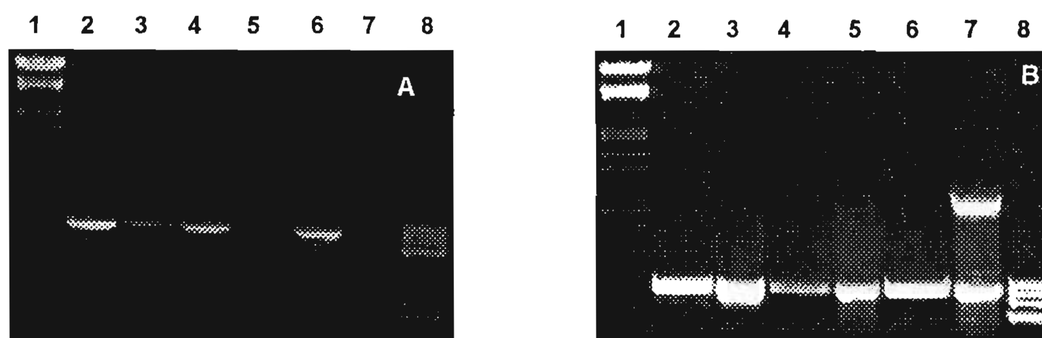


Figure 3.5: Agarose gel electrophoresis of amplified PCR (A) and RT-PCR (B) products generated from transgenic plant RNA extracts using primer pair StoxF / StoxR. (A) Lanes: (1) DNA molecular marker III, (2) to (7) RNA from plants #7.20, #7.47, #7.33, #6.8, #6.12 and #18.1 respectively, (8) DNA molecular marker V; (B) Lanes: as for (A). A band of approximately 543 bp was expected after RT-PCR, with no band after PCR.

DNase I and RNase A treatment of templates. To test whether the results in figure 3.5 were due to DNA contamination, all samples were treated with either RNase A or DNase I prior to RT-PCR using the Titan kit. Uncontaminated RNA samples treated with RNase A were not expected to produce a band after RT-PCR as the enzyme should have destroyed the RNA. If a band was observed, it would indicate DNA contamination. In contrast, a band was expected for samples treated with DNase I since this enzyme should not degrade RNA but destroy DNA. As a positive control, RT-PCR was performed on non-enzyme treated samples. Figure 3.6A shows the results obtained. Samples #6.12 and #18.1 gave the expected results, while sample #7.20 produced no bands after all 3 treatments. Samples #7.33 and #6.8 produced no bands after RNase- and DNase treatments, but did produce one after the no enzyme treatment. Sample #7.47 did the opposite to what was expected by producing a band after RNase treatment and no band after DNase treatment.

To test whether the results shown in figure 3.6A were reproducible, RT-PCR was repeated on DNase-, RNase- and no enzyme treated samples using the Titan RT-PCR kit. This time bands were observed for RNase treated samples of #7.20, #7.47, #6.8 and #18.1 (figure 3.6B). None of the DNase treated samples produced bands. This proved that the results were unpredictable.

RNA extraction protocol as a source of contamination. A possible cause of sample contamination with DNA was the RNeasy spin columns, provided with the RNeasy Plant Mini Kit, being overloaded by the use of too much plant material during RNA extraction. Three extractions were done using sample #6.8 with i) 200 mg, ii) 100 mg and iii) 50 mg leaf tissue. Thereafter, RT-PCR and PCR were performed. The 200 mg and 50 mg samples produced the expected band after RT-PCR and no band after PCR, but the 100 mg sample produced a slight band after RT-PCR and a darker band after PCR (figure 3.7A). This suggested that DNA contamination was present in that sample. The above experiment was repeated to confirm the result. This time only the 50 mg sample appeared uncontaminated. The 200 mg and 100 mg samples produced bands after RT-PCR and PCR (figure 3.7B). Since results were not reproducible, inconsistent purification of the sample RNA during extraction was suspected and the suspicion of column overload was confirmed.

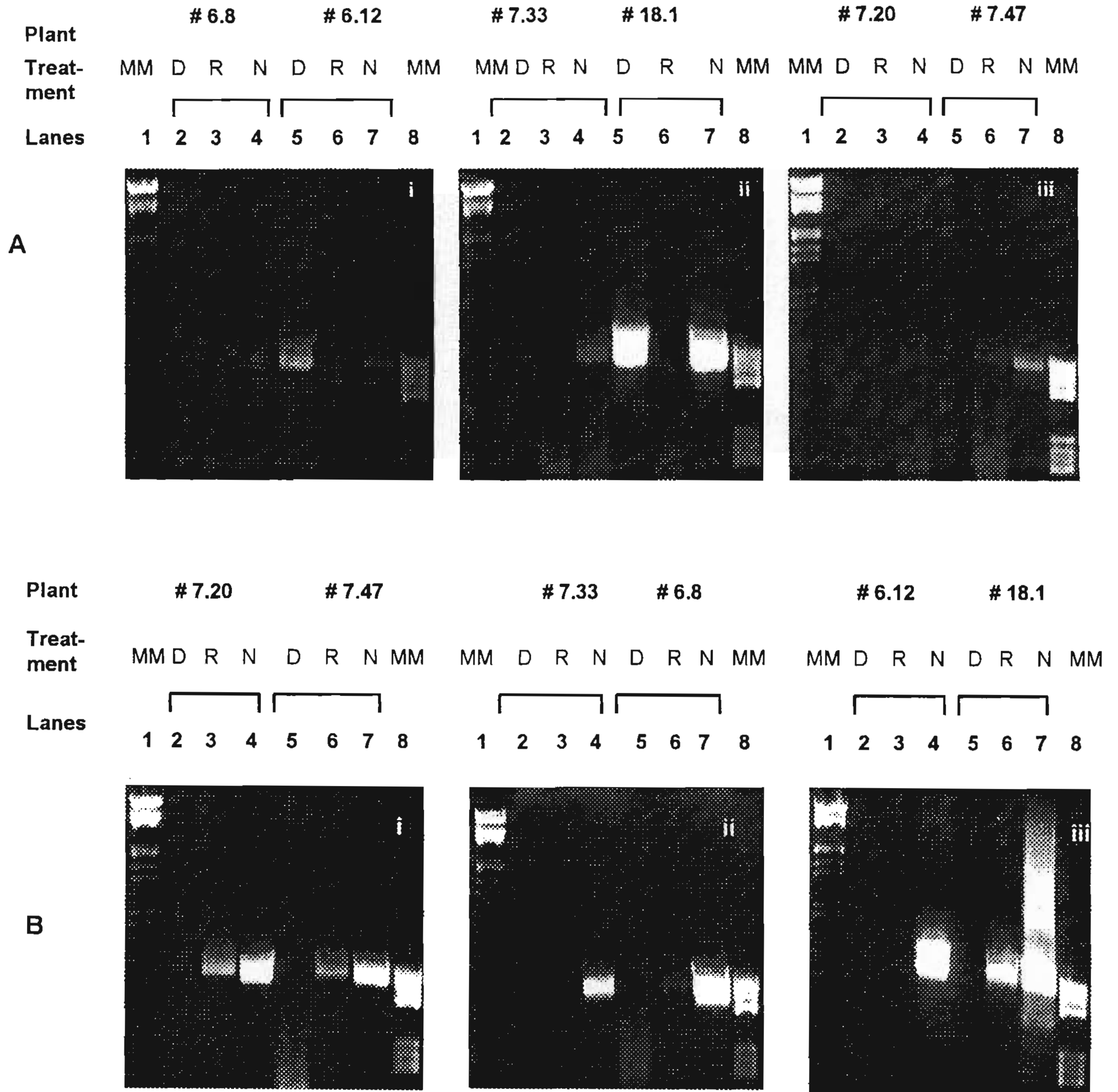


Figure 3.6: Effect of DNase I and RNase A treatment of RNA extract on RT-PCR. Agarose gel electrophoresis of amplified RT-PCR products generated from plant RNA subjected to three treatments, namely DNase I (D), RNase A (R) and no enzyme treatment (N). MM represents the DNA molecular size markers. A 543 bp band after RNase treatment indicates DNA contamination of the RNA sample.

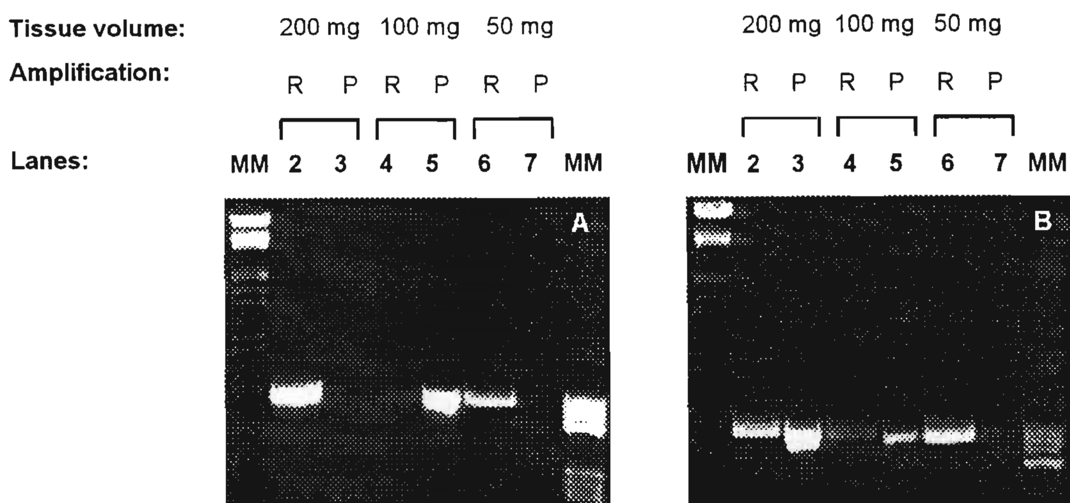


Figure 3.7: Effect of tissue load on DNA contamination of RNA extracts. Agarose gel electrophoresis was performed on amplified PCR (P) and RT-PCR (R) products after RNA extraction from sample #6.8 using the RNeasy Plant Mini Kit with varying amounts of starting material. A band of approximately 543 bp was expected after RT-PCR, with no band after PCR. (B) shows the results of the replicated experiment done in (A). MM refers to the DNA molecular weight marker.

Modification of RNA extraction protocol. In an attempt to eliminate sample contamination, the RNeasy RNA extraction protocol was modified by using 225 µl RPE wash buffer and washing four times, instead of twice with 500 µl RPE as recommended in section 3.2.3.2. Sample #6.8 was used and i) 100 mg tissue using the protocol provided with the kit, ii) 100 mg tissue using the modified protocol and iii) 200 mg tissue using the modified protocol tested. Thereafter, RT-PCR and PCR were performed. Results showed that all samples had DNA contamination, with samples obtained after using the modified protocol being more contaminated than those obtained using the protocol provided with the kit (figure 3.8). This proved that an alternate protocol for RNA extraction was required in order to obtain DNA-free RNA.

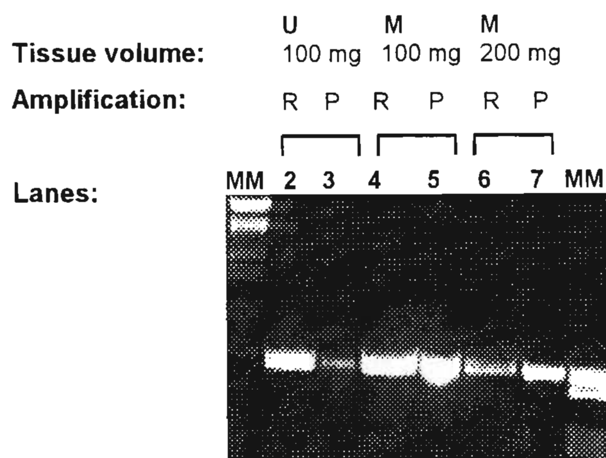


Figure 3.8: Agarose gel electrophoresis of amplified PCR (P) and RT-PCR (R) Bt products after RNA extraction from sample #6.8 using the RNeasy Plant Mini Kit with varying amounts of starting material and having modified the extraction protocol. (U) refers to the unmodified protocol and (M) refers to the modified one.

Revised RNA extraction protocol. After an extensive catalogue search for an extraction protocol capable of producing pure, high quality RNA, the SV Total RNA Isolation System (Promega) was chosen. RNA was extracted from samples #7.20, #7.47, #7.33, #6.8, #6.12 and #18.1. Figure 3.9 shows the results obtained after RT-PCR and PCR reactions. All samples produced the expected 543 bp band after RT-PCR and no band after PCR. The slight band observed in lane 13 of figure 3.9 was suspected to be spill-over from the RT-PCR product during gel loading since the result was not reproducible upon re-running of the gel. Yields with this kit were not as high as with the RNeasy kit, but the samples appeared completely uncontaminated. In addition, results were consistent.

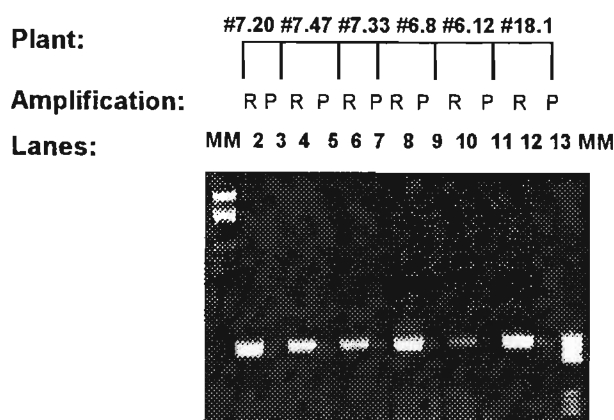


Figure 3.9: Agarose gel electrophoresis of amplified RT-PCR (R) and PCR (P) products generated from transgenic plant RNA extracted using the SV Total RNA Isolation Kit.

RT-PCR protocol. During RT-PCR optimisation, the efficacy of the Titan RT-PCR kit was tested by comparing results obtained using this kit with results obtained when using a different one, namely the GeneAmp RNA PCR Kit (Perkin Elmer). Samples tested were #7.20, #7.47, #7.33 and a positive control (pAW109) provided with the GeneAmp kit. Every sample failed to produce a band after all 3 treatments. To test whether the enzymes from the GeneAmp RT-PCR kit were inactive, pAW109 was used during RT-PCR using both GeneAmp and Titan kits. While the GeneAmp kit failed to produce a band, the Titan kit did produce one (figure 3.10). This confirmed the suspicion of enzyme inactivity.

Figure 3.10: Agarose gel electrophoresis of amplified RT-PCR product generated from sample pAW109 (positive control RNA from the GeneAmp RT-PCR kit) using either the GeneAmp- or Titan One Tube RT-PCR kits. Lanes: (1) DNA molecular marker III, (2) RT-PCR product after using the GeneAmp RT-PCR kit, (3) RT-PCR product after using the Titan RT-PCR kit, (4) DNA molecular marker V.

Separate reverse transcription, using Expand Reverse Transcriptase (Boeringher Mannheim), followed by PCR was also attempted during RT-PCR optimisation. The results obtained were identical to those obtained when using the Titan One Tube RT-PCR System.

These results proved that the Titan One Tube RT-PCR System was reliable and, as such, this kit was chosen for further analysis of mRNA. Since DNA-free RNA is a prerequisite for RT-PCR, the SV Total RNA Isolation System was chosen for the extraction of RNA to be utilised during this technique. In addition to the requirement for large quantities of RNA, Northern analysis is not as sensitive to DNA contamination as RT-PCR. For this reason the RNeasy Plant Mini kit was chosen for the extraction of RNA to be utilised during this technique.

3.3.2 Optimisation of Northern Analysis

Initial Northern protocol. The first protocol used for Northern analysis made use of a positively charged nylon membrane (Amersham), prehybridised in Rapid-Hyb buffer (Amersham) at 42°C for 4 hours. The probe, labeled using the Prime-It II labeling kit (Stratagene), was added directly to the prehybridisation solution and incubated overnight at 42°C. A high stringency wash was used to remove unbound probe, namely 1 x SSC; 0.1% (w/v) SDS at room temperature, followed by 0.2 x SSC; 0.1% (w/v) SDS for 20 min at 68°C. No bands were obtained (results not shown). One possibility is that the washes were too stringent.

Table 3.1 illustrates the parameters that were changed, the source of the materials and the results obtained for each protocol attempted during the optimisation of Northern analysis. Four protocols were used, all making use of the downward capillary blotting technique described by Chomczynski and Mackey (1994). All four protocols made use of a positively charged nylon membrane and all used a random labeling reaction to radioactively label the double-stranded DNA probe.

Prehybridisation temperature and stringency of washes altered. Protocol 2 used the same membrane (Amersham) and prehybridisation solution (Rapid-Hyb buffer) as the initial protocol. Temperature of prehybridisation was, however, raised to 65°C for 4 hours. Probe labeling was as before, but the hybridisation temperature was raised to 65°C. The membrane was washed with 2 x SSC; 0.1% (w/v) SDS at 65°C for 20 min. Two distinct ribosomal RNA bands (25S and 16S) were obtained (figure 3.11), indicating non-specific hybridisation. The Bt band was not observed.

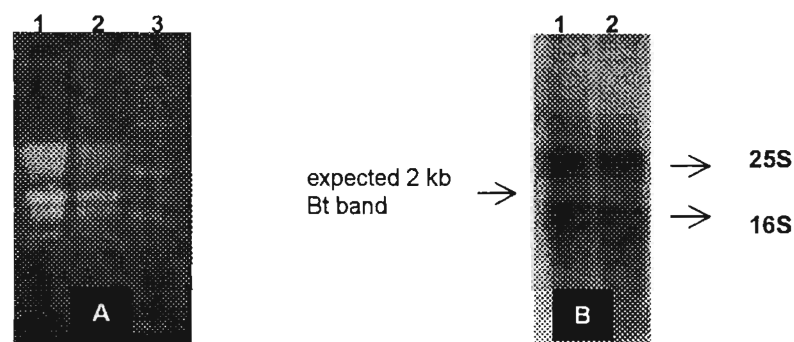


Figure 3.11: Result of agarose gel electrophoresis (A) and Northern blot after Bt probing (B) of total RNA using protocol 2. Lanes: (1) #7.20, (2) #6.8, (3) RNA ladder.

Table 3.1: Parameters varied in Northern optimisation

Protocol number	Prehyb. buffer	Prehyb/Hyb. incubation temperature (°C)	Prehyb. incubation time (h)	Wash solutions and temp.	N^+ membrane source	Probe labeling kit source	Result
1 (initial)	Rapid-Hyb	42	4	1xSSC; 0.1% SDS and 0.2xSSC; 0.1% SDS at 68°C	Amersham	Prime-It II	no bands
2	Rapid-Hyb	65	4	2xSSC; 0.1% SDS at 65°C	Amersham	Prime-It II	25S and 16S rRNA bands
3	50% deionised formamide, 5xSSC, 5xDenhart's solution, 200µg denatured salmon sperm DNA, 0.1% SDS	42	5	2xSSC; 0.1% SDS and 0.5xSSC; 0.1% SDS at 42-65°C	Amersham	Prime-It II	25S and 16S rRNA bands
4 (final)	0.36M Na_2HPO_4, 0.14M NaH_2PO_4, 1mM EDTA, 7% SDS	65	24	2xSSC; 0.1% SDS and 0.5xSSC; 0.1% SDS at 65°C	Boeringher Mannheim	Mega-prime	Bt band of 2 kb

Formulation of prehybridisation solution changed. The third protocol attempted during Northern optimisation made use of a different solution for prehybridisation. A formulation consisting of 50% (v/v) deionised formamide, 5 x SSC, 5 x Denhart's solution, 200 μg denatured salmon sperm DNA and 0.1% (w/v) SDS was used. Prehybridisation for 5 h and hybridisation overnight were at 42°C. Wash solutions used were 2 x SSC; 0.1% (w/v) SDS and 0.5 x SSC; 0.1% (w/v) SDS at 42 and 65°C respectively. Once again, non-specific hybridisation to the 25S and 16S ribosomal RNAs were obtained (results not shown). As for protocols 1 and 2, no Bt band was evident.

Final Northern hybridisation protocol. The final protocol made use of a positively charged nylon membrane obtained from Boehringer Mannheim. The prehybridisation solution was changed to 0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA and 7% (w/v) SDS. Prehybridisation incubation was increased to 24 h. Prehybridisation and hybridisation temperatures were also increased to 65°C. The probe was labeled using the Megaprime Labeling System (Amersham). Wash solutions used were 2 x SSC; 0.1% (w/v) SDS and 0.5 x SSC; 0.1% (w/v) SDS for 20 min each. This protocol proved successful, producing a Bt band of the expected 2.0 kb (figure 3.12) for all samples except #18.1, which appeared contaminated with DNA. The nontransgenic plant (lane 4) produced no bands, confirming the expression of Bt mRNA in the other samples.

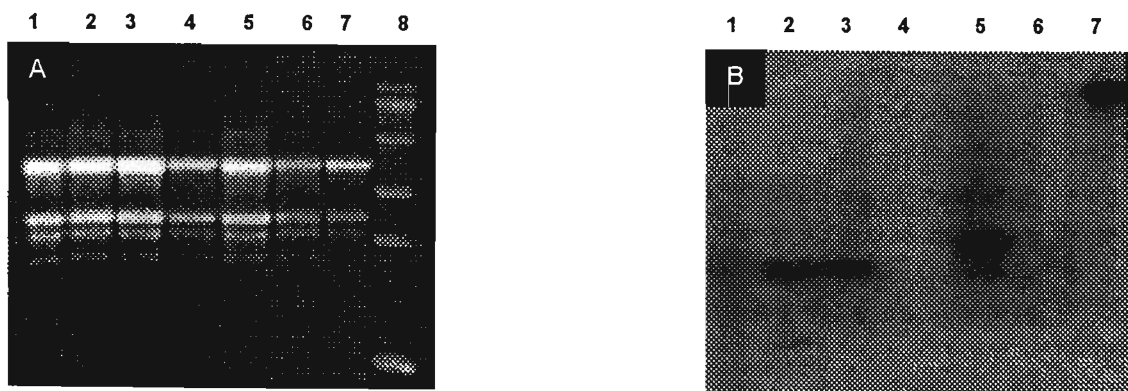


Figure 3.12: Optimised protocol for Northern analysis of Bt gene transcription. Agarose gel electrophoresis (A) and Northern hybridisation (B) of total RNA. The Northern Blot is an identical copy of the denaturing agarose gel. Lanes: (1)#7.20, (2) #7.33, (3) #7.47, (4) negative control (nontransgenic), (5) #6.8, (6) #6.12, (7) #18.1, (8) RNA molecular weight marker.

3.4 CONCLUSION

Figure 3.13 is an illustration of the final protocols for RNA isolation, RT-PCR and Northern analysis, obtained after optimisation of these techniques.

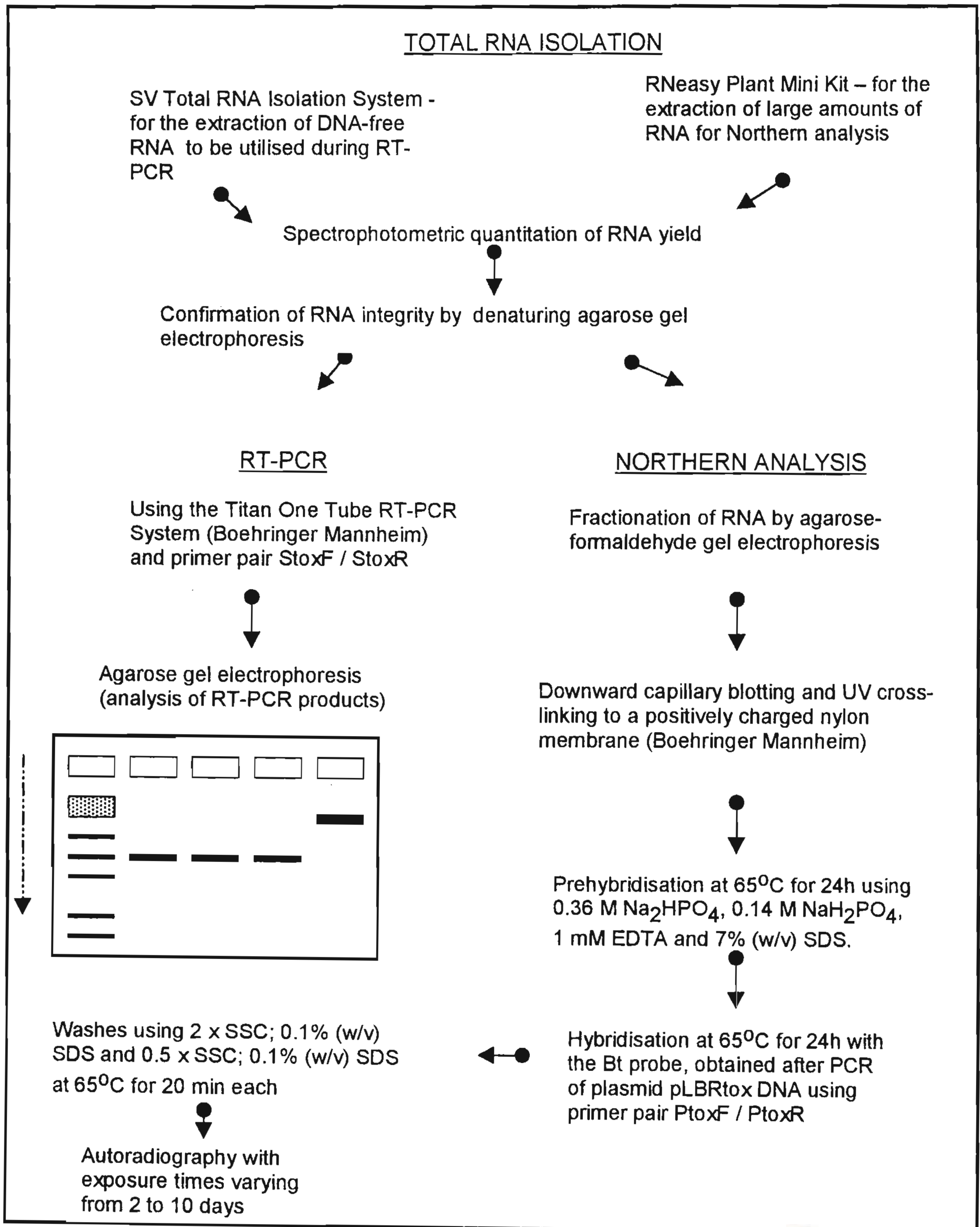


Figure 3.13: Final RNA isolation, RT-PCR and Northern hybridisation protocols.

CHAPTER 4: DEVELOPMENT OF SUITABLE WATERING REGIMES FOR THE INDUCTION OF PLANT STRESS

4.1 INTRODUCTION

The primary objective of the investigation reported in this chapter was to create a sufficient degree of stress in transgenic sugarcane plants containing the Bt gene in order to study the stress response of UBI and SUC-1 promoters. This would necessarily involve developing suitable techniques for the physiological measurement of stress in the plants. Water was chosen as the parameter to vary in the induction of plant stress since it is well documented that water stress is one of the most significant environmental factors limiting crop yield (Larson and Eastin, 1970; Viets, 1970; Schonfeld *et al.*, 1988; Bohnert, Nelson & Jensen, 1995; Shinozaki & Yamaguchi-Shinozaki, 1997). In addition, it is one of the easier stress inducing factors to control under glasshouse conditions.

The significance of water for crop growth is manifold (Cosgrove, 1986). Water is the medium required for the maintenance of living structures and for the many biochemical reactions taking place in a plant. An adequate water supply is also required to maintain turgor pressure, which in turn is responsible for providing firmness to the plant and which is the motor for extension growth of cells. Circulation of water through the plant enables transport of nutrients, assimilates and hormones from root to shoot and vice versa. Transpiration of leaves contributes to the reduction of leaf temperature under conditions of high radiation and high air temperature (Cosgrove, 1986). This chapter focuses on the establishment of suitable watering regimes, as well as techniques used to measure the stress response of transgenic sugarcane plants.

Kramer and Brix (1965) listed five desirable characteristics of a method for measuring plant stress. These characteristics were all taken into account when choosing methods to measure water stress of the sugarcane plants in the present study. Among these were requirements that the results obtained should correlate well with rates of physiological processes, that the stress measurement should be applicable to a wide range of plants and soil with the same degree of significance, and that the method should be simple and inexpensive and require a minimum

amount of tissue. Three techniques considered to be likely candidates, namely relative water content (RWC) determination, infra red gas analysis (IRGA) and leaf elongation measurements, were compared in this study.

4.1.1 Relative water content (RWC) determination

RWC determination is a simple technique and a minimum amount of equipment is involved. It is a laboratory method, but, if properly handled, field samples can be transported short distances. During RWC determination, the fresh weight of leaves are compared to their weight after standing in water in a humid container (turgid weight). Dry weight is then measured after the leaves have been dried out in an oven.

Difficulties arise in interpreting RWC measurements since they may change in relative meaning with plant age, tissue part, season and plant species (Knipling, 1967). Errors may arise from infiltration of water into the cut edges and intercellular spaces, cell growth may occur during saturation time, and weight may be lost due to respiration (Weatherley, 1950). The temperature at which the determination is made may also cause marked effects (Millar, 1966). Thorough blotting of the wet leaf pieces may also be difficult in some cases.

4.1.2 Infra red gas analysis (IRGA)

Infra red gas analysis of carbon dioxide is the most widespread contemporary method of determining photosynthetic and respiratory CO_2 exchange in plants. Its popularity stems from the reliability, accuracy and simplicity of this technique compared to others. The principle of IRGA is based on the fact that heteratomic gas molecules, i. e. gas molecules composed of two different atoms, absorb radiation at specific sub-millimeter infra red wavebands, each gas having a characteristic absorption spectrum. Gas molecules consisting of two identical atoms (e.g. O_2 and N_2) do not absorb infra red radiation and thus do not interfere with determination of the concentration of heteratomic molecules (Coombs *et al.*, 1985).

An infra red gas analyser consists of three basic parts, namely, an infra red source, a gas cell and a detector. The rate of CO_2 assimilation by a leaf enclosed in the

gas cell is determined by measuring the change in the CO_2 concentration of the air flowing across the chamber. CO_2 in the gas cell will decrease the radiation reaching the detector, causing a decrease in detector output signal.

Measurement of CO_2 uptake provides an alternative and direct method of measuring the reduction in productivity associated with stress, with important advantages over measurements of dry weight change. It is instantaneous, non-destructive, allows separate investigation of individual leaves and allows separation of photosynthetic gain from respiratory losses (Coombs *et al.*, 1985). IRGA has also been used for a wide range of heteratomic gas molecules, including H_2O, NH_3, CO, N_2O, NO and gaseous hydrocarbons.

4.1.3 Leaf elongation measurements

Reduced or suppressed growth, deviating from what is expected judging by past performance, is the basic response of a plant to an adverse or stress situation and the first indication that something is wrong (Treshow, 1970). Growth responses, however, are often slight and difficult to detect. The normal growth rate must be known before any suppression can be recognised. The amount of growth a plant should make also depends a great deal on the opinion of the observer.

The rate of growth in length may be observed by making measurements of the lengths of whole plants or plant members, or of marked zones in growing members (Thomas, 1960). As may be readily observed, growth in length runs its course in a characteristic way. For any plant or part of a plant, at any time, there is a maximum rate of elongation, determined by inherited genetical factors, which cannot be exceeded whatever the environmental conditions may be. At any time in the grand period, external conditions affect the rate of growth by determining how near to or how far from the inherent maximum rate for that time the actual growth rate will be. Consistently favorable external conditions over a whole growing season, a rare occurrence in nature, would cause the average rate to approach the inherent maximum and relatively tall specimens of the species would be produced. Conversely, consistently unfavorable conditions would stunt growth.

4.1.4 Factors affecting the interpretation of water stress experimental data

Much controversy has arisen in the past over the interpretation of experimental results, as insufficient attention has been paid to the criteria used for measuring the responses of plants to soil moisture conditions (Salter and Goode, 1967). Care is therefore needed when comparing the results of experiments not only to draw a clear distinction between the different effects of soil moisture stress on plant processes such as transpiration and growth, but also to differentiate between vegetative growth, total yield and marketable yield. Distinction has also to be made between immediate effects on perennial crops and long-term effects; for example, an immediate effect may be to increase or reduce vegetative growth without an apparent immediate effect on the fruiting of apple trees, but the long-term effect on cropping may be directly related to the growth effect.

One further aspect relating to the measurement of plant processes concerns the time when the measurement is made (Salter and Goode, 1967). The rate of many processes fluctuates throughout the day as a result of diurnal changes in the moisture content of the tissues caused by internal water deficits developing in the plant. Such fluctuations need to be borne in mind when interpreting experimental results, so that the real effects of the soil moisture treatments are not obscured or confused by these transient changes. The above mentioned factors were all taken into account during the measurement of plant stress in the present study.

4.2 MATERIALS AND METHODS

4.2.1 Experimental plant material

Plants were maintained in the glasshouse at the South African Sugar Association Experiment Station in Mt Edgecombe. Experimental material consisted of non-transgenic, as well as transgenic (#7.20, #7.47, #7.33, #6.8, #6.12, #18.1) NCo310 plants. Plant sizes ranged from 500-600 mm (small plants), 1.0-1.5 cm (medium plants) and 2.5-3.0 m (large plants). The feeding regime during plant maintenance, prior to water stress experiments, was as follows: 1.5 g urea plus 1.5 g hydroponic nutrient mix (9.1% N, 2.6% P, 12.2% K, 9.6% Ca, 2.6% Mg, 4.4% S, 0.3% Fe, 0.024% Mn, 0.024% Zn, 0.005% Cu, 0.10% B, 0.10% Mo). Plants were fertilised once a week. They were sprayed every two weeks with 2 ml/l Dursban 2E which is an insecticide containing chlorpirifos (240 g/l) and chlorpyrifos (240 g/l) as active

ingredients. The temperature in the glasshouse was maintained at 27-32°C.

4.2.2 Watering systems

During routine maintenance, a spraying system was used for plants in small (90 mm) and medium (175 mm) pots, while a drip system was used for plants in large (35 cm) pots. Plants received water twice a day, at 7.30 am and 4 pm. During experiments, non-stressed (control) plants were watered manually, twice a day, at 7.30 am and 4 pm. Plants in large pots were given 500 ml per watering, while those in medium- and small-sized pots were given 250 ml per watering. Stressed plants were not given any water (except for RWC determination where there were different degrees of water stress; see 4.3.1.1). Light and air temperature were the same for control and stressed plants. Water availability was the only factor that differed.

4.2.3 Stress measurements

4.2.3.1 Relative Water Content (RWC) determination

RWC measurements were made between 11h00 and 13h00. Test samples were collected on identical sampling dates in the water-stressed and well-watered pots. Immediately after cutting a 4 cm section of each leaf, these were sealed in a plastic bag. Experimental material for medium and small plants were pooled, i.e. a section from each of the 4 medium plants was cut and weighed together, while sections from each of the 2 small plants were weighed together. Fresh weight was determined within 60 minutes after excision. Turgid weight was obtained after soaking the leaves for 16-18 hours in distilled water at room temperature (about 20°C). After soaking, leaves were quickly and carefully blotted dry with tissue paper prior to determination of turgid weight. Dry weight was obtained after oven-drying the leaf samples for 72 hours at 70°C. RWC was then calculated using the following equation:

$$RWC (\%) = (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100$$

Once all the data had been collected, statistical analysis was carried out.

4.2.3.2 Leaf elongation measurements

The elongation rate of the third youngest leaf on each plant (i.e. both stressed and control plants) was measured at 9 am daily. Leaf rolling and leaf senescing were

also noted at this time.

4.2.3.3 Infra Red Gas Analysis (IRGA)

IRGA measurements were performed at noon with the aid of an LCA-3 (ADC), which is a battery portable integrated open system, on 3 control and 3 water-stressed plants, by placing a 4 cm piece of the third youngest leaf in the leaf chamber (refer to figure 4.1). Stomatal conductance, photosynthesis and transpiration of the non-stressed and stressed plants were measured. Measurements were made in triplicate for each leaf. The results obtained were computer processed using Microsoft^R Excel '97. Analysis of variance was deduced.

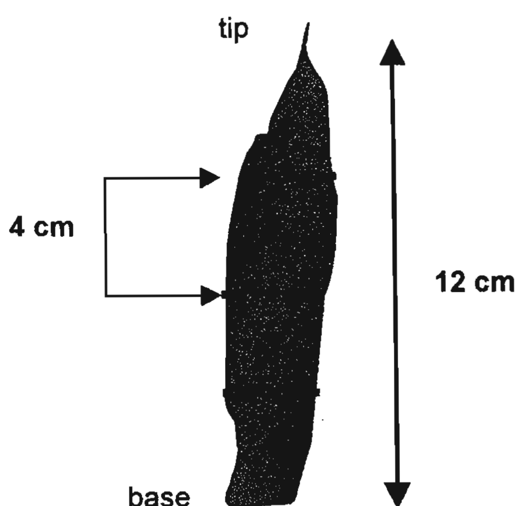


Figure 4.1 Representation of the third youngest leaf of a sugarcane plant divided into 4 sections. Section 2 was used for IRGA analysis and leaf sampling for RNA extraction.

4.3 RESULTS AND DISCUSSION

4.3.1 Stress determined by Relative Water Content (RWC)

An initial experiment was conducted which consisted of water stressing non-transgenic NCo310 plants at different stages of development, viz. large (1.0 - 2.5 m), medium (0.5 - 1 m) and small (500 - 600 mm) plants. In order to achieve differing degrees of water stress, these plants were further subdivided into 4 categories, viz. plants watered twice a day (controls), plants watered once a day, plants watered once every 2 days and plants watered once every 4 days. To each category 7 plants were assigned: 1 large, 2 medium and 4 small. Since Fischer and Sanchez (1979) showed that water potential is reasonably stable

between 11h00 and 13h00, leaf samples were taken during this time and RWC measured.

As can be seen in figure 4.2, the RWC was high for all plants at the beginning of the experiment, but declined as water was withheld from the plants. However, as soon as the plant received water, the RWC rose. This cycle continued throughout the investigation. No observable differences were thus evident between the control and stressed plants when using RWC as a measure of water stress.

Another important observation was that the large plants were much more stressed than the medium and small plants even though they were presumed to have all received the same treatment (figure 4.3). Even the large control plants showed signs of leaf rolling and accelerated leaf senescing (i.e. not senescing because of age, but due to stress). This could have been as a result of the amount of water supplied to these plants (500 ml) being insufficient. In contrast, medium and small plants did not show adequate symptoms of being stressed. At the beginning of the experiment, it was anticipated that the plants watered once every 4 days would show severe symptoms of stress, but this was not the case. At the end of the experiment, these plants still looked relatively healthy when compared to the control plants. A possibility is that the degree of stress imposed on the plants was not sufficient to cause differences between treatments. These results showed that the watering regime was not precisely suitable to establish different degrees of water stress.

4.3.2 Stress determined by leaf elongation and Infra Red Gas Analysis

In a second set of experiments, leaf elongation and IRGA measurements were used to measure stress. The aim of this investigation was to compare the simple method of measuring leaf elongation rates of the third youngest leaf with the more sophisticated IRGA measurements which give information about the photosynthetic and transpiration rates, as well as stomatal conductance of the plants.

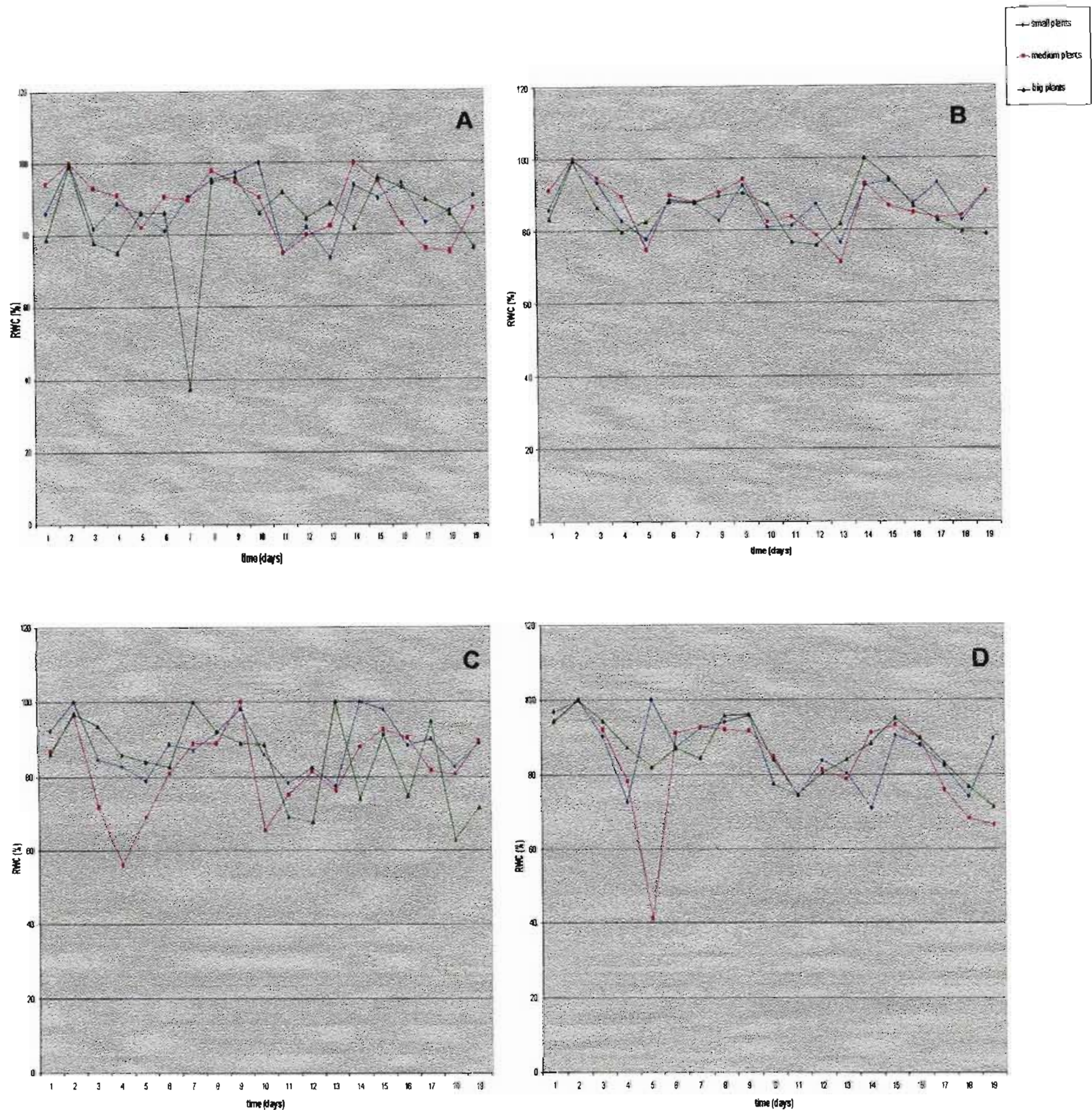


Figure 4.2: Water stress in non-transgenic NCo310 plants as measured using relative water content. (A) illustrates plants watered twice a day, (B) plants watered once a day, (C) plants watered once every two days and (D) plants watered once every four days. Plants were subjected to differing degrees of water stress. RWC was high at the beginning of the experiment, declined as water was withheld and then rose when plants were watered.

Figure 4.3: Comparison of plants used for RWC determination at the start of the experiment (A) and after 20 days of stress (B). After 20 days of water stress, medium- and small-sized plants still looked relatively healthy, indicating that the degree of stress imposed was not sufficient to cause differences between treatments. Large plants were adversely affected, with controls (well-watered) also appearing stressed.

In an initial experiment in which simple leaf elongation was studied, 12 non-transgenic NCo310 plants were used (6 water-stressed and 6 non-stressed). In contrast to the RWC experiment, there was only one degree of stress in this experiment, viz. severe stress (no water). Control plants were watered twice a day as described in section 4.2.2. Figure 4.4(A) illustrates the leaf elongation rates of the well-watered and water-stressed plants. An analysis of variance was carried out (two-sample assuming equal variances) on the data. P(T<=t) was calculated to be 0.00078. Since $P < 0.05$, it could be concluded that there were significant differences between the two treatments. Both sets of plants had a higher rate of leaf elongation at the beginning of the experiment compared to at the end. However, control plants grew more rapidly than stressed plants. This is evident by observing the steeper initial slope of the graph for control plants in figure 4.4(A). Furthermore, stressed plants ceased growth by day 7, whereas control plants continued to grow throughout the experiment.

Figure 4.4(B) compares the appearance of plants before (i) and after (ii) the stress treatment. All plants were healthy at the start of the experiment as evidenced in figure 4.4B(i). As can be seen in figure 4.4B(ii), no leaf rolling was evident at the end of the stress period in the control plants, showing that they were not stressed. On the other hand, marked leaf rolling and leaf senescing was observed in stressed plants. The slight senescing observed in control plants (1 out of 6 leaves for duration of experiment) could be attributed to leaf age. A very important observation not easily discernable in the figure was that plants that were placed (unintentionally) in the shade were less stressed than those placed in direct sunlight. Plants in the sun died at a faster rate and could not be recovered at the end of the experiment, whereas those in the shade were recovered. This observation was taken into account when setting up the second experiment of this kind in which all plants were given the same amount of light.

Since the technique of measuring leaf elongation seemed promising, this technique was compared to the more sophisticated Infra Red Gas Analysis. In a second experiment, six plants were used (3 stressed and 3 non-stressed) and both leaf elongation and IRGA measurements were conducted (figure 4.5).

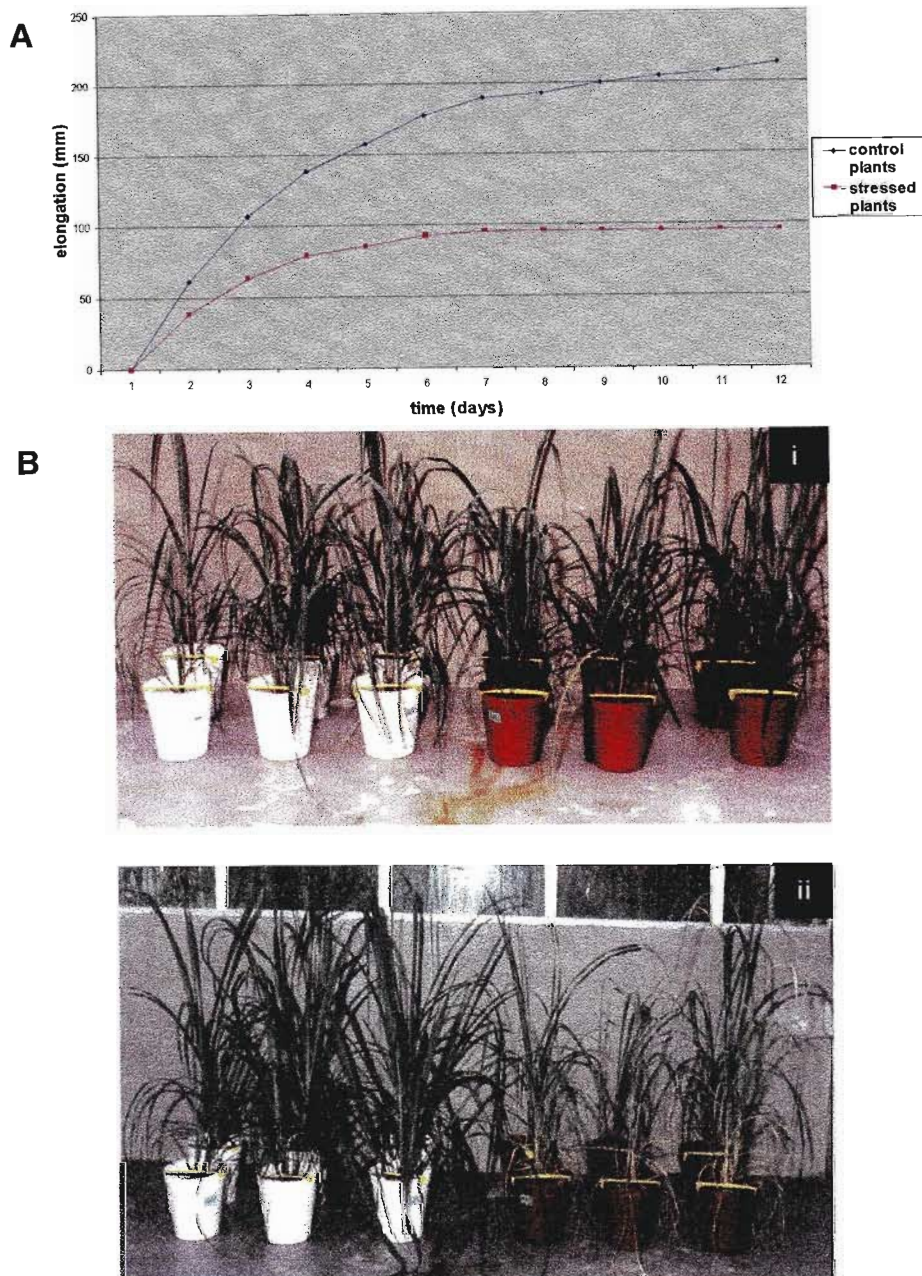


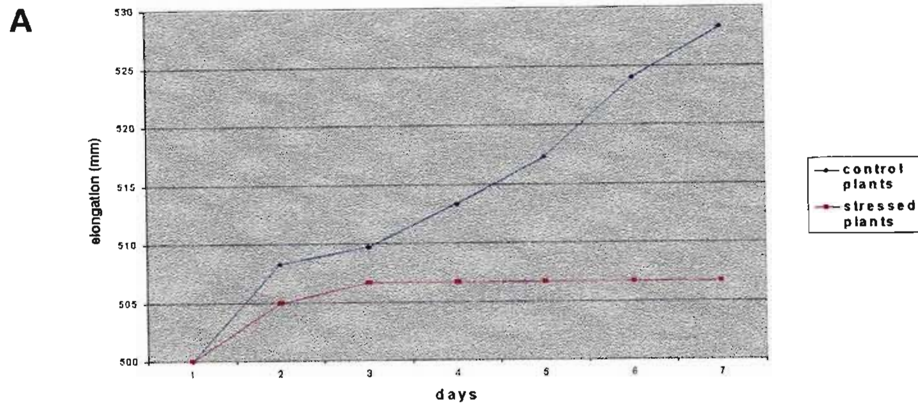
Figure 4.4 Leaf elongation of control (well-watered) and water-stressed non-transgenic NCo310 plants. In this initial experiment, control plants were watered twice a day (250 ml per watering) while stressed plants were not given any water throughout the experiment. Elongation of the third youngest leaf was measured daily at 9 am (A). The mean for the 6 plants per day (per treatment) was plotted. B(i) shows the appearance of plants before the commencement of stress, while B(ii) shows the appearance after 12 days of treatment. Plants in white pots represent the controls (watered), while those in red pots were the experimentals (no water). After 12 days of treatment, experimental plants showed severe signs of stress (leaf rolling and leaf senescing) while controls remained healthy.

Figure 4.5: Infra red gas analysis of plants in glasshouse during water stress treatment.

Once again marked differences in leaf elongation rate between control and stressed plants were evident [figure 4.6(A)]. However, the stressed plants in this experiment died sooner (day 7) than those in the previous experiment (day 12). It is clearly evident in figure 4.6(A) that stressed plants ceased growth by day 3, while control plants continued growth throughout the experiment. As in the previous experiment, the rate of initial growth was also higher for the control plants when compared to stressed plants. An analysis of variance carried out on the above data showed that $P(T<=t)$ was less than 0.05 (0.018033) and it could thus be concluded that there were significant differences between the two treatments.

Figure 4.6(B) provides a visual comparison of control and experimental plants at the end of the water stress experiment. Leaf rolling and leaf senescing was evident in stressed plants by day 3 and became severe with duration of the experiment [figure 4.6B(i)]. No leaf rolling and very slight leaf senescing (attributed to leaf age) occurred in the control plants [figure 4.6B(ii)].

A



B

Figure 4.6: Leaf elongation of control (watered) and water-stressed plants in a second experiment. As in the initial experiment, control plants were watered twice a day (250 ml per watering) while stressed plants were not given any water throughout the experiment. (A) illustrates the elongation of the third youngest leaf which was measured daily at 9 am. (B) is a comparison of a control (i) and experimental (ii) plant after water stress treatment. Note the severe senescence of leaves in the experimental plant compared to the control plant.

Figure 4.7 shows the IRGA results obtained for this experiment. Marked differences between control and stressed plants on day 3 for photosynthetic rates (figure 4.7A), as well as for stomatal conductance (figure 4.7B) were evident. For both measurements, statistical analysis confirmed these significant differences ($P<0.05$). Transpiration rates (figure 4.7 C) on the other hand showed no significant differences between control and stressed plants ($P>0.05$).

Days 3, 5, 6 and 7 show distinct differences in photosynthetic rates between control and stressed plants, with stressed plants hardly photosynthesising at all on those days. Throughout the experiment, control plants had a higher stomatal conductance than stressed plants. This was most pronounced on days 3, 5 and 7. The decrease in stomatal conductance suggests that the stomata of the stressed plants were closing. Consequently, the amount of CO_2 entering the plant would be decreased because of the greater resistance to gaseous diffusion. Thus, photosynthesis would decrease. This in turn should affect leaf elongation, since there will be less energy reserves available for cell division. Also, the soil water potential of stressed plants would have declined with duration of the experiment and as such the plant water potential would have declined. As a result cell turgor would have decreased. In this way cell expansion would not have been possible and thus no growth could take place, since growth consists of both cell division and expansion.

In contrast to photosynthetic rates and stomatal conductance, stressed plants had an apparent higher transpiration rate than the control plants for five out of the seven days. This is contradictory since it was previously deduced that the stomata were closed by the end of the experiment (as evidenced by the extremely low stomatal conductance) and this should have increased resistance to gaseous diffusion. Thus, less water should have been transpired by the stressed plants. Also, it was expected that photosynthesis and transpiration would follow a similar trend since both rely on the same pathway for gaseous diffusion. However, Bull and Glaziou (1975) claim that in many varieties of sugarcane, transpiration rate per unit of green leaf area (as calculated by the IRGA) remains unaltered. This could have been the case with the NCo310 plants used in this study.

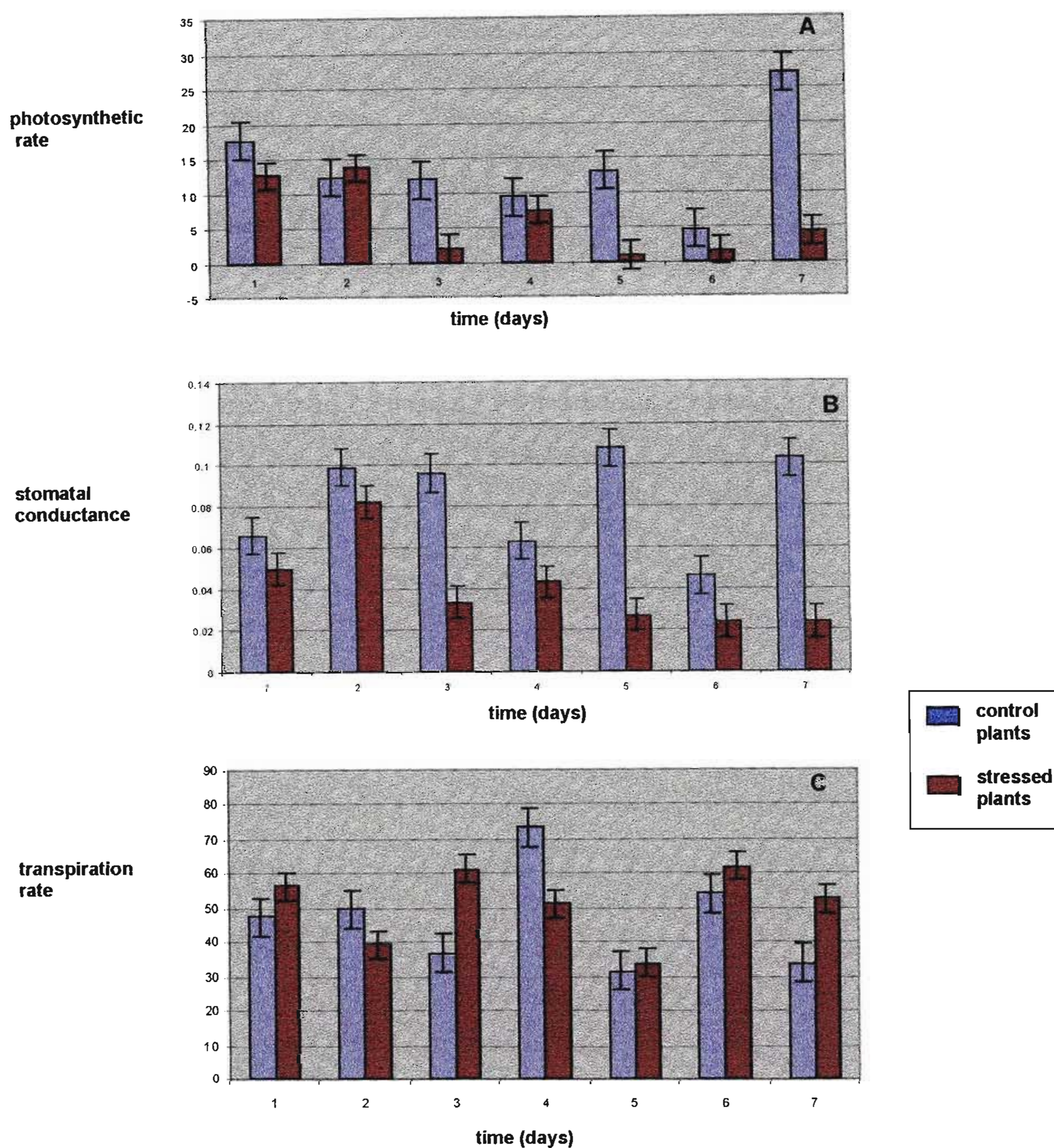


Figure 4.7: <u>Photosynthetic rates (A), stomatal conductance (B) and transpiration rates (C) of watered and water-stressed plants</u>. Control plants were watered twice a day (250 ml per watering), while stressed plants did not receive water throughout the experiment. IRGA measurements were made at noon, daily, on the third youngest leaf using an LCA-3 (ADC). The mean for the 3 plants per day (per treatment) was plotted.

As can be seen when comparing IRGA readings with leaf elongation measurements, leaf elongation is a more accurate measure of water stress. In addition, leaf elongation measurements are easier to interpret than IRGA readings.

4.4 CONCLUSIONS

These preliminary experiments show that leaf elongation, which is simple and non-destructive, is a better measure of water stress than IRGA measurements, which are more time-consuming and require intensive processing of data. Furthermore, it is evident that differing degrees of water stress are difficult to measure. Severe stress, induced by withholding water completely, proved to be more efficient. Thus, when setting up the experiment which involved sampling for RNA extraction and measurement of promoter activity during stress of transgenic NCo310 plants, described in chapter 5, stress was induced by withholding water supply to experimental plants and measured by observing leaf elongation.

CHAPTER 5: EFFECT OF WATER STRESS ON THE ACTIVITIES OF UBI AND SUC-1 PROMOTERS

5.1 INTRODUCTION

Once sufficient plants had been micropropagated (chapter 2) and the techniques of RT-PCR and Northern hybridisation developed (chapter 3), as well as suitable water stress parameters established (chapter 4), the effect of water stress on UBI and SUC-1 promoter activity could be ascertained in a final experiment. This chapter focuses on the final experiment in which transgenic as well as nontransformed sugarcane plants were subjected to periods of water deficit and the effects of stress measured. Leaf elongation was used as a physiological measure of plant stress, while Bt mRNA expression was used to deduce promoter activity during stress.

5.2 MATERIALS AND METHODS

5.2.1 Experimental plant material

Plants were maintained in the glasshouse at the South African Sugar Association Experiment Station as described in section 4.2.1.

5.2.2 Watering systems

Plants were watered according to the protocol described in section 4.2.2.

5.2.3 Measurement of stress

The elongation of the third youngest leaf on each plant (both stressed and non-stressed) was measured at 9 am daily. Leaf rolling and leaf senescing were also noted at this time.

5.2.4 Leaf sampling for RNA extraction

Leaf samples were collected daily between 11 am and noon from both control and stressed plants, immediately frozen in liquid nitrogen and stored at –80°C until needed. The same portion of the leaf that was used for IRGA analysis in chapter 4 (fig. 4.1) was chosen for RNA extractions.

5.2.5 RNA extraction

For RT-PCR, RNA was extracted using the SV Total RNA Isolation Kit (Promega) as described in section 3.2.3.1, while the RNeasy Plant Mini Kit (Quiagen) was used to extract RNA for Northern blotting as described in section 3.2.3.2.

5.2.6 RT-PCR

RT-PCR was carried out according to the protocol described in section 3.2.5.2 using the Titan One Tube RT-PCR System.

5.2.7 Northern hybridisation

Northerns were carried out according to the protocol described in section 3.2.7.

5.3 RESULTS

During this study both transgenic and nontransformed NCo310 plants were water-stressed. Half the transgenic plants contained the Bt gene driven by the ubiquitin promoter (genotypes #6.8, #6.12 and #18.1), while the other half contained the Bt gene driven by the SUC-1 promoter (genotypes #7.20, #7.47 and #7.33). The aim of the experiment was to compare the activity of UBI and SUC-1 promoters in stressed and non-stressed transgenic plants.

In order to achieve uniformity among samples, only plants in medium-sized (160 mm x 175 mm) pots were used. This determined how many plants were assigned to each treatment per genotype. In this way, soil volume was similar for all plants. Thus, approximately the same volume of water could be given to each non-stressed plant, namely 300-350 ml per watering, and drying out of soil for stressed plants was consistent. This uniformity reduced variability among replicates. All plants were placed in the same glasshouse chamber so that environmental conditions, such as light intensity, temperature and humidity could be kept constant. Differences observed between treatments were therefore assumed to be due to the treatment and not other experimental conditions. Stressed plants were placed at one end of the chamber and did not receive any water throughout the duration of the experiment. Non-stressed plants were placed at the other end of the chamber and received water twice a day at 9 am and 4 pm.

Leaf elongation measurements were made at 9 am daily on the third youngest leaf of 6 plants per genotype, while leaf samples for molecular analysis were taken from 3 randomly chosen plants per genotype, between 11 am and noon daily. Figure 5.1 illustrates the above experimental design.

5.3.1 Development of plant stress over time

Figure 5.2 illustrates the water stress observed for respective transgenic and nontransformed (control) NCo310 plants over 9 days. Leaf elongation measurements represent the average of data for all clones of an event. As can be seen, all watered plants continued to show leaf growth throughout the experiment, while stress-treated plants had ceased growth between days 3 and 4. On average, watered plant leaves grew approximately 5-10 cm per day. Also evident in figure 5.2 is that not all plants showed the same stress trends. There were marked differences between the leaf elongation rates of watered and water-stressed #6.12, #18.1, #7.33 and control plants. This was confirmed statistically, with $P(T<=t)$ being less than 0.05 for these plants (table 5.1). Initial rates of growth for watered plants from genotypes #6.12, #18.1, #7.33 and control plants were higher than their stress-treated counterparts, whereas the opposite was true for #7.20, #7.47 and #6.8 plants. $P(T<=t)$ was greater than 0.05 for #6.8, #7.20 and #7.47 plants, indicating that there were no significant differences between the watered and stress-treated plants in these series.

Table 5.1 T-test results for transgenic and control NCo310 plants. A positive stress response is indicated by +, while – indicates a negative one.

Genotype	**Promoter**	**P(T<=t)**	**Stress significance**
#6.8	UBI	0.41387	–
#6.12	UBI	0.005884	+
#18.1	UBI	0.000949	+
#7.33	SUC-1	0.003195	+
#7.47	SUC-1	0.317962	–
#7.20	SUC-1	0.28104	–
Nontransformed plants		0.018031	+

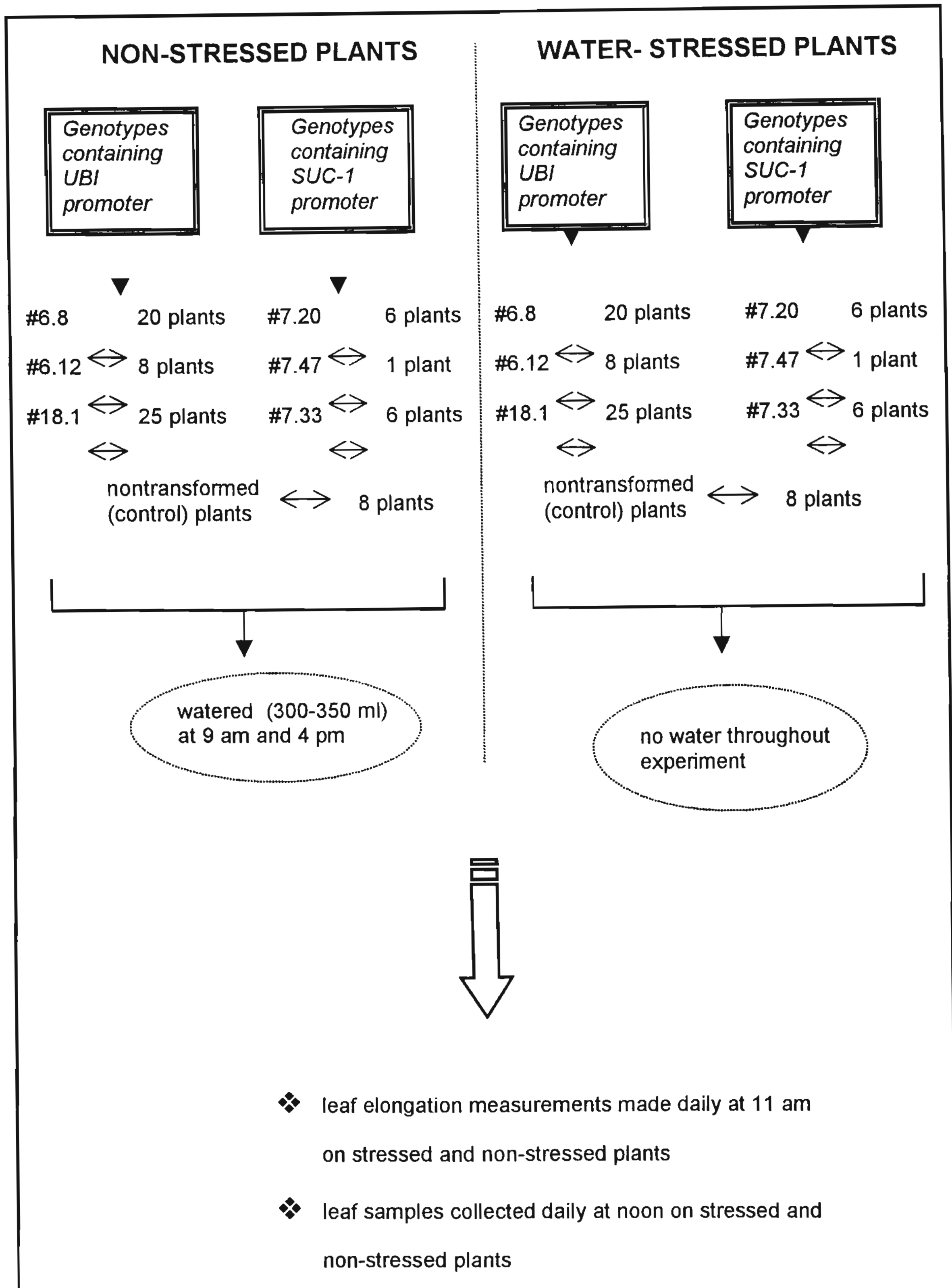


Figure 5.1: Design of stress experiment on transgenic and nontransformed NCo310 sugarcane plants containing the Bt gene. In order to obtain uniformity among samples, plants of similar sizes were chosen. This determined the number of plants per genotype assigned to each treatment. The duration of the stress experiment was 9 days.

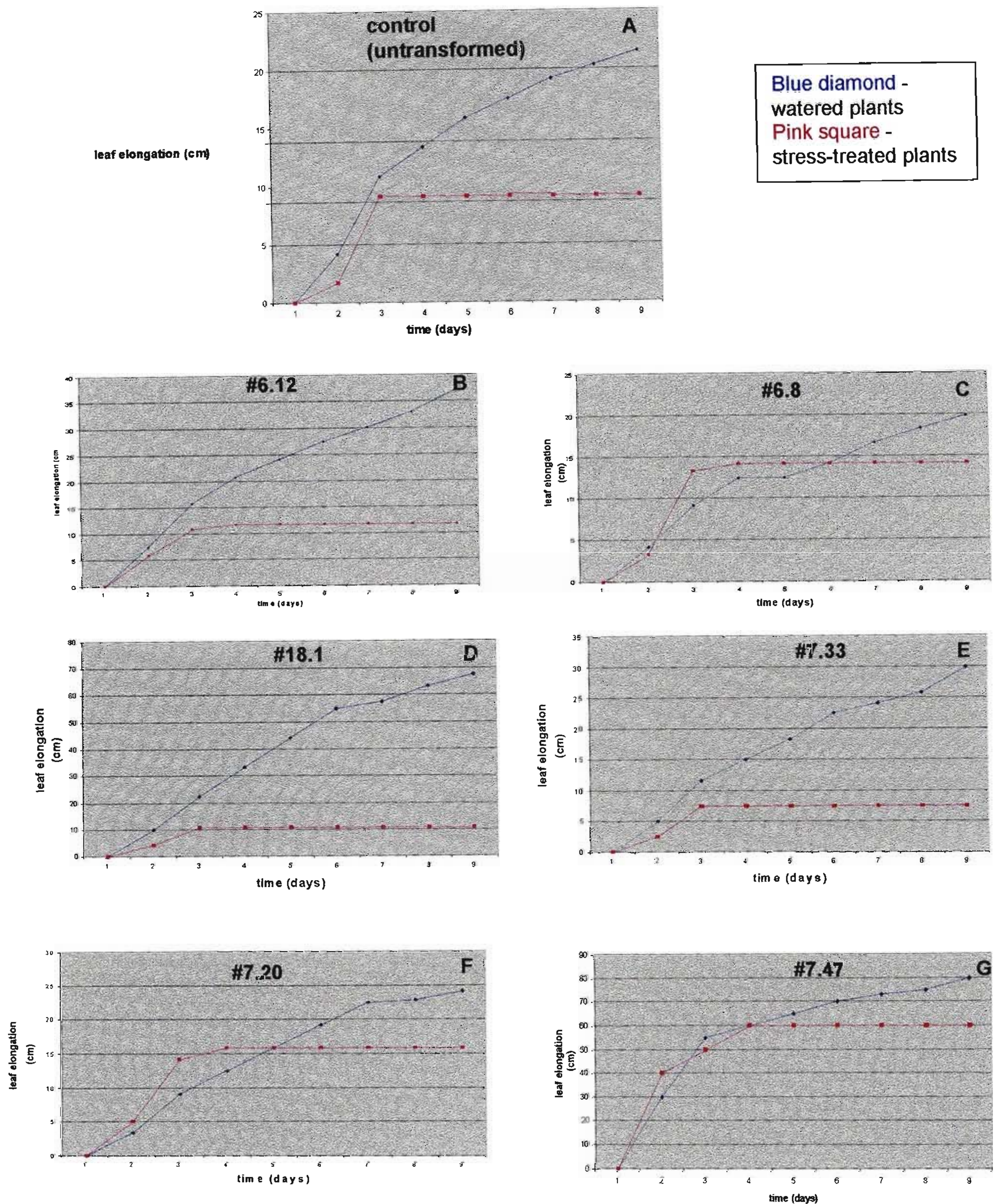


Figure 5.2 Plant stress as measured by leaf elongation of transgenic and nontransformed plants. (A) represents the nontransformed (control) plant, while (B) to (G) represent transgenic genotypes #6.12, #6.8, #18.1, #7.33, #7.20 and #7.47 respectively.

Figure 5.3 provides a visual comparison of representative SUC-1 (A) and UBI (B) plants, as well as watered (C) and stress-treated (D) plants . In general, most stress-treated SUC-1 and all stress-treated control plants appeared relatively healthy throughout the experiment, with very little leaf senescing occurring. Slight leaf rolling, however, became evident in these plants on day 4. Most stress-treated UBI plants on the other hand appeared severely stressed. The #6.8 series in particular showed the earliest and severest symptoms of stress, with the leaves first appearing to lose chlorophyll (milky appearance) by day 2, and thereafter turning completely yellow by day 6. Genotypes #18.1 and #6.8 also exhibited severe leaf rolling by day 6.

A striking observation concerned watered plants from the genotype #6.8 (figure 5.3B). These plants started rolling from day 1 and were severely rolled by day 2. This phenomenon is hard to explain, as no additional signs of stress were evident. The leaves of these watered #6.8 plants did not senesce, nor did they cease growth throughout the experiment. Furthermore, these plants were spread out randomly in the chamber and, consequently, location could not have been an adverse factor affecting the plants, since the other watered plants showed no signs of leaf rolling (figure 5.3C). In addition, all plants (stressed and watered) received the same treatment during routine maintenance prior to the experiment and should therefore all have been non-stressed at the beginning of the experiment.

The physical appearances of transgenic plants correlate positively with the leaf elongation measurements described above. As mentioned, t-test results for genotypes #6.12, #18.1 and #7.33 plants confirmed significant stress responses, while results for #6.8, #7.20 and #7.47 suggest no significant differences between stressed and watered plants (table 5.1). In contrast, control (nontransformed) plants produced a t-test result less than 0.05, suggesting significant stress, while their physical appearance at the end of the experiment did not indicate this since their stress-treated plants exhibited very little leaf senescence and only slight leaf rolling.

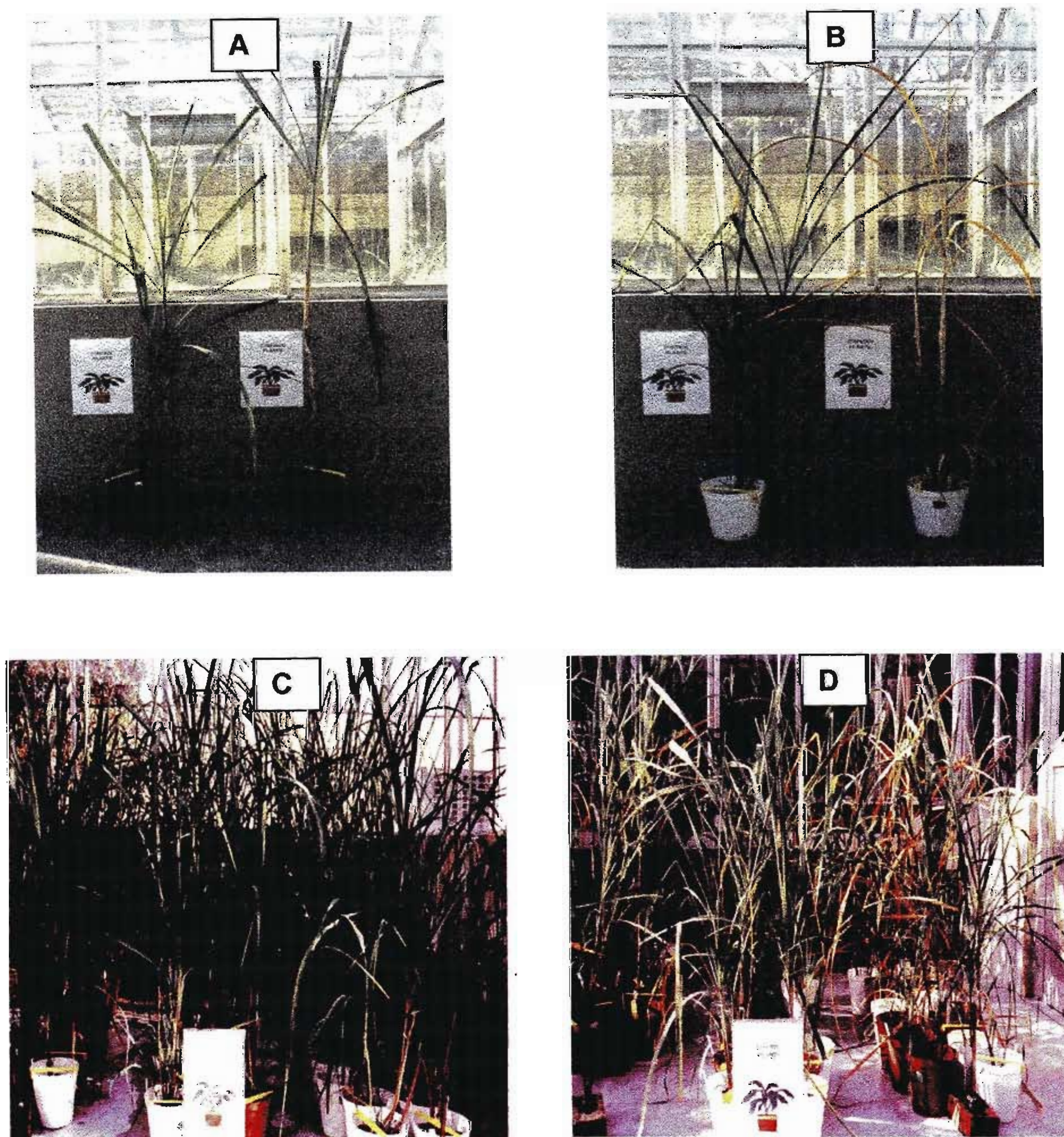


Figure 5.3 Comparison of representative SUC-1 (A) and UBI (B) plants, as well as watered (C) and stress-treated (D) plants on day 9 of stress experiment. (A) illustrates genotype #7.20 containing the SUC-1 promoter, while (B) illustrates genotype #6.8 containing the UBI promoter. Plants on the left of (A) and (B), as well as all plants in (C) were watered twice a day, while those on the right of (A) and (B) and all plants in (D) were water-stressed by completely withholding water.

From the leaf elongation measurements and physical appearance of experimental plants, it was realised that genotypes #6.12, #18.1 and #7.33 would be the only plants to indicate differences in promoter activity (if any) when comparing stress-treated and watered plants. As such, these genotypes are focused on in section 5.4.1 when comparing SUC-1 and UBI promoter activity.

5.3.2 Promoter activity during course of stress response

For molecular analysis, leaf samples were collected from three randomly chosen plants per genotype per treatment between 11 am and noon daily (section 5.2.4). These were immediately frozen in liquid nitrogen, to minimise RNase degradation, and thereafter stored at –80°C until needed. RNA was extracted for RT-PCR and Northern analysis. RT-PCR was performed to establish whether Bt mRNA was being transcribed in stressed and non-stressed plant material. Northern analysis was carried out to investigate transcription of the Bt gene, a sugarcane stress-response gene (homologous to a heat shock protein gene from rice [*Oryza sativa*]) and a sugarcane housekeeping gene (60S ribosomal protein gene).

5.3.2.1 Bt gene transcription as measured by RT-PCR analysis

RT-PCR analysis revealed that the Bt gene was transcribed in all plants (Figure 5.4), stressed and non-stressed, suggesting that both UBI and SUC-1 promoters were active at all times. However, the extent of mRNA expression could not be determined from these results since the bands obtained were of similar intensity for most plants tested. Genotypes #7.47 and #7.33 were the exceptions, both producing a slightly lighter band for day 9 of the stress treatment. This suggests a possible decrease in gene expression for these genotypes by the end of the experiment.

Leaf elongation measurements (section 5.3.1) for genotype #7.33 correlate positively with the above RT-PCR results, since statistical analysis showed significant differences between stress-treated and watered plants. However, #7.47 results are negatively correlated. T-test data (table 5.1) suggest no significant differences between #7.47 stress-treated and watered plants, while RT-PCR results suggest a stress response by the end of the experiment, shown by a decrease in gene expression (figure 5.4). This discrepancy leads to the

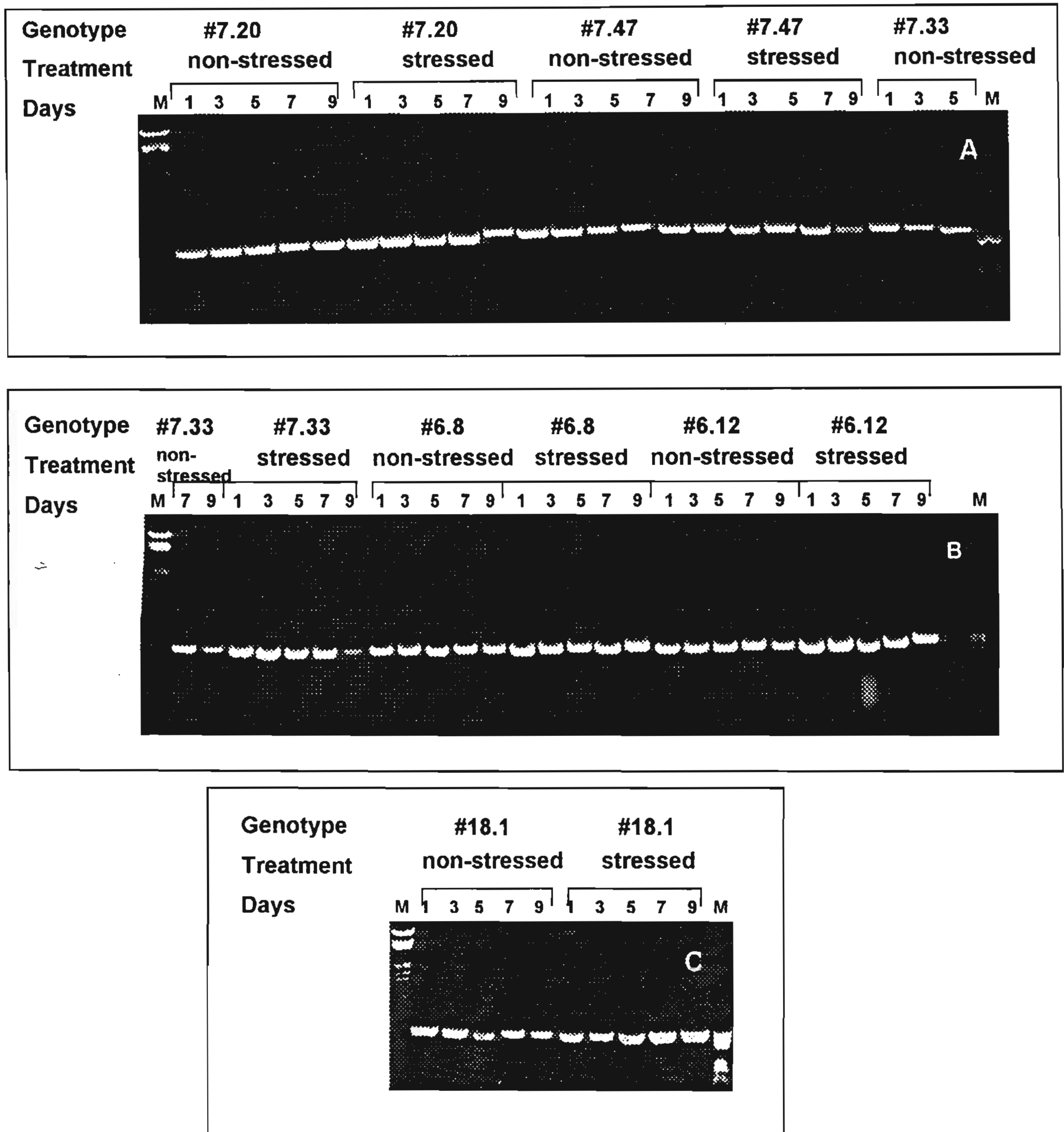


Figure 5.4 Specific detection of Bt gene transcription from leaves of transgenic sugarcane plants by reverse transcription-polymerase chain reaction. Two oligonucleotide primers (StoxF and StoxR) were used for the amplification of a 543 bp fragment (within the toxin core sequence of the Bt gene) from nucleotides 108 to 652, downstream of the initiation codon. All plants expressed the transgene during stress and stress-free conditions, confirming promoter activity.

possibility that the results obtained for genotypes #7.33 and #7.47 could be due to differences in PCR amplification efficiency as a result of template concentration.

5.3.2.2 Establishment of loading parameters for Northern hybridisation

Prior to Northern analysis, it was imperative that the exact amount of RNA per sample be loaded onto a denaturing agarose gel (section 3.2.6 of chapter 3), which was later to be blotted onto a positively charged nylon membrane. In this way, differences in band intensity (or gene expression) observed between samples would not be due to differences in sample concentration, but the result of the treatment imposed. Figure 5.5 illustrates a representative denaturing agarose gel containing 15 μg total RNA per sample. The uniform brightness of the bands confirmed even loading of samples. Also, the distinct rRNA bands and absence of smearing indicate that the samples were not degraded.

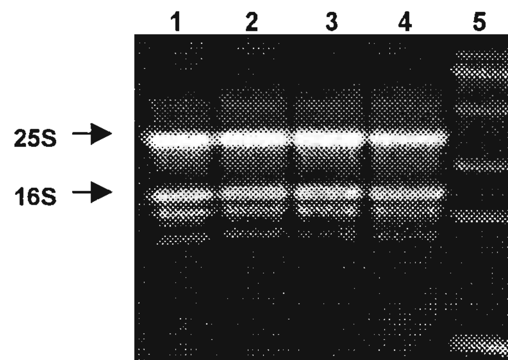


Figure 5.5 Agarose gel electrophoresis of RNA samples prior to Northern analysis of Bt mRNA transcription. Samples were randomly chosen to check loading efficiency. Lanes: (1) #7.20, (2) #7.33, (3) #6.8, (4) #6.12 and (5) RNA ladder.

Northern analysis of the transcription of a housekeeping gene was also attempted as a further means of confirming sample loading consistency. A sugarcane 60S ribosomal protein gene was chosen. Autoradiographs were left for 13 days to expose. No results were obtained, however, and membrane damage was suspected.

5.3.2.3 Northern analysis of Bt gene transcription

Northern hybridisations were performed in order to further characterise Bt gene transcription in both stressed and non-stressed transgenic plants. A denaturing

agarose-formaldehyde gel containing 15 μg total RNA per sample was blotted onto a positively-charged nylon membrane and probed at 65°C with a double-stranded DNA probe. The probe was prepared by PCR-amplifying the Bt gene from plasmid pLBRtox, using specific primers PtoxF and PtoxR (as described in section 3.2 of chapter 3), which amplify the 2 kb Bt insert. The probe was then labeled using random priming of the PCR-amplified Bt gene. Autoradiographs were left to expose at –80°C for 96h.

General features of Bt gene transcription. Figure 5.6 is a representation of the autoradiographs obtained after Northern analysis of Bt gene transcription. A multiple-banding pattern for Bt mRNA was observed in all transgenic plants tested (Figure 5.6A-F). In contrast, the control plants exhibited no bands whatsoever (results not shown). This confirmed the specificity of the probe as well as the identity of the Bt mRNA in transformed samples. Banding patterns were similar for all transgenics, with 5 distinct bands of approximately 2.0, 1.6, 1.2, 1.0 and 0.35 kb respectively. The fact that multiple bands were evident in both stressed and non-stressed plants indicated that this was not a stress-related phenomenon. A possible reason for these multiple transcripts could be the presence of polyadenylation signals in the coding region of the wild-type Bt gene, as suggested by Adang *et al.* (1987), Murray *et al.* (1991) and Diehn *et al.* (1998), leading to premature termination of the message.

Differences in Bt gene expression between individual transformants. Band intensity may be used as a measure of Bt gene expression, with dark bands suggesting high expression and lighter bands low expression. In figure 5.6, distinct differences between individual transformants in overall expression of the Bt gene could be observed. Genotype #7.20 (figure 5.6A) had the lowest overall Bt gene expression, while #6.12 (figure 5.6E) had the highest. Such differences are likely to be a reflection of random differences between transformants due to copy number or positional effects, i. e. the position at which the transgene is inserted in the sugarcane genome (Broer, 1996).

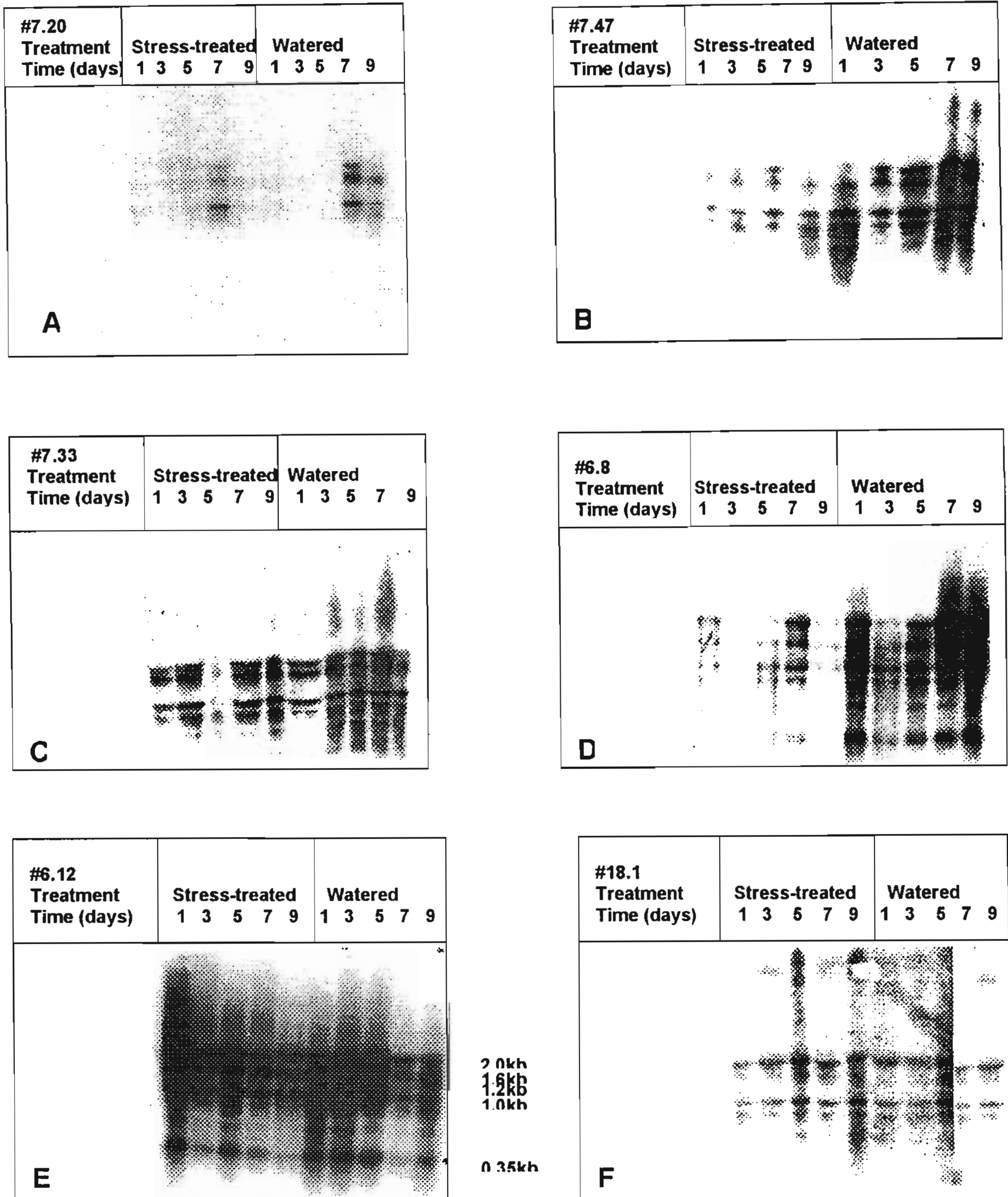


Figure 5.6 Bt gene transcripts. Representative autoradiographs were obtained using 15 μg total RNA isolated from transgenic sugarcane plants containing the Bt gene where (A) #7.20, (B) #7.47, (C) #7.33, (D) #6.8, (E) #6.12 and (F) #18.1. The specific activity of the probe used was 1.9 X 10^9 cpm μg DNA^{-1}. Autoradiographs were exposed for 96h (4 days). Banding patterns were similar for all transgenics with 5 distinct bands of approximately 2.0, 1.6, 1.2, 1.0 and 0.35 kb respectively.

Stress response of the UBI promoter. In genotype #6.12 (figure 5.6E) the UBI promoter remained active in both stressed and watered plants, with activity being particularly high at the beginning of the experiment for both treatments. However, promoter activity declined in both stress-treated and watered #6.12 plants as the experiment proceeded. Since t-test results show that there was significant stress in the stress-treated plants (table 5.1), the above result suggests that the UBI promoter decreases activity under stress. In contrast to genotype #6.12, in genotype #6.8 (figure 5.6D) the promoter was highly active in watered plants compared to stress-treated plants, activity being more pronounced towards the end of the experiment. No conclusions can be drawn from this, since t-test results show no significant differences between stress-treated and watered #6.8 plants. Genotype #18.1 (figure 5.6F) had low overall promoter activity. This was relatively constant throughout the experiment in both stressed and watered plants. As for genotype #6.12, t-test results for #18.1 also suggest significant stress in stress-treated plants. As mentioned above, the differences in expression between transformants with the same promoter could be due to positional effects of the transgene in the plant genome.

Stress response of the SUC-1 promoter. In genotype #7.47 (figure 5.6B) the SUC-1 promoter appeared more active in watered plants compared to stress-treated plants. This result is in contrast to t-test results showing no differences between stress-treated and watered #7.47 plants. Genotype #7.20 (figure 5.6A) had the lowest overall promoter activity of all samples tested, as previously mentioned, with activity being at its highest on day 7 for both treatments. Like genotype #7.47, t-test results for #7.20 also show no significant differences between stress-treated and watered plants. Therefore, no conclusions about promoter activity can be drawn from results obtained for these genotypes. In contrast, genotype #7.33 had a 'significant' stress result, as determined by t-test analysis, and thus deductions about promoter activity could be made from this genotype. The SUC-1 promoter maintained constant activity throughout the experiment in both stress-treated and watered #7.33 plants. This suggests that the SUC-1 promoter is able to maintain sufficient levels of activity, without being affected by stress. Out of all the transformants, overall Bt gene expression was intermediate in this genotype.

5.3.2.4 Northern analysis of heat-shock gene transcription

In order to provide a means of comparison for UBI- and SUC-1 driven Bt gene expression, analysis of the expression of a stress-related gene was attempted. A probe was prepared by radioactively labelling a sugarcane gene homologous to the heat-shock protein in *Oryza sativa* (rice). Membranes containing the Bt probe were stripped and thereafter incubated with the new probe. Autoradiographs were left for 48h at –80°C to expose.

General features of heat-shock gene expression. Figure 5.7 shows the transcription product of the heat-shock protein gene. A single band of approximately 2.7 kb was observed in all samples (figure 5.7A-G). In general, watered plants appeared to have higher expression levels compared to stress-treated plants. This was most pronounced in genotypes #7.33 (figure 5.7C), #6.8 (figure 5.7D) and #6.12 (figure 5.7E). Control plants (figure 5.7G) had the highest heat-shock gene expression, while genotype #7.20 (figure 5.7A) had the lowest.

Comparison of Bt and heat-shock gene expression during stress treatments. The most striking difference between the two genes was their expression patterns. The Bt gene produced multiple mRNA species (figure 5.6) compared to the single mRNA transcribed by the heat-shock gene (figure 5.7). mRNA sizes were also distinctly different for the two genes, confirming their identity. The absence of Bt gene expression in control plants compared to contrastingly high heat-shock gene expression in these plants confirmed their nontransformed status. Genotype #7.20 had the lowest expression of all samples for both genes. Both genes were generally highly expressed in watered plants compared to stress-treated plants. This result was surprising for the heat-shock gene since this is a stress-related gene and expression was therefore expected to increase under stress conditions (Ashburner and Bonner, 1979; Edelman *et al.*, 1988).

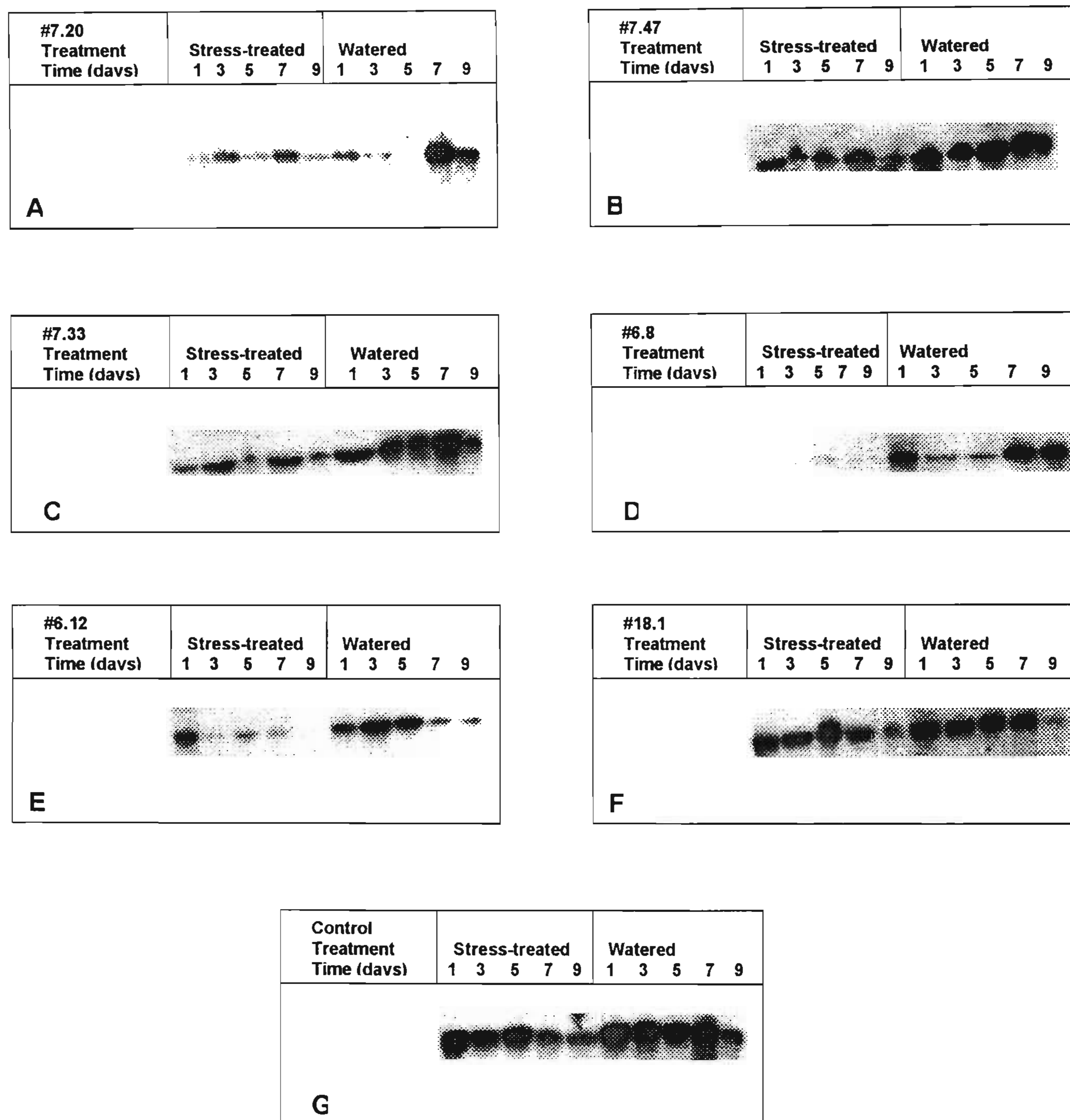


Figure 5.7 Transcription of a putative heat shock gene. Representative autoradiographs were obtained using DNA probe made from sugarcane gene homologous to rice heat shock gene where (A) #7.20, (B) #7.47, (C) #7.33, (D) #6.8, (E) #6.12, (F) #18.1 and (G) nontransformed plant. Specific activity of the probe was 1.9 X 10^9 cpm μg DNA^{-1}. Exposure times were 48h. A single band of approximately 2.7 kb was observed in all plants.

5.4 DISCUSSION

5.4.1 Limitations of transgenic experimental plants for comparison of promoter activity

The great variation in overall Bt gene expression levels, observed in figure 5.6, illustrates the limitations of using transgenic plant material during investigation of UBI and SUC-1 promoter activities. Methods currently employed to accomplish stable plant transformation result in the random integration of foreign DNA throughout the genomes of recipient cells (Weising *et al.*, 1988), as was the case in the present study. The inserted transgenes are thus exposed to influences of varying chromosomal environments (Broer, 1996). They are influenced by *cis*-acting factors not carried by the transgene but present at the insertion site (position effect). Expression will vary depending on where the transgene has integrated into the genome. Thus, if the inserted gene is localised in a highly repressed domain, expression will be reduced as in genome #7.20. If, however, the transgene has inserted into a region which does not adopt a repressive chromatin conformation, then expression should be high as in genome #6.12. To date, this impact cannot be prevented (Broer, 1996) and may be the cause of the differing levels of gene expression observed in the individual transformants used in the present study. As can be seen in figure 5.6, expression levels differed even between transformants carrying identical promoter elements. For example, even though genomes #7.20 and #7.33 both carried the SUC-1 promoter, #7.20 exhibited lower overall Bt gene expression compared to #7.33.

One of the major disadvantages of direct gene transfer, the transformation technique employed in the present study, is the fact that vector DNA may be subjected to cleavage resulting in rearrangement, truncation, tandem formation and linkage of co-transformed genes either during or prior to integration (Webb and Morris, 1992). This fact may be responsible for some aspects of transcriptional regulation that still remain unknown, and more importantly, uncontrollable (Fisk and Dandekar, 1993). It also appears that positional effects may not only be responsible for differences in the amount of expression, but also differences in tissue specificity directed by any particular promoter (Fisk and Dandekar, 1993). It has been suggested that one of the contributors to positional effects is the activity of endogenous promoters in the proximity of the foreign gene insertion site (Weising *et al.*, 1988). Transcription of flanking genes

may affect the transgene promoter by causing torsional stress. Methylation of DNA has also been shown to impair transcription in plants (Matzke *et al.*, 1989; Hobbs *et al.*, 1990; Bochardt *et al.*, 1992; Ottaviani *et al.*, 1993). This process may contribute to some of the observed positional effects.

Finally, depending on which tissue culture method was used for clonal propagation (chapter 2), the possibility exists that the physiological state of the plants growing in the glasshouse were different. This in turn could have affected promoter activity. For the above reasons, SUC-1 and UBI promoter activities could not be directly compared.

5.4.2 Possible inadequacy of stress treatment

Upon analysis of heat shock gene transcription in figure 5.7, it became clear that there was no dramatic change in expression levels from watered to stressed plants, especially in genotypes #7.47, #18.1 and nontransformed control plants. Although t-test results for #18.1 and control plants suggested possible stress in the non-watered plants, Northern analysis of heat shock gene expression suggest that this might not be so. It was expected that the stress response of the heat shock gene would be pronounced since this is a stress-related gene (Ashburner and Bonner, 1979; Edelman *et al.*, 1988). Therefore, the variations in Bt gene expression could be due solely to positional effects and not as a result of the stress-treatment. On the other hand, the fact that the heat shock gene did not show induction in response to water stress illustrates that assumptions cannot be made based on sequence similarity.

5.4.3 Indirect comparison of UBI and SUC-1 promoter activities

For the comparison of promoter activity, genotypes #7.33 (containing the SUC-1 promoter) and #6.12 (containing the UBI promoter) will be focused on, since these genotypes had t-test values suggesting significant differences between stress-treated and watered plants (table 5.1). From Northern hybridisation results, UBI promoter activity appeared to decrease under stress (figure 5.6E), while SUC-1 promoter activity remained constant (figure 5.6C). Bt mRNA was more abundant in watered UBI plants compared to their stressed counterparts, while SUC-1 plants had the same proportion of Bt mRNA in both stressed and watered plants. In addition, genotype #6.12 had higher overall Bt gene

expression compared to #7.33, suggesting that the UBI promoter was more active than the SUC-1 promoter, particularly under stress-free conditions. This, however, could be due to position of insertion of the transgene in the plant genome, as suggested above, with the Bt gene being in a more favourable position in genotype #6.12. The fact that SUC-1 activity remained constant throughout the experiment, while UBI activity decreased under stress, suggests that SUC-1 is superior to UBI in terms of driving transgene expression under stressful environmental conditions.

Similar results were obtained in a study carried out at the Institute for Plant Biotechnology (IPB) in Stellenbosch (1998), in which an existing expression vector (pUBI 920) containing the cauliflower mosaic virus 35S promoter (35S) and the complete maize ubiquitin (UBI - including the first exon and intron) promoter in tandem was modified by insertion of the GUS reporter gene. The resulting plasmid was designated pGUS 510. The aim was to investigate the efficiency of pUBI 510 as a sugarcane expression vector. Since the GUS gene was driven only by the UBI promoter in a control vector, pAHC 27, it was expected that gene expression in pGUS 510 would be at least as effective as expression in pAHC 27. Studies confirmed that the tandem promoter construct was approximately twice as active as the UBI promoter alone, which would make it an ideal constitutive promoter for high level expression in transgenic sugarcane.

The differences in promoter activity found in the present study could not be determined from RT-PCR results (figure 5.4). Only Northern analysis gave an indication of differential promoter performance. Quantitation of mRNA by conventional RT-PCR is difficult due to different reverse transcription and PCR amplification efficiencies and sample-to-sample and tube-to-tube variations (Wang *et al.*, 1989; Chang *et al.*, 1993; Souaze *et al.*, 1996). Specific procedures for quantitation by RT-PCR use coamplification of a different reporter gene product (Chelly *et al.*, 1988; Chelly *et al.*, 1990; Frye *et al.*, 1989; Rappolee *et al.*, 1988) or coamplification of a competitor DNA fragment that differs in size from the cDNA of interest but is amplified with the same pair of primers (Gilliland *et al.*, 1990). Chang *et al.* (1993) report a method for the analysis of mRNA expression by RT-PCR using a synthetic RNA that serves as an internal control

for both reverse transcription and PCR amplification. Quantitative RT-PCR is performed with a wide range of concentrations of internal standard to a fixed amount of target mRNA to determine the amount of internal standard that gives a signal of equal intensity to the signal due to the target mRNA. The results obtained are then used to estimate the amount of target mRNA molecules in the sample by interpolation (Chang *et al.*, 1993). In the present study, RT-PCR was useful in confirmation of Bt gene transcription, and thus promoter activity, in the individual transgenic sugarcane plants.

5.4.4 Occurrence of short Bt transcripts

The most striking observation from Northern analysis of Bt gene transcription was the occurrence of short Bt transcripts in addition to the full length transcript. This is interesting, since only a single 2.0 kb Bt band was observed during optimisation of Northern hybridisation, as reported in chapter 3 (figure 3.12). The distinct nature of the multiple bands observed in figure 5.6 confirmed that this was a 'plant-related' phenomenon as opposed to experimental error. Furthermore, the results reported in the present chapter are in agreement with those observed by Adang *et al.* (1987), Murray *et al.* (1991) and Diehn *et al.* (1998). Since multiple mRNA transcripts of smaller size (1.6, 1.2, 1.0 and 0.35 kb) than expected (2.0 kb) were obtained, it is possible that the wild-type Bt gene contains sequences in its coding region that are recognised as plant polyadenylation signals. This could be interfering with the expression of full-length Bt mRNAs in the transgenic sugarcane plants used in the present study.

Adang *et al.* (1987) observed that tobacco plants expressing a *cry1A(c)* protoxin gene or a 3'-truncated version produced a polyadenylated 1.7 kb transcript. The size of this transcript was shorter than expected for either gene, prompting the suggestion that the Bt coding region contains plant polyadenylation signals. However, the transcript disappeared as the plants matured (Murray *et al.*, 1991). Therefore, the mechanism responsible for the production of the 1.7 kb transcript could not be determined and the disappearance of the transcript during development was not explained. In another study, transcripts of 1.6 and 0.9 kb were detected in the poly(A+) RNA fractions of plants expressing a *cry1A(b)* gene. However, these transcripts were believed to be degradation intermediates (Murray *et al.*, 1991). Diehn *et al.* (1998) were the first to show that sequences

within the coding region of the Bt gene can be recognised as polyadenylation signals by plants. Hybridisation, RT-PCR and RNase-H-mapping experiments carried out by those authors revealed that nearly every tobacco cell line stably transformed with the *cry1A(c)* gene accumulated polyadenylated transcripts of approximately 900 to 600 nucleotides in length.

mRNA polyadenylation is a common feature of gene expression, having been observed in eukaryotic nuclei (Proudfoot, 1991), human mitochondria (Ojala *et al.*, 1981) and some bacterial genes (Cao and Sarkar, 1992). Virtually all plant genes have multiple poly(A) induction sites and these are composed of many distinct *cis* elements (Hunt, 1994). These elements can be grouped into three classes, namely far-upstream elements (FUEs), near-upstream elements (NUEs) and the actual cleavage / poly(A) sites (CSs). An interesting characteristic of the different *cis* elements is their lack of strict sequence conservation in plants (Hunt, 1994). For this reason it was difficult in the present study to identify sequences common to all the short Bt transcripts which could be responsible for premature polyadenylation and cleavage of the Bt gene.

5.4.5 Analysis of Bt coding sequence

The coding sequence for the Bt234 gene is shown in figure 5.8. Upon inspection of the Bt sequence, a number of motifs rarely present in plant genes could be found repeatedly. The most striking were the numerous A/T-rich stretches. Bases A and T made up 31.05 and 31.37% of the Bt gene, while C and G only accounted for 17.14 and 20.43%, respectively. A/T-rich sequences are normally only found in nontranslated regions of plant genes, in particular in introns (Goodall and Filipowicz, 1989). These sequences could be recognised as regulatory signals and lead to internal transcription in a sense or antisense orientation (Yenofsky *et al.*, 1994), or as introns, causing missplicing and degradation of the corresponding mRNA (van Aarssen *et al.*, 1995).

Also striking was the presence of the ATTTA motif, which occurred at ten sites within the Bt gene, namely 338-342, 508-512, 514-518, 595-599, 704-708, 810-814, 1286-1290, 1394-1398, 1486-1490 and 1822-1826 bp regions respectively. The role of these motifs as messenger destabilisation elements has been demonstrated for various unstable transcripts in eukaryotic cells (Shaw and

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        10         20         30         40         50         60
ATGGAGGATC CCCATGGATA ACAATCCGAA CATCAATGAA TGCATTCCTT ATAATTGTTT
        70         80         90        100        110        120
AAGTAACCCT GAAGTAGAAG TATTAGGTGG AGAAAGAATA GAAACTGGTT ACACCCCAAT
       130        140        150        160        170        180
CGATATTTCC TTGTCGCTAA CGCAATTTCT TTTGAGTGAA TTTGTTCCCG GTGCTGGATT
       190        200        210        220        230        240
TGTGTTAGGA CTAGTTGATA TAATATGGGG AATTTTTGGT CCCTCTCAAT GGGACGCATT
       250        260        270        280        290        300
TCTTGTACAA ATTGAACAGT TAATTAACCA AAGAATAGAA GAATTCGCTA GGAACCAAGC
       310        320        330        340        350        360
CATTTCTAGA TTAGAAGGAC TAAGCAATCT TTATCAAATT TACGCAGAAT CTTTTAGAGA
       370        380        390        400        410        420
GTGGGAAGCA GATCCTACTA ATCCAGCATT AAGAGAAGAG ATGCGTATTC AATTCAATGA
       430        440        450        460        470        480
TGAACAGTGC CCTTACAACC GCTATTCCTC TTTTTGCAGT TCAAAATTAT CAAGTTCCTC
       490        500        510        520        530        540
TTTTATCAGT ATATGTTCAA GCTGCAAATT TACATTTATC AGTTTTGAGA GATGTTTCAG
       550        560        570        580        590        600
TGTTTGGACA AAGGTGGGGA TTTGATGCCG CGACTATCAA TAGTCGTTAT AATGATTTAA
       610        620        630        640        650        660
CTAGGCTTAT TGGCAACTAT ACAGATTATG CTGTACGCTG GTACAATACG GGATTAGAAC
       670        680        690        700        710        720
GTGTATGGGG ACCGGATTCT AGAGATTGGG TAAGGTATAA TCAATTTAGA AGAGAATTAA
       730        740        750        760        770        780
CACTAACTGT ATTAGATATC GTTGCTCTGT TCCCGAATTA TGATAGTAGA AGATATCCAA
       790        800        810        820        830        840
TTCGAACAGT TTCCCAATTA ACAAGAGAAA TTTATACAAA CCCAGTATTA GAAAATTTTG
       850        860        870        880        890        900
ATGGTAGTTT TCGAGGCTCG GCTCAGGGCA TAGAAAGAAG TATTAGGAGTCCACATTTGA
       910        920        930        940        950        960
TGGATATACT TAACAGTATA  ACCATCTATA CGGATGCTCA TAGGGGTTAT TATTATTGGT
       970        980        990       1000       1010       1020
CAGGGCATCA AATAATGGCT TCTCCTGTCG GTTTTTCGGG GCCAGAATTC ACGTTTCCGC
      1030       1040       1050       1060       1070       1080
TATATGGAAC CATGGGAAAT GCAGCTCCAC AACAACGTAT TGTTGCTCAA CTAGGTCAGG
      1090       1100       1110       1120       1130       1140
GCGTGTATAG AACATTATCG TCCACTTTAT ATAGAAGACC TTTTAATATA GGGATAAATA
      1150       1160       1170       1180       1190       1200
ATCAACAACT ATCTGTTCTT GACGGGACAG AATTTGCTTA TGGAACCTCC TCAAATTTGC
      1210       1220       1230       1240       1250       1260
CATCCGCTGT ATACAGAAAA AGCGGAACGGTAGATTCGCTGGATGAAATA CCGCCACAGA
      1270       1280       1290       1300       1310       1320
ATAACAACGT GCCACCTAGG CAAGGATTTA GTCATCGATT AAGCCATGTT TCAATGTTTC
      1330       1340       1350       1360       1370       1380
GTTCAGGCTT TAGTAATAGT AGTGTAAGTA TAATAAGAGC TCCTATGTTC TCTTGGATAC
      1390       1400       1410       1420       1430       1440
ATCGTAGTGC TGAATTTAAT AATATAATTG CATCGGATAG TATTACTCAA ACCCTGCAGT
      1450       1460       1470       1480       1490       1500
GAAGGGAAAC TTTCTTTTTA ATGGTTCTGT AATTTCAGGA CCAGGATTTA CTGGTGGGGA
      1510       1520       1530       1540       1550       1560
CTTAGTTAGA TTAAATAGTA GTGGAAATAA CATTCAGAAT AGAGGGTATA TTGAAGTTCC
      1570       1580       1590       1600       1610       1620
AATTCACTTC CCATCGACAT CTACCAGATA TCGAGTTCGT GTACGGTATG CTTCTGTAAC
      1630       1640       1650       1660       1670       1680
CCCGATTCAC CTCAACGTTA ATTGGGGTAA TTCATCCATT TTTTCCAATA CAGTACCAGC
      1690       1700       1710       1720       1730       1740
TACAGCTACG TCATTAGATA ATCTACAATC AAGTGATTTT GGTTATTTTG AAAGTGCCAA
      1750       1760       1770       1780       1790       1800
TGCTTTTACA TCTTCATTAG GTAATATAGT AGGTGTTAGA AATTTTAGTG GGACTGCAGG
      1810       1820       1830       1840       1850       1860
AGTGATAATA GACAGATTTG AATTTATTCC AGTTACTGCA ACACTCGAGT GATAA
```

Figure 5.8: Nucleic acid sequence for the Bt234 gene.

Kamen, 1986; Laird-Offringa, 1992). Such sequences are recognised by proteins that bind specifically, causing transcript degradation (Savant-Bhonsale and Cleveland, 1992).

Finally, numerous potential polyadenylation sites, which are normally found in the 3' untranslated region of plant genes (Dean *et al.*, 1986) were also present in the Bt coding sequence. Table 5.2 lists the polyadenylation motifs present in Bt234 (underlined sequences in figure 5.8), as analysed by DNASIS for Windows, Version 2.1 (1994), Hitachi Software Engineering Co., Ltd. As mentioned, these sequences can lead to the synthesis of prematurely terminated and non-polyadenylated unstable transcripts (Mogen *et al.*, 1990).

Table 5.2: Plant polyadenylation motifs present in Bt234 gene

Polyadeny-lation Motif	**# times motif occurs in 2.0 kb Bt gene**	**Position in Bt gene**	**# times motif occurs in 0.35 kb Bt transcript**	**# times motif occurs in 1.0 kb Bt transcript**	**# times motif occurs in 1.2 kb Bt transcript**	**# times motif occurs in 1.6 kb Bt transcript**
AATAAT	3	971-976, 1137-1142, 1398-1403	0	1	2	3
AATGAA	0		0	0	0	0
AATTAT	2	465-470, 756-761	0	2	2	2
AGTATA	3	488-493, 915-920, 1347-1352	0	2	2	3

CONCLUDING REMARKS

Characterisation of UBI and SUC-1 promoters, both driving expression of the Bt transgene, was undertaken in the present investigation. Since ubiquitin has been documented to be a stress-related protein, it was hypothesised that the activity of UBI would at least be maintained, or even increased, under water stress conditions. A second hypothesis was that SUC-1 might enhance overall gene expression since the CaMV 35S component is a widely and successfully used constitutive promoter.

The following were revealed:

1. UBI activity decreases somewhat under stress, while SUC-1 activity appears to remain constant. SUC-1 would thus seem to be the promoter of choice for driving Bt gene expression in transgenic sugarcane plants in future, since expression of the gene is most important under stressful conditions when eldana infestations are more prevalent.
2. The wild-type (unmodified) Bt gene is unsuitable for sugarcane transformation, since a large proportion of incomplete transcripts are produced, probably due to premature polyadenylation. Future work will require the use of a modified version of the Bt gene.
3. An alternate model system for studying promoter activity in whole plants should also be explored, since transgenic plants have the limitation of 'positional effects' affecting transgene expression, making direct, controlled comparison between plants containing different promoters difficult.

Future Directions

Modification of the Bt gene to a more 'plant like' form could be of great value to the sugar industry by increasing toxin expression in transgenic sugarcane plants containing this gene and in this way increasing plant protection against sugarcane pests. Perlak *et al.* (1990; 1991) made modifications to key regions of the *cryIA(b)* and *cryIA(c)* structural genes that resulted in a 100-fold increase in expression in cotton plants compared to the wild-type gene sequences. The modifications eliminated ATTTA sequences and almost all potential plant polyadenylation sequences, greatly increased the G+C nucleotide content of the gene, and

minimised the use of rare plant codons. They reported the level of CryIA(b) or CryIA(c) proteins in cotton expressing the modified sequences to range from 0.05 to 0.1% of total soluble protein. Elucidating the mechanisms responsible for the low accumulation of Bt234 mRNA in sugarcane may make it easier in the future to engineer novel foreign genes and other Bt genes for high expression in sugarcane. It may also provide insight into natural gene expression mechanisms in plants.

In addition to the coding sequences, availability of regulatory sequences or promoters to target expression to appropriate cells, tissues or developmental stages is an essential component for engineering traits in transgenic plants (Gruber and Crosby, 1993; Datla, Anderson and Selvaraj, 1997). The isolation of novel promoters from sugarcane and the characterisation of already existing promoters from other sources are of key importance.

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