DEVELOPMENT OF A TRANSFORMATION SYSTEM FOR SUGARCANE (*SACCHARUM* SPP. HYBRIDS) IN SOUTH AFRICA USING HERBICIDE RESISTANCE AS A MODEL SYSTEM

Sandra Jane Snyman

Dissertation presented for the degree of Doctor of Philosophy at the University of Stellenbosch, South Africa.

December 2002

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

ABSTRACT

Conventional sugarcane breeding is limited by the complexity of the sugarcane genome, the relatively narrow genetic base of modern sugarcane germplasm and the time it takes to screen and release a new cultivar. Genetic engineering offers the potential to enhance conventional breeding by the rapid introduction of new characteristics into sugarcane. However, it is recognised that the molecular and developmental processes involved in transformation vary greatly with the recipient material. In this work, tissue culture protocols and a microprojectile bombardment-based transformation system were developed for a range of South African sugarcane cultivars. The *pat* gene, which confers resistance to the compound glufosinate ammonium and the commercial herbicide Buster®, was used as a model transgene. A co-bombardment system was used, with the selection gene *nptII* delivered on a separate vector construct.

Type 3 embryogenic callus is commonly used as target material for microprojectile bombardment in sugarcane and it was found that this form of callus was optimally produced when cycled on MS medium containing 1 and 3 mg/l 2,4-D. Although there was no genotype-specific response in mass of callus produced, NCo310 was chosen as the recipient cultivar for transient expression studies, as it showed the highest transient expression levels when compared to other cultivars. Transient expression using either GUS or anthocyanin gene constructs was used quantitatively to optimise microprojectile bombardment parameters and choice of promoter. A helium pressure of 1000 kPa and tungsten particles, as opposed to gold, were established for use in the gene delivery system. Out of four promoters tested, the ubiquitin promoter was shown to drive gene expression most effectively. Regeneration efficiency was improved by the addition of kinetin (0.5 mg/l) to the regeneration medium. To optimise selection of stable transformants, a geneticin kill curve was established and the concentration that killed 100% of untransformed cells was found to be 45 mg/l.

NCo310 was used as the recipient cultivar in stable gene expression studies. Plants were regenerated via conventional indirect embryogenesis from bombarded callus. From a total of 200 bombardments, seven plants regenerated on selection medium containing 45 mg/l geneticin. Among these, PCR analysis indicated that one line was an escape, one contained only the *nptII* gene and five were transformants. Southern blot analysis indicated that transgene copy number ranged from one to nine. Three of the transgenic plants were identical as they had

the same integration profiles and arose from the same transformation event. Both RT-PCR and phenotypic analysis as determined by glasshouse spraying with Buster (4 l/ha), confirmed that lines 22.2 and 1.7 were stably transformed, and line 22.1, which contained a single copy of the *pat* gene, was silenced. A low transformation efficiency of 1% (1 transgenic plant per 100 bombardments) was calculated.

A novel regeneration route was developed and characterised for use in a sugarcane transformation programme. It was envisaged that induction of somatic embryos directly on immature leaf roll discs on medium containing low auxin levels (0.3 mg/l 2.4-D), would facilitate shorter *in vitro* culture times. In a preliminary study, not involving transformation, a lower regeneration efficiency was observed directly from leaf discs when compared to indirect embryogenesis. However, the decreased exposure to high auxin concentrations and shorter time frame for regeneration could reduce the likelihood of somaclonal variation in plants derived from direct morphogenesis.

The use of leaf roll discs as target material for bombardment is a novel approach in sugarcane. The efficiency of transformation from bombarded leaf discs, regenerated via either direct or indirect embryogenesis, was compared to the more conventionally used embryogenic callus route. The stringency of the selection regime was reduced in an attempt to increase regeneration frequency, despite the chance of more 'escapes' surviving the process. Consequently, 15 mg/l geneticin was incorporated in the selection medium, as this inhibited growth of 75% of untransformed cells. Improved regeneration efficiencies of 28 and 23% were observed from bombarded leaf discs, regenerated via direct and indirect embryogenesis, respectively, compared to that of 6% from callus. Plants regenerated on selection medium directly from leaf discs in 13-22 weeks, and indirectly from bombarded discs in 18-26 weeks. Both approaches resulted in a more rapid production of plants than the conventional callus-based route, where plants were regenerated in 24-36 weeks. PCR, Southern and phenotypic analysis indicated that regardless of the regeneration route, the majority of plants were escapes. This could be attributed to the low stringency selection procedure. In addition, a large proportion (45%) of plants regenerated via direct somatic embryogenesis were chimaeric. Although initial PCR and phenotypic analyses of these plants indicated that they were transformed, none of the mature plants contained the *pat* gene. When the selection pressure was increased from 15 to 45 mg/l geneticin, a single transformed line was obtained via direct embryogenesis (1.7% transformation efficiency). However, once again the majority of plants

regenerating on selection medium where escapes, indicating that further optimisation of this route in a transformation system is necessary. A promising transformation route is the bombardment of leaf discs followed by indirect embryogenesis. This approach resulted in a transformation frequency of 2.5%, which is 2.5 x higher than that observed when embryogenic callus was used as target material for bombardment. However, chimaeric plants were obtained, as well as a large proportion of escapes, although the latter could be due to the low stringency used for selection (15 mg/l geneticin). Further investigation into this route is necessary.

A single transgenic line, 22.2, was field-tested to establish the stability of the *pat* transgene over several rounds of vegetative propagation. In addition, the agronomic performance of transgenic plants was compared to that of untransformed sugarcane. A preliminary field trial was carried out to determine rates of herbicide application. Line 22.2 was resistant to Buster applied at rates of up to 7 l/ha. A large-scale field trial was undertaken to compare the efficacy of four weed control treatments applied to transformed and untransformed sugarcane. Transgenic sugarcane stalk characteristics (diameter, population density, height and fibre content) were not significantly different from untransformed controls. Susceptibility to the diseases smut and SCMV were unchanged in transgenic cane. Transgenic sugarcane was less susceptible to the stalk borer, *Eldana saccharina*, although it was not apparent why this was so. The plant/herbicide combinations that were highest yielding in terms of cane mass and sucrose content were transgenic cane treated with a pre-emergent herbicide cocktail, followed by Buster application (5 l/ha), and untransformed cane treated with the conventional weed control programme. When a cost comparison was carried out, it was clear that the most economical treatments were transgenic cane treated with Buster, and transgenic cane treated with pre-emergent herbicides plus Buster. However, the pricing of the herbicide to which resistance is engineered is crucual if herbicide resistant sugarcane is to be commercially viable.

OPSOMMING

Suikerriet teling word deur die kompleksitiet van die suikerriet genoom, die relatiewe nou genetiese basis van moderne suikerriet en die tyd wat dit neem om 'n nuwe variëteit te evalueer en vry te stel. Genetiese manipulering kan konvensionele planteteling versnel deurdat nuwe eienskappe in suikerrietgenoom ingevoeg kan word. Dit is egter 'n bekend dat die transformeringspotensiaal tussen verskillende teikenmateriaal (species en variëteite) grootliks kan varieer. In hierdie studie is weefselkultuur metodes en 'n nuwe transformerings sisteem vir 'n reeks van Suid Afrikaanse suikerriet kultivars ontwikkel. Die *pat* geen, wat weerstandbiedendheid teen die chemiese stof ammoniumglufosinaat en die kommersiële onkruiddoder Buster® bied, is as 'n model oordragsgeen gebruik. 'n Ko-bombarderings sisteem is gebruik waartydens die seleksie geen *nptII* wat op 'n aparte vektor konstruksie teenwoordig was.

Tipe 3 embriogeniese kallus word algemeen as teiken materiaal vir mikroprojektiel bombardering in suikerriet gebruik. Die studie toon dat hierdie tipe kallus optimaal geproduseer word wanneer dit die weefsel afwisselend op MS-medium wat onderskeidelik 1 en 3 mg/l 2,4D bevat, gekweek word. Alhoewel kallus vorming nie genotipe spesifek was nie is NCo310 gekies as die teiken kultivar vir oorgangsgeen-uitdrukking studies gebruik. Die besluit is geneem omdat die hoogste oorgangsgeen-uitdrukking by die kultivar voor gekom het. Oorgangsgeen-uitdrukking waar óf die GUS-geen óf antosianientranskripsiefaktore gebruik is, is kwantitatief gebruik om mikroprojektiel bombarderings parameters en die keuse van promotor te optimiseer. 'n Helium druk van 1000 kPa en wolframstaal deeltjies, in plaas van goud deeltjies, is gebruik. Uit die vier promotors wat getoets is, het die ubiquitin promotor die beste resultate gelewer om geen uitdrukking aan te dryf. Regenerasie doeltreffendheid is met die byvoeging van kinetien (0.5 mg/l) verbeter. 'n Genetisien afsterwingskromme het aangetoon dat 45 mg/l die optimale seleksietoestande was.

NCo310 is ook vir stabiele geenuitdrukking studies gebruik. Plante is deur middel van konvensionele indirekte embriogenese van gebombardeerde kallus geregenereer. Uit 'n totaal van 200 bombarderings is sewe plante geregeneer op seleksie medium wat 45 mg/l genetisien bevat het. Met behulp van die polimerase ketting reaksie (PKR) is aangetoon dat een lyn geen transgeen bevat het nie, dat een lyn net die *nptII* geen bevat het en dat vyf lyne beide die transgene bevat het. Gebaseer op Southern kladanalise het die transgeniese lyne tussen een en

tien kopiee van transgeen bevat. Die ontleding het verder aangetoon dat drie van die transgeniese plante identies was m.a.w. dat hulle uit 'n enkele getransformeerde sel ontstaan het. Beide RT-PKR en fenotipiese analise deur die plante met Buster in glashuise te bespruit (4 l/ha), het bevestig dat lyne 22.7 en 1.7 stabiel getransformeer was. Geenuitdrukking in lyn 22.1, wat 'n enkele kopié van die *pat* geen bevat het, is egter uitgedoof. 'n Lae transformeringsdoeltreffendheid (1 transgeniese plant per 100 bombarderings) is behaal.

'n Nuwe regenerasie roete vir gebruik in 'n suikerriet transformerings-program is ontwikkel. In hierdie benadering was die veronderstelling dat induksie van somatiese embrio's direk op onvolwasse blaarrol-snitte, op 'n medium wat lae ouksien vlakke (0.3mg/l 2.4.D) bevat tot 'n korter *in vitro* kultuur periode sal lei. Alhoewel regenerasie doeltreffendheid direk van blaarrol-snitte af laer was as vanaf indirekte embriogenese, het die verminderde blootstelling aan hoë ouksien konsentrasies en korter generasie periodes die moontlikheid van somatiese variasie in plante waarskynlik verminder.

Die gebruik van blaarrol-snitte as teiken materiaal vir bombarderings is 'n nuwe benadering in suikerriet. Die transformeringsdoeltreffendheid van gebombardeerde blaarrol-snitte, wat óf deur direkte óf indirekte embriogenese geregenereer is, is vergelykbaar met die meer konvensionele embriogeniese kallus roete. Om die regenerasie to probeer verbeter is die seleksiedruk na 15 mg/l genetesien verminder. Dit het tot 'n regenerasie doeltreffendheid van onderskeidelik 28 en 23 % by gebombardeerde blaarrol-snitte wat deur middel van direkte en indirekte embriogenese gelei. In vergeleke, is daar net 6 % regenerering vanaf kallus verkry. Plante wat direk van blaarrol-snitte afkomstig was, het 13-22 weke geneem om te ontwikkel. Die wat indirek van gebombardeerde snitte afkomstig was, het 18-26 weke geneem. Beide hierdie benaderings het tot gevolg dat 'n vinniger produksie van plante moontlik is in vergelyking met die konvensionele kallus-gebaseerde roete wat 24-36 weke neem. PKR, Southern kladanalise en fenotipiese analises het getoon dat ongeag die regenerasie roete, die meeste plante wat geproduseer was nie transgenies was nie. Die lae seleksiedruk waarskynlik die rede hiervoor. 'n Hoë persentasie (45 %) van plante wat deur direkte somatiese embriogenese geregenereer is, is chimeries. Ten spyte van die feit dat die aanvanklike PKR en fenotipiese analises aangedui het dat hierdie plante getransformeer was, het nie een van die volwasse plante die *pat* geen bevat nie. Nadat seleksiedruk verhoog is van 15 tot 45 mg/l genetisien, is 'n enkele getransformeerde lyn deur middel van direkte embiogenese (1.7 % transformeringsdoeltreffendheid) gekry. Ondanks verhoging van seleksiedruk was die meeste

plante weereens nie transgenies nie. Dit toon duidelik op dat verdere optimalisering van hierdie roete in 'n transformerings sisteem nodig is. Dit wil voorkom asof bombardering van blaarrol-snitte opgevolg deur indirekte embriogenese 'n belowende transformasie roete mag wees. Hierdie benadering het tot 'n transformasie frekwensie van 2.5 % gelei, wat 2.5 keer hoër is as wat met die embriogeniese kallus verkry is. Ongelukkig is daar nog baie plante was deur die proses kom wat nie getransformeer is nie of wat chimeras is. Dit is waarskynlik toe te skryf aan te lae seleksiedruk (15 mg/l genetisien) wat in die studie toegepas is.

'n Enkele transgeniese lyn, 22.2, is onder veldtoestande getoes om vas te stel of uitdrukking van die *pat*-transgeen stabiel is gedurende opeenvolgende vegetatiewe voortplantingsiklusse. 'n Voorlopige veldproef is uitgevoer om die optimale konsentrasie van onkruidmiddels te bepaal. Lyn 22.2 was weerstandbiedend teen Buster wat teen 'n konsentrasie van tot 7 l/ha toegedien is. 'n Grootskaalse veldproef om die doeltreffendheid van vier onkruiddoders te toets op getransformeerde en ongetransformeerde suikerriet is uitgevoer. Die transgeneiese suikerriet stingels se kenmerke (deursnit, populasie digtheid en veselinhoud) was nie betekenisvol verskillend as dié van ongetransformeerde kontrole plante nie. Transgeniese suikerriet se vatbaarheid teen siektes soos brandswam en mosaïek was onveranderd. Transgeniese riet was minder vatbaar vir die stingelboorder *Eldana saccharina*, alhoewel die rede hiervoor nie duidelik is nie. Die plant/onkruiddoder kombinasies wat die hoogste rietmassa en sukrose inhoud gelewe het was transgeniese riet wat behandel is met 'n voor-opkoms onkruiddoder mengsel gevolg met die toediening van Buster (5 l/ha) en ongetransformeerde suikerriet wat behandel is met 'n konvensionele onkruid beheer program. 'n Koste analise toon dat die transgeniese riet wat behandel is met die voor-opkoms onkruiddoder Buster, die mees ekonomiese kombinasie is. Ooglopend sal die prys van die onkruiddoder waarteen die weerstand verkry is, 'n kritieke faktor wees in besluitneming on die transgeniese suikerriet kommersieël beskikbaar te stel.

ACKNOWLEDGEMENTS

A heartfelt thanks to my supervisors Drs Frikkie Botha and Barbara Huckett for their unflagging advice, support and encouragement over the last four years.

Sincere thanks to Dr Peter Hewitt, Director SASEX, for permitting me to submit research carried out at the Experiment Station for a PhD thesis.

To Noel Leibbrandt, formerly of the Agronomy Department, SASEX, a special word of thanks for his enthusiasm and interest during this study and for conducting the trial on agronomic performance of transgenic sugarcane reported in Chapter 6.

In addition, I would like to extend a special word of thanks to the following people:

Gwethlyn Meyer of the Biotechnology Department, SASEX for her laboratory assistance, encouragement and spiritual upliftment,

The Tissue Culture group of the Biotechnology Department at SASEX for excellent technical assistance in the laboratory and glasshouse,

Dr Paula Watt, Assistant Dean: Faculty of Science, University of Natal, Durban, for useful discussions and the use of facilities in the School of Life Sciences,

Nishen Govender and Murt Murdock of the Biometry Department at SASEX for their advice on statistical analyses,

Rob Donaldson of the Agronomy Department, SASEX, for useful discussions, especially with regard to agronomic performance of sugarcane,

Raj Balbador of the Public Relations Department at SASEX for his assistance with photography,

Sharon MacFarlane of the Pathology Department for providing the photographs in Figures 6.3A and 6.3C,

Graham Leslie of the Entomology Department at SASEX for providing the photograph in Figure 6.4A,

Julie Barnes of the Biotechnology Department at SASEX for proof-reading the manuscript,

Tania van Antwerpen, SASEX, for translating the Abstract into Afrikaans,

Sagie Dorasamy and Ronnie Padaychee, SASEX, for their assistance with printing,

And last, but not least, thanks to Terry Snyman, my husband, for years of patience and understanding, and putting Gareth and Justin to bed on all those nights when I was 'climbing my mountain'!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2,4-D	2,4-dichloro-phenoxyacetic acid
35S	Cauliflower mosaic virus 35 ribosomal subunit
ANOVA	analysis of variance
ATP	adenosine tri-phosphate
BAP	benzyl-amino purine
bar	bialaphos resistance
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
Kb	kilo base pair
CaMV	cauliflower mosaic virus
CAT	chloramphenicol transferase
cDNA	complementary DNA
CPA	4-chlorophenoxyacetic acid
CTAB	cetyl triethylammonium bromide
DNA	deoxyribonucleic acid
dCTP	deoxycytosine triphosphate
dNTP	deoxynucleoside triphosphate
DPX	microscopy mountant consisting of distyrene (a polystrene), plasticiser (tricersyl phosphate) and xylene
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbant assay
Emu	Emu synthetic monocotyledonous promoter
ERC	estimated recoverable crystal
FAA	formalin-acetic-alcohol
G418	geneticin
GFP	green flourescent protien
GUS	β-glucuronidase
ha	hectare
IBA	3-indolebutyric acid
LSD	least significant difference
MAR	matrix attachment region

Mg	megagrams (=1000 kg)
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
MS0.3	Murashige and Skoog medium with 0.3 mg/l 2,4-D
MS1	Murashige and Skoog medium with 1mg/l 2,4-D
MS3	Murashige and Skoog medium with 3 mg/l 2,4-D
NAA	naphthyleneacetic acid
nos	nopaline synthase
NPTII	neomycin phosphotransferase
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PIG	particle inflow gun
PPT	phosphinothricin
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RM	regeneration medium
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SA	South Africa
SASEX	South African Sugar Association Experiment Station
SCMV	sugarcane mosaic virus
SDS	sodium dodecyl sulphate
SE	standard error
SED	standard errors of differences of means
spp.	species
SSPE	sodium, sodium phosphate and EDTA buffer
TDZ	thidiazuron
TE	Tris-EDTA buffer
Tn5	transposon 5 from *E.coli*
Tris	tris(hydroxymethyl)-aminomethane
Ubi-1	maize polyubiquitin-1 promoter
UV	ultra violet
X-Gluc	5-bromo-4-chloro-3-indolyl-β-glucuronic acid

CHAPTER 1
GENERAL INTRODUCTION

1.1 SUGARCANE AS A CROP

Sugarcane is a hybrid (*Saccharum* species hybrids) belonging to the Gramineae. It is best grown commercially in tropical and sub-tropical areas that are characterised by warm temperatures, high incident solar radiation and annual rainfall, and deep fertile soils (Barnes, 1974). Sugarcane is cultivated in over 120 countries and is the source of approximately 50% of the world's sugar (Anonymous, 2000a). In addition, it is used as a raw material for ethanol production in Brazil, which is the largest producer of cane sugar in the world (http://apps.fao.org). South Africa ranks 13th in world-production of cane sugar, currently manufacturing 2.5 million Mg sugar per annum (Anonymous, 2000a).

The phenomenon of yield or productivity plateau that has been observed globally in many crop plants, has been well researched for sugarcane in Australia (Bull and Tovey, 1974; Garside, 1997; Garside *et al.*, 2001). Due to the loss of productive capacity of soils under sugarcane monoculture, sugar yield increased at a rate of 0.01 million Mg sugar/ha/annum for the period 1970-1990. This, combined with factors such as the sucrose storage limits of 14% (g sucrose/100 g fresh mass plant) reached by the genotypes currently in use (Heinz, 1987) and increasing world sugar production figures of 130 million Mg for 2001, continue to drive sugar industries to increase output. Another major economic factor world-wide affecting yield is crop damage caused by insects. Large stands of single crop species have long been recognised as favouring dramatic increases in pest species, despite the use of chemical pesticides, insect parasitoids and modified cropping practices (Thomas *et al.*, 1995a, 1995b).

Sugarcane was first grown in South Africa in 1848, and by 1900 there were thirty mills producing 16 000 Mg of sugar from 2 600 ha of land (O'Reilly, 1998). The South African sugar industry is located in the region of latitude 30° South and longitude 30° East, with 90% of the sugarcane being grown under mainly rain-fed conditions in the province of KwaZulu Natal, and the balance under irrigation in the more northerly province of Mpumalanga (Figure 1.1). Sugarcane in KwaZulu Natal is grown under fairly adverse conditions in that the area is the furthest south that sugarcane is found in the world, and its viability is only possible due to the influence of the warm Mozambique current which flows 15 km offshore (O'Reilly, 1998).

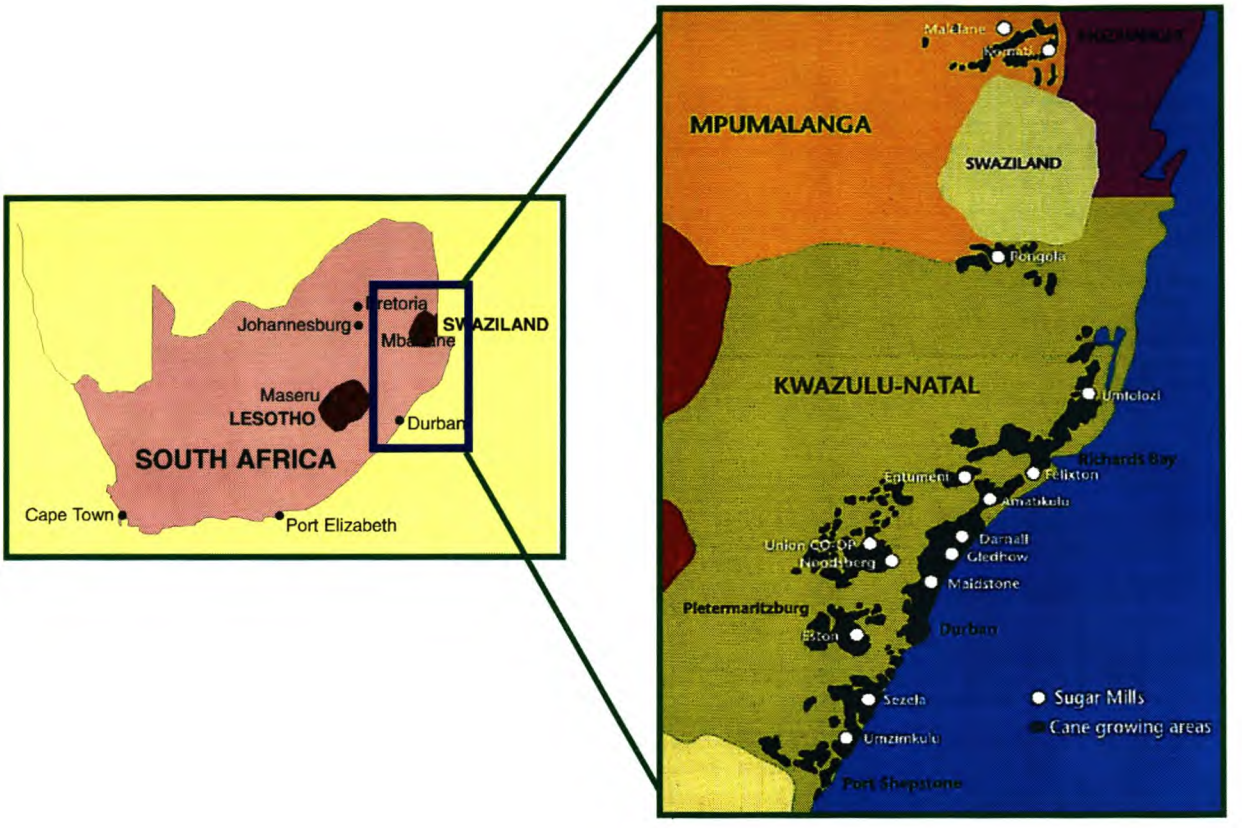


Figure 1.1 Map showing South African sugarcane growing regions.

The South African Sugar Association Experiment Station (SASEX) at Mount Edgecombe is responsible for producing the commercial sugarcane cultivars grown in South Africa and much of southern Africa. Cultivars released in the industry have been selected primarily for sucrose yields of 10-14%, but traits such as disease and pest resistance, and the improvement of yields in marginal environments are also seen as important criteria in the breeding programme (Nuss, personal communication) (Table 1.1).

1.2 LIMITATIONS OF CONVENTIONAL SUGARCANE BREEDING

Sugarcane breeding as we know it today began only 100 years ago in Java in response to the need for resistance to sereh disease. One of a few locally avaliable *Saccharum spontaneum* clones, Glagah (2n=112), was used in an interspecific hybridisation with *S. officinarum*, known as 'noble cane'. A selected hybrid was backcrossed to noble types (nobilisation), and the process was repeated until in 1921 the first 'supercane' POJ2878 was produced (Barnes, 1974). Several of the POJ canes are ancestors of the N and NCo cultivars currently widely grown in the South African sugar industry. This means that modern commercial sugarcane is derived from a narrow genetic base, consisting of the germplasm of about 20 *S. officinarum* clones and

less than 10 *S. spontaneum* derivatives (Arceneaux, 1965). Nowadays, the outcome of crosses is unpredictable because of the highly polyploid genome of commercial parents (2n=40-120) (Price, 1967). This is as a result of the 2n+n chromosome transmission that occurred in early nobilisations due to a phenomenon known as 'female restituition', where the number of chromosomes contributed by the female parent, *S. officinarum,* increased with respect to the parent.

Table 1.1 Agronomic characteristics of selected South African sugarcane cultivars.

Cultivar	*Year of release*	*Proportion (%) of 2001 crop*	*ERC % cane*	*Best features*	*Shortfalls*
NCo376	1955	18.7	13.8	Ratooning ability and adaptability	Susceptible to drought, SCMV and smut
N12	1979	31.9	14.7	High sucrose	Susceptible to eldana
N19	1986	14.2	14.2	High sucrose, resistance to smut	Susceptible to eldana and SCMV
N31	1997	0.1	12.5	Resistance to rust	Low sucrose content, susceptible to smut
N38	2001	N/a	12.8	Resistance to rust, leaf scald and smut	Very susceptible to eldana, low fibre content

ERC % cane (estimated recoverable crystal, expressed as a percentage of the fresh mass of the sugarcane plant; gives an estimate of the sucrose content).
Eldana (most prevalent insect pest: *Eldana saccharina* Walker Lepidoptera)
SCMV (sugarcane mosaic virus)
Fibre (the insoluble portion of a sugarcane stalk; can be used as an indication of how easy it is to mill)
N/a (data not available)
Smut (causal agent *Ustilago scitaminea*)
Rust (causal agent *Puccinia melanocephala*)
Leaf scald (causal agent *Xanthomonas albilineans*)

In South Africa, crossing has to be carried out in specially constructed glasshouses, as the external environmental conditions inhibit the production of viable pollen and seed (Brett, 1974). Once seed has been produced, screening and selection takes place over a 14-year period before a new variety is released (Butterfield and Thomas, 1996). In addition to the long selection period, breeding for disease or pest resistance is a very imprecise process. Sugarcane is therefore a prime candidate for the application of genetic engineering, which may provide an alternative to the conventional but somewhat problematic sugarcane breeding.

1.3 GENETIC ENGINEERING

Genetic engineering has opened new avenues to modify crops. Genetic transformation permits the use of exotic genetic material, as well as the modification of the expression of genes within the plant's own genome. In principle, transformation permits the directed introduction of characters not possible by other techniques. There is the potential to enhance productivity

through disease, pest and herbicide resistance, and environmental stress reduction. Transgenic plants are also being used to produce pharmaceutical products (Giddings *et al.*, 2000) and edible vaccines (Johnson, 1996; Daniell *et al.*, 2001; Richter *et al.*, 2001). This has been possible by introducing novel genes via techniques such as *Agrobacterium*–mediated gene transfer and microprojectile bombardment, which have provided genotype-independent systems for transformation of a wide range of species.

The natural ability of *Agrobacterium* to transform plants has resulted in the successful transformation of most dicotyledonous species. Traditionally, monocotyledons have been recalcitrant to transformation by this bacterium, but there are some reports of the use of this approach in wheat (Cheng *et al.*, 1997), rice (Dong *et al.*, 1996), maize (Kemper *et al.*, 1996), barley (Tingay *et al.*, 1997) and sugarcane (Arencibia *et al.*, 1998b; Elliot *et al.*, 1998; Enriquez-Obregon *et al.*, 1998) that suggest this may no longer be a limitation. However, this technique is not routinely used for the genetic engineering of sugarcane.

A gene transfer technique which has been successfully used in dicotyledonous plant species is direct DNA uptake by protoplasts which have been isolated from the mesophyll layer in leaves. In the graminaceous monocots, however, protoplasts from leaves are not capable of sustained cell division (Vasil, 1987), so embryogenic suspension cultures have been used as a source of totipotent protoplasts (Bhaskaran and Smith, 1990; Bommineni and Jauhar, 1997). Although it is difficult and labour-intensive to initiate and maintain these liquid cultures, and regenerative capacity has been observed to decline gradually (Jahne *et al.*, 1995), early work in sugarcane reported the recovery of transgenic plants from suspension culture cells (Sun *et al.*, 1993).

The use of embryogenic callus cultures for DNA transfer, particularly by microprojectile bombardment has increased the ability to transform members of the Gramineae (Bower and Birch, 1992; Bommineni and Jauhar, 1997). The advantage of using regularly maintained callus is that cells are in an undifferentiated state and are capable of regenerating into plants. Sugar industries world-wide have employed the use of embryogenic callus as target material for microprojectile bombardment in genetic engineering programmes for the production of transgenic sugarcane (Bower and Birch, 1992; Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996; Ingelbrecht *et al.*, 1999; Falco *et al.*, 2000). Despite this common approach, transgenic plant production ranges widely from 1 to 20 plants per bombardment (Falco *et al.*, 2000, Bower *et al.*, 1996, respectively). Although factors such as the choice of cultivar, genotypic

responses *in vitro* and selection regime may account for the differences in efficiency reported, these observations suggest that this approach cannot be universally applied.

Aside from varying levels in tranformation efficiency in sugarcane, the use of embryogenic callus as target material for bombardment has other limitations. Establishing, developing and maintaining callus cultures is labor intensive and the recovery of plants ready for transfer to the glasshouse may take as long as 36 weeks (Bower *et al.*, 1996). In addition, reports of field trials of transgenic sugarcane stands in Australia revealed a high incidence of phenotypic variability (Hansom *et al.*, 1999; Grof, 2001; Grof and Campbell, 2001). This may be attributed to somaclonal variation or collateral damage due to the transformation process. Recent literature has focused on this occurrence in transgenic oat (Choi *et al.*, 2000b) and barley (Bregitzer *et al.*, 1998b). Curiously, little has been published on the field performance of transgenic sugarcane. In addition, information about transgene expression in this highly polyploid crop is scant.

For commercial applications, and because transgene expression may be affected by both genetic backgrounds and environmental conditions, it is important to assess and select desirable transformants. Transgenic evaluation can be carried out at two levels. One is at the molecular level of transgene expression and the other at the level of transgenic traits (product) of interest.

1.4 HERBICIDE RESISTANCE AS A MODEL SYSTEM

Herbicide resistant crops represent one of the first and most highly publicised applications of plant biotechnology. Of the many traits that can be altered or conferred through genetic engineering, herbicide resistance was chosen to optimise a system to produce genetically transformed sugarcane in South Africa. A simple gene expression system was needed to assess both transformation efficiency in the laboratory and stable gene expression in the field. Herbicide resistance as a model system has a number of advantages: the genes responsible for resistance have been isolated, characterised and inserted into several major crops, it is easy to test phenotypic expression of the introduced gene, and plasmid constructs are readily available from agrochemical companies. In addition to these technical considerations, the agronomic importance of herbicides is a driving force behind the development of herbicide resistant plants.

Herbicide resistance using the *pat* gene was chosen as a model system for the South African sugarcane transformation programme. L-Phosphinothricin (PPT) or the ammonium salt, glufosinate, is the active compound of the non-selective herbicide Basta (or Buster® in South Africa; Aventis). The enzyme PAT, encoded by the *bar* or *pat* genes (same function, but from different sources), inactivates PPT by acetylating it (Botterman and Leemans, 1988). Transgenic plants expressing the *pat* gene are resistant to the herbicide Buster. Some of the first transgenic crops to be field-tested and released commercially are oilseed rape, chicory, maize and soybean which are resistant to glufosinate ammonium (Lehmann, 1999).

Many monocotyledonous transformation systems use PPT resistance as a means of selecting stable transformants (Spencer *et al.*, 1990; Somers *et al.*, 1992; Toki *et al.*, 1992; Hartman *et al.*, 1994; Dennehey *et al.*, 1994; Asano *et al.*, 1998; Iser *et al.*, 1999; Uze *et al.*, 1999; Petolino *et al.*, 2000; Sorokin *et al.*, 2000). However, in this study it was decided that the *nptII* selectable marker was to be used, as the work of Bower *et al.* (1996) indicated that this was a superior selection system in sugarcane.

1.5 AIMS OF THE STUDY

Using *pat* as a transgene and a procedure similar to that developed by Bower *et al.* (1996) and Gallo-Meagher and Irvine (1996) for microprojectile bombardment of embryogenic callus in sugarcane, a protocol for transformation of South African cultivars was developed [Chapters 3 and 4]. However, due to low transformation efficiency compared to other published reports, a novel approach was developed which involves bombardment of leaf discs followed by regeneration via direct somatic embryogenesis [Chapter 5]. Due to the reduced costs, labour input and time in culture, it is argued that this may lend itself to being used as an alternative transformation system in other sugar industries.

Herbicide resistant plants were analysed at a molecular level by PCR and Southern analysis to determine the presence of the transgene and copy number in the sugarcane genome. In addition, phenotypic assessment by glasshouse spraying was carried out [Chapters 4 and 5]. A single clone was field-tested to establish the stability of the transgene over several rounds of vegetative propagation as well as agronomic performance in terms of morphological characteristics, pest and disease resistance and yields [Chapter 6]. In addition, the economic advantages of growing transgenic, herbicide-resistant sugarcane are presented.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

Genetic improvement of crops has traditionally been achieved through sexual hybridisation between related species, which has resulted in numerous cultivars with high yields and superior agronomic performance. More recently, through the introduction of new tools of biotechnology, crossing barriers have been overcome, and genes from unrelated sources have become available to be introduced asexually into plants. The first reports dealing with attempts to express engineered foreign genes in plants were published in the late 1960's (Ledoux and Huart, 1968, 1969). Despite the promise of biotechnological methods, significant problems arose in the practical application of these methods for crop improvement, particularly in the graminaceous monocotyledons. This group of plants was initially difficult to engineer, mainly due to their recalcitrance to *in vitro* regeneration and their resistance to *Agrobacterium* infection (reviewed by Vasil, 1987; Jahne *et al.*, 1995; Repillin *et al.*, 2001). Systematic screening of cultivars and explant tissues for regeneration potential, development of various DNA delivery methods and optimisation of gene expression casettes have produced transformation protocols in the major cereals and graminaceous monocots, although some elite cultivars still remain recalcitrant (reviewed by Christou, 1995; Jahne *et al.*, 1995; Birch 1997; Kempken, 2001). Most transgenic plants released commercially exhibit herbicide and/or insect resistance, traits that are controlled by a single gene (reviewed by Fisk and Dandekar, 1993; Estruch *et al.*, 1997; Hansen and Wright, 1999). The introduction of more complex traits, such as an improvement in the nutritional quality of rice (Tyagi and Mohanty, 2000), are underway.

This review will focus on techniques (and associated problems) used to insert foreign DNA in to monocot genomes and the regeneration of transgenic plants from cultured cells. The current challenges for genetic engineers such as somaclonal variation, transgene silencing, instability and rearrangement will be discussed. Although the response of graminaceous monocots will be highlighted, reference will be made to dicotyledonous plants where appropriate.

2.2 SUGARCANE BREEDING

2.2.1 History of breeding

Modern sugarcane varieties are complex hybrids derived from *Saccharum officinarum*, the noble sugarcanes; *S. barberi*, the Indian sugarcanes; *S. sinense*, the Chinese sugarcanes; and

two wild species *S. spontaneum* and *S. robustum* (Daniels and Roach, 1987; Irvine, 1999). The genes for sucrose accumulation are derived from *S. officinarum*, *S. barberi* and *S. sinense*. *S. officinarum* has the qualities of high sucrose content and low fibre (fibre is the insoluble portion of the dry matter). This, together with their large size and good appearance, evidently led to their being referred to as the 'noble canes'. However, these varieties were limited in number and yield potential, and were susceptible to diseases and pests. The wild species *S. spontaneum*, while possessing low levels of sucrose, has the attributes of pest and disease resistance, tolerance to environmental stress and greater yield potential through higher biomass production (Sreenivasan *et al.*, 1987).

It was the pioneering work of the Dutch breeders in Indonesia in the late 1800's that provided the basis for breeding high-yielding cultivars. Sugarcane breeding as we know it today began in Java in response to the need for resistance to sereh disease (Sreenivasan *et al.*, 1987). The simultaneous discovery of the fertility of sugarcane in both Java and Barbados prompted the early sugarcane breeders to make interspecific crosses between *S. officinarum* and the wild *S. spontaneum* (which was resistant to sereh) (Stevenson, 1965). These interspecific hybrids were continually backcrossed to *S. officinarum* in a process called nobilisation (Stevenson, 1965). Nobilisation thus became established as a method of retaining the desirable qualities of *S. officinarum*, retaining the hardiness and disease resistance of *S. spontaneum*, while diluting the negative effects of the wild germplasm (Berding and Roach, 1987). As a result of nobilisation, a limited degree of genetic diversity exists between sugarcane cultivars, which limits the gene pool for introgression into new germplasm (Berding and Roach, 1987).

2.2.2 Genetic complexity of sugarcane

Cyological studies by Bremer (1961) showed that nobilisation is characterised by asymmetric chromosome transmission. In a cross between *S. officinarum* (2n=80) as the female parent and *S. spontaneum* (2n=40-128) as the male parent, *S. officinarum* generally transmits two haploid chromosome sets while *S. spontaneum* transmits one. This 2n+n transmission is continued up to the second backcross. A consequence of this is that modern cultivars have chromosome numbers ranging from 100-130, of which only 5-10% come from *S. spontaneum* (D'Hont *et al.*, 1996). In addition to ploidy levels that range from 5X to 14X (X=5, 6, 8, 10, 12, or 14), chromosomal mosaicism has been reported (Burner and Legendre, 1994; D'Hont *et al.*, 1998).

Sugarcane is among the most genetically complex crops. Commercial cultivars are interspecific

hybrids of up to five parental species, and many are cytogenetic aneuploids having a large and variable chromosome number (Bennet and Smith, 1976). The elevated ploidy levels and the cytogenetic complexity of interspecific hybrids contributed to the failure of classical genetic studies to establish a single Mendelian-inherited trait in sugarcane until 1991 (Moore and Irvine, 1991).

2.2.3 Narrow genetic base of sugarcane cultivars

Although the interspecific hybridisation programmes that were initiated in the 1890's in Java revolutionised sugarcane breeding, the genetic base of most modern sugarcane cultivars is narrow in relation to the potential range of genetic material available. During the early work on nobilisation, only a few of the large number of potential clones of the noble canes, *S. officinarum*, were used for breeding purposes, while the use of 'non-*officinarum*' sources was even more restricted (Berding and Roach, 1987). During the early 1900's a limited amount of material was available for setting up the early crosses and a wild cane Kassoer (originating from Java, and thought to be a spontaneous interspecific hybrid of *S. officinarum* and *S. spontaneum*) was used in several crosses with *S. officinarum* (Bremer, 1961; Sreenivasan *et al.*, 1987). This resulted in the production of different seedlings given the designation POJ. Line POJ2878 played an important role in breeding programmes in Hawaii and at Coimbatoire, India. As the South African sugar industry relied heavily on Co (Coimbatore) seedlings derived from POJ crosses in India, the NCo (selected in Natal from Coimbatore seedlings) crosses which form the basis of the local breeding programme, can trace their ancestry to the POJ plants. Consequently, there is a narrow genetic base from which most commercial international cultivars arise.

The technique of backcross breeding is widely used when breeders wish to transfer genes from exotic germplasm in to cultivated varieties. In backcross breeding, the hybrid derived from a cross between the donor and recurrent parent is crossed back to the recurrent parent and the progeny are screened for the target character. The selected individuals are crossed again to the recurrent parent and the process is repeated. After several cycles, plants are obtained that are nearly identical genetically to the recurrent parent, with the exception that the genes for the target character have been added. However, two persistent problems with conventional backcrossing in sugarcane are 1) the rate of return to the recurrent genotype in those regions of the genome unlinked to the gene/s being introgressed, and 2) elimination of undesirable genes closely linked to the desirable genes (Moore and Irvine, 1991). In addition, backcrossing is

time consuming and ineffective in transferring complex genetic characters (Nuss, personal communication).

2.2.4 Current status of the South African breeding programme

The South African Sugar Association Experiment Station (SASEX) Plant Breeding Department is responsible for producing the commercial sugarcane cultivars grown in South Africa and much of southern Africa. Every year 180 000 candidate cultivars obtained from seed produced after cross pollination of selected parents, enter the selection programme (Butterfield, personal communication). These potential new commercial cultivars undergo a six-stage selection programme, which takes between 12 and 15 years to complete. During the selection process, lines are chosen for their sucrose content, sucrose yields per hectare, and resistance to the diseases and pests that occur within the industry. There is a large effect of disease on the programme, with up to 40% of the best cultivars at each of the later selection stages being rejected due to susceptibility to various diseases and pests (Table 2.1) (Butterfield and Thomas, 1996). This is a significant hidden cost to the South African sugar industry, as many potentially high yielding cultivars are not released because of disease problems.

Table 2.1 Number of South African cultivars that are discarded from the SASEX breeding programme. These cultivars ranked high for sucrose yield, but were discarded at an advanced stage of the selection programme due to disease susceptibility (from Butterfield and Thomas (1996)).

	Number of cultivars discarded		
Disease	*Stage 3*	*Stage 4*	*Stage 5*
Mosaic	17	2	2
Smut	29	11	3
Rust	23	6	1
Gumming	11	5	0
Leaf scald	1	2	0
other	7	3	-
eldana	-	-	3
Total	88/230 (38%)	29/64 (45%)	9/26 (35%)

In the context of classical breeding, the application of genetic engineering methods to commercially important sugarcane cultivars could play an increasingly important role in solving some fundamental challenges that face crop production. This could include elevating yields by improving agronomic traits such as enhancing pest, stress and herbicide resistance. The advent of molecular technologies provides an important adjunct for classical breeding to widen the gene pool to accomplish these tasks.

2.3 ADVANCES IN BREEDING SHOWN BY GENETIC ENGINEERING TO DATE

The genetic engineering of plants has allowed genes to be transferred to the target species from a wide variety of sources, including higher and lower plants, bacteria and animals. In many cases there was no source of the desired trait in the gene pool for conventional breeding. Since genes from nearly any source can be engineered to express in plants, the range of potential products that can be produced by transgenic plants is extensive (reviewed by Dunwell, 1999) and is beyond the scope of this review. However, below is an indication of the range of genes that have been transferred to plants:

- insect resistance from *cry* genes of *Bacillus thuringiensis* has resulted in commercial releases in maize (Koziel *et al.*, 1993), cotton and potato (reviewed by Estruch *et al.*, 1997; Bhau and Koul, 1998; de Maagd *et al.*, 1999),
- an array of disease resistance genes such as antifungal proteins (chitinases, β-1,3-glucanases and phytoalexins) (Cornelissen and Melchers, 1993; Zhou *et al.*, 1994; Jach *et al.*, 1995; Cary *et al.*, 2000; Dahleen *et al.*, 2001), antimicrobial reactive oxygen species, secondary metabolites, proteins and peptides (reviewed by Ryan, 1990; Horn *et al.*, 1996; Gao *et al.*, 2000; Kempken, 2001) and hydrolytic enzymes (Rajaskaran *et al.*, 2000),
- virus resistance via several strategies (reviewed by Baulcombe *et al.*, 1986; Beachy *et al.*, 1990; Fitchen and Beachy, 1993; Kavanagh and Spillane, 1995; Baulcombe, 1996; Dawson, 1996) and
- herbicide resistance to many compounds (reviewed by Botterman and Leemans, 1988; Mullineaux, 1992; Tsaftaris, 1996; Owen, 2000).

An important factor in the development of a successful genetic engineering system involves the ability to recognise transformants. An efficient selection system is imperative (section 2.6), but establishing whether a novel gene is expressing at satisfactory levels, especially if the gene encodes pest or disease resistance, is more complicated as it involves an interaction between the plant and invading pest. Herbicide resistance therefore offers a great advantage for confirmation of stable gene integration, especially once plants are transferred to the soil. Herbicide resistance can be monitored by spraying plants or seedlings in the soil or by a localised application of herbicide to the leaves with a brush. The herbicide resistance gene can be physically linked to other genes conferring agronomically useful traits that do not provide selectable or directly screenable phenotypes during the initial stages of transformation.

2.3.1 Herbicide resistance as a model system

The development of herbicide resistance in crop plants using genetic engineering was one of the first applications of this technology to a commercially important objective (reviewed by Botterman and Leemans, 1988; Hathway, 1989; Burnside, 1992; Kishore *et al.*, 1992; Mullineaux, 1992; Tsaftaris, 1996; Freyssinet and Cole, 1999). In the USA, the use of herbicide resistant crops has significantly reduced the total amount of herbicide used on agricultural crops, and the ecological advantage is that biodegradable herbicides such as glyphosate and phosphinothricin can be used (Kempken, 2001). In addition, yield increases have been observed in transgenic herbicide-resistant crops such as sugarcane under controlled, small-scale field trials (Leibbrandt and Snyman, 2001) and in commercially-grown canola, chicory, cotton, maize and soybean (Nap, 1999).

Herbicide resistance has also served as a model system for many of the methods that are now routine in experiments using plant transformation. There are a number of reasons for this. First, the mechanisms of herbicide resistance were partly understood prior to the first attempts to genetically engineer resistance in plants. This arose from the isolation in the laboratory of resistant strains of bacteria, the selection of herbicide-tolerant plant cells in tissue culture and the study of field-selected, herbicide-tolerant weed and crop species (Hathway, 1989). Second, from such studies it was clear that the introduction of a single gene into a plant was likely to result in the desired phenotype (i.e. herbicide tolerance) which could be readily recognised and was well within the scope of the technology existing in the 1980's (Botterman and Leemans, 1988). Third, the main supporters of this work were (and still are) agrochemical companies (Mullineaux, 1992).

Herbicide resistance introduced in to crops by genetic modification currently involves mainly two herbicides: phosphinothricin (or glufosinate) marketed by Aventis (France) under various brand names such as Basta, Buster (in South Africa), Finale and Liberty; and glyphosate marketed by the USA-based company Monsanto as Roundup (Nap, 1999). Both chemicals are broad-spectrum herbicides that make no distinction between crops and weeds.

2.3.1.1 Phosphinothricin resistance

Bialaphos is a tri-peptide compound consisting of two alanine residues, which are removed in the plant cell by peptidases and an analogue of L-glutamic acid called phosphinothricin (Thompson *et al.*, 1987). L-Phosphinothricin (PPT), also known as glufosinate, is an analog of

glutamate and acts as a competitive inhibitor of the enzyme glutamine synthetase in plants and bacteria. Glutamine synthetase catalyses ATP-dependant incorporation of ammonium into the amide position of glutamate, resulting in the formation of glutamine. This is the major route for capturing the toxic ammonium produced during photorespiration, inorganic nitrogen assimilation and both catabolic and anabolic processes (De Block *et al.*, 1987). The inhibition of glutamine synthetase by PPT results in an accumulation of ammonium in plants, which is derived mainly from photorespiration. High concentrations of ammonium interfere with the electron-transport system of both chloroplasts and mitochondria, resulting in the production of free radicals, and, ultimately leading to the death of the plant (Tachibana *et al.*, 1986).

Resistance to the herbicide is conferred by the enzyme phosphinothricin-N-acetyltransferase (PAT), which inactivates PPT by acetylation, thereby detoxifying it. Two similar genes encode PAT, the *bar* gene of *Streptomyces hygroscopicus* (Murakami *et al.*, 1986; Thompson *et al.*, 1987; Wehrmann *et al.*, 1996), named because of resistance conferred to Basta, and the *pat* gene isolated from *S. viridiochromogenes* (Wohlleben *et al.*, 1988). The nucleotide homology for the two genes is 87% (Wohlleben *et al.*, 1988) and although most reports describe the use of the *bar* gene, it appears as though similar levels of herbicide resistance can be obtained in plants using the *pat* gene (Wolleben *et al.*, 1988; Gless *et al.*, 1998; Snyman *et al.*, 1998; Jiang *et al.*, 2000). PPT is synthesised chemically (Basta; Aventis) or by fermentation of *Streptomyces hygroscopicus*.

In order to obtain phosphinothricin resistance in plants, chimaeric genes have to be constructed (De Block *et al.*, 1987; Wohlleben *et al.*, 1988). These have been successfully expressed in several graminaceous monocot plants such as wheat (Weeks *et al.*, 1993; Zhou *et al.*, 1993; Iser *et al.*, 1999; Uze *et al.*, 1999; Sorokin *et al.*, 2000), rice (Cao *et al.*, 1992; Datta *et al.*, 1992b; Gopalakrishnan *et al.*, 2000), maize (Spencer *et al.*, 1990; Toki *et al.*, 1992; Dennehey *et al.*, 1994; Petolino *et al.*, 2000), oat (Somers *et al.*, 1992; Gless *et al.*, 1998), barley (Koprek *et al.*, 1996), turfgrasses (Hartman *et al.*, 1994; Asanao *et al.*, 1998), switchgrass (Richards *et al.*, 2001) and sugarcane (Gallo-Meagher and Irvine, 1996; Snyman *et al.*, 1998; Falco *et al.*, 2000).

Whether hebicide resistance is used as the target trait or as a means of selecting for various other introduced traits, transgenic plants can be selected directly on phosphinothricin-containing medium in the laboratory phase of genetic modification. Further details of

phosphinothricin as a selective agent will be dealt with in section 2.6.2.2.

2.4. TISSUE CULTURE SYSTEMS OF MONOCOTYLEDONOUS CEREAL CROPS AND GRASSES

Numerous studies have been conducted on various aspects of *in vitro* culture of graminaceous crop plants and grasses for use in micropropagation, pathogen-free plant production, germplasm storage, cryopreservation and genetic transformation (Thorpe, 1990). The range of *in vitro* culture systems for plants is extensive and research in this area spans several decades. As early as 1939, White reported controlled differentiation of shoots from tissue cultures of tobacco. Skoog and Miller (1957) suggested the concept of hormonal control of organ formation, according to which root and shoot differentiation is a function of the auxin-cytokinin ratio. An important contribution to the field of *in vitro* morphogenesis was made by Steward (1958), who observed the differentiation of embryos in somatic (asexual) cell cultures of carrot. The development and appearance of these embryos were comparable to zygotic embryos of this plant. In 1965 it was shown for the first time that entire plants could be obtained from a single cell (Vasil and Hildebrandt, 1965). By empirically manipulating the growth medium and other environmental factors, it has been possible to induce shoot differentiation from explants, calli, cells and protoplasts of over 100 species (Thorpe, 1990).

Until the 1980's both the induction and maintenance of totipotent callus cultures of the graminaceous monocots were found to be extremely difficult (Vasil, 1987; Bhaskaran and Smith, 1990; Futterer and Potrykus, 1995). Fortunately in sugarcane, this type of research began in the late 1960's (Heinz and Mee, 1969) and the pathways of regeneration by somatic embryogenesis or organogenesis have been well characterised and documented (Ho and Vasil, 1983; Chen *et al.*, 1988a; Guiderdoni and Demarly, 1988).

2.4.1 An overview of *in vitro* culture systems in sugarcane

Although sugarcane is vegetatively propagated commercially, a substantial amount of work has been carried out on *in vitro* culture systems for the purposes of somatic cell improvement through culture-induced mutations (Heinz and Mee, 1969; Heinz *et al.*, 1977; Larkin and Scowcroft, 1981; Liu, 1990), the production of disease-free plants (Irvine and Benda, 1985, 1987), *in vitro* micropropagation (Lee, 1987; Grisham and Bourg, 1989; Moutia and Dookun, 1999) and more recently, genetic transformation (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996; Arencibia *et al.*, 1997, 1999; 2000; Falco *et al.*, 2000; Snyman *et al.*, 2000,

2001). It is evident from these reports that sugarcane can be regenerated via several pathways, and these are summarised in Figure 2.1.

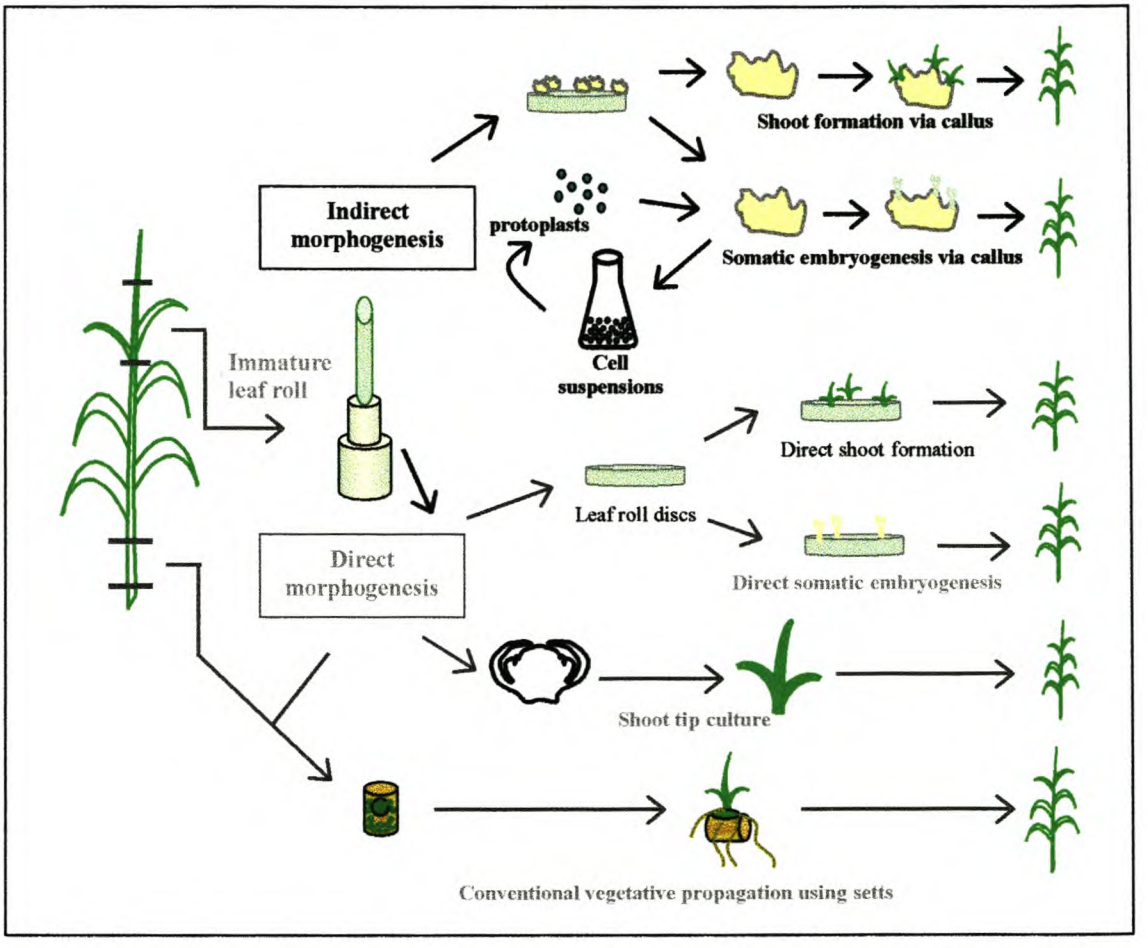


Figure 2.1 Diagrammatic representation of the different routes by which sugarcane plants can be regenerated *in vitro* and *in vivo*.

Regeneration of sugarcane can occur through two main routes, direct and indirect morphogenesis. With direct morphogenesis, plants are regenerated directly from tissue such as immature leaf roll discs. Indirect morphogenesis involves the initial culturing of leaf roll sections or inflorescences on an auxin-containing medium to produce an undifferentiated mass of cells, or callus. The source of auxin used in sugarcane tissue culture is normally 2,4-dichlorophenoxyacetic acid (2,4-D) at a concentration of 3 mg/l (Nadar *et al.*, 1978; Lee, 1987; Taylor *et al.*, 1992b; Fitch and Moore, 1993), but other synthetic auxins such as picloram (Fitch and Moore, 1990) and dicamba have been used (Brisibe *et al.*, 1994).

Direct organogenesis (or shoot morphogenesis) is the way in which sugarcane is propagated

vegetatively on a commercial basis. Sections of stalk or setts containing a bud and root primordia give rise to plantlets following the inhibition of apical dominance (Moutia and Dookun, 1999). Direct organogenesis *in vitro* can be achieved by shoot tip culture (Lee, 1987; Grisham and Bourg, 1989) or shoot multiplication from leaf discs (Irvine and Benda, 1987; Grisham and Bourg, 1989; Gambley *et al.*, 1993, 1994).

Direct somatic embryo formation on sugarcane leaf discs (i.e. without callus formation) *in vitro* was first reported by Irvine and Benda (1985) during the process of rapid regeneration of plantlets in an attempt to rid sugarcane of sugarcane mosaic virus (SCMV). It was recently reported again during studies on suspension cultures for protoplast isolation (Aftab and Iqbal, 1999), and in a transformation study using leaf discs as target material, with plantlet regeneration via embryo formation directly on leaf discs (Snyman *et al.*, 2000, 2001).

During the process of indirect somatic embryogenesis, embryos develop from somatic cells within a callus mass under exogenous hormonal control. These embryos germinate to form plants when auxin is removed from the medium and generally no other hormones are added to the medium (Heinz and Mee, 1969; Nadar *et al.*, 1978; Ho and Vasil, 1983; Irvine *et al.*, 1983; Chen *et al.*, 1988a; Bower and Birch, 1992; Bower *et al.*, 1996).

Indirect organogenesis involves the *de novo* organisation of shoot meristems from unorganised cells in callus cultures (George, 1993). In contrast to indirect somatic embryogenesis, indirect organogenesis requires hormonal induction. Hormones such as kinetin and naphthyleneacetic acid (NAA) are used for shoot formation and 3-indolebutyric acid (IBA) for rooting (Nadar and Heinz, 1977; Lee, 1987; Gallo-Meagher and Irvine, 1996). Shoots produced are transferred to new culture medium to stimulate root formation. In general, a balance between auxin and cytokinin growth regulators needs to be established for shoot initiation, and rooting occurs on media containing a different source of auxin or a reduced cytokinin concentration (Evans *et al.*, 1981; George, 1993).

Early work on dicotyledonous plants suggested that shoot meristems derived from *in vitro* callus cultures were multicellular in origin (Steward, 1958; Norris *et al.*, 1983; Skene and Barlass, 1983), and, if used for genetic transformation, may result in the production of chimaeric plants. Chimaerism is of particular concern in genetic transformation, clonal propagation, mutation research, genetic analysis and breeding. A chimaera is a plant composed

of two or more types (usually genetically different) that are capable of a congruent and integrated existence. Chimaeras in sugarcane have been observed when plants were regenerated via indirect organogenesis (Irvine, 1984). Despite this report, stably transformed sugarcane plants have been regenerated using this pathway (Gallo-Meagher and Irvine; 1996; Ingelbrecht *et al.*, 1999). However, chimaeric transformed plants with GUS sectors were observed after bombarding exposed shoot meristems (Gambley *et al.*, 1994). This emphasises the need to identify an appropriate pathway of regeneration, as well as to screen transformants for chimaerism.

Liquid suspension cultures have been initiated from sugarcane callus (Chen *et al.*, 1987; Chowdhury and Vasil, 1992; Taylor *et al.*, 1992a, 1992b) and these were used for protoplast isolation as a source of single cells for early transformation studies (Chen *et al.*, 1987; Chowdhury and Vasil, 1992; Rathus and Birch, 1992; Rathus *et al.*, 1993). Suspension cultures and protoplasts can be regenerated via either organogenesis or somatic embryogenesis. However, protoplast regeneration in sugarcane is problematic and appears to be confined to particular cultivars and laboratories.

For the purposes of sugarcane micropropagation, both somatic embryogenic and organogenic pathways of regeneration have been successfully used, and choice of the morphogenic route is based on the particular application required of *in vitro* culture.

2.4.2 Somatic embryogenesis

Somatic embryogenesis has been demonstrated as a common method of plant regeneration in tissue cultures of the Gramineae (reviewed by Vasil, 1987; Bhaskaran and Smith, 1990). It has been used for both micropropagation and transgenic studies, and is preferred to organogenesis because embryos arise from single cells (Dunstan *et al.*, 1978; Vasil, 1988), thereby ensuring the production and proliferation of identical clones. Interestingly, plant regeneration from callus and shoot buds, indicating regeneration via both organogenesis and somatic embryogenesis, has been observed in rice (Jones and Rost, 1989), maize (Lowe *et al.*, 1985; Zhong *et al.*, 1992), sorghum (Wernicke *et al.*, 1982) and sugarcane (Chen *et al.*, 1988a). This may pose problems in transgenic systems if a proportion of the regenerated plants are chimaeric.

Because they are not formed on the tissues of the original mother explant, shoots or other

organs are said to be regenerated indirectly when they are formed on previously unorganised callus, or in cell cultures (Ammirato, 1987). In *in vitro* cultures, the dedifferentiation of somatic cells to produce callus, suspension cultures or protoplasts, and the success in turning the dedifferentiated cells back to the state of meristematic cells constitutes the basis for the regeneration process (Bhaskaran and Smith, 1990; Cachita-Cosma, 1991).

Somatic embryogenesis is the production of embryogenic–like structures bearing both root and shoot meristems from somatic cells (Steward, 1958). A somatic embryo is an independent bipolar structure, is not physically attached to the tissue of origin, and can develop directly or indirectly (following the formation of a mass of proembryogenic cells), and germinate to form plants in a manner analagous to germination of zygotic embryos in seeds (reviewed by Ammirato, 1987; Merkle *et al.*, 1990; Zimmerman, 1993; Litz and Gray, 1995; Dodeman *et al.*, 1997; Mordhorst et al, 1997). This analagy in development is shown diagrammatically in Figure 2.2.

In all systems, the embryogenic cells from which embryoids are derived show a number of common features that are characterised by rapidly dividing meristematic cells. These include small size, dense cytoplasmic contents, large nuclei with prominent enlarged nucleoli, small vacuoles and many starch grains (Ammirato, 1987). Embryogenic calli are characteristically compact, organised and white to pale yellow in colour (Green and Philips, 1975; Ho and Vasil, 1983; He *et al.*, 1990; Taylor *et al.*, 1992b; Snyman *et al.*, 1996). These cultures can grow rapidly, can be maintained for long periods of time and form large numbers of somatic embryos which can be regenerated into plants (Armstrong and Green, 1985; Cachita-Cosma, 1991).

Direct (adventitious) embryogenesis, the formation of somatic embryos on explanted tissues without any intervening callus phase, is observed much more frequently in dicotyledonous plants than in monocots (George, 1993), occurring on gametophyte tissues (such as pollen microspores), immature zygotic embryos or young seedlings (Maheswaran and Williams, 1984; Smith and Krikorian, 1989). However, adventive somatic embryos have been observed on immature leaf tissue of orchardgrass (Conger *et al.*, 1983; Denchev *et al.*, 1997), barley (Pasternak *et al.*, 1999) and sugarcane (Aftab and Iqubal, 1999; Snyman *et al.*, 2000, 2001), on shoot meristems of maize (Zhong *et al.*, 1992; Zhong and Sticklen, 2000) and on immature zygotic embryos of rice (Mariani *et al.*, 2000) and millet (Vikrant and Rashid, 2001). Certain

culture conditions may allow this type of embryo formation, including the presence of maltose instead of sucrose in the growth medium (Conger *et al.*, 1983), the specific light regime to which the culture was exposed (Aftab and Iqbal, 1999) and the concentration of 2,4-D in the growth medium (Snyman *et al.*, 2000, 2001; Vikrant and Rashid, 2001).

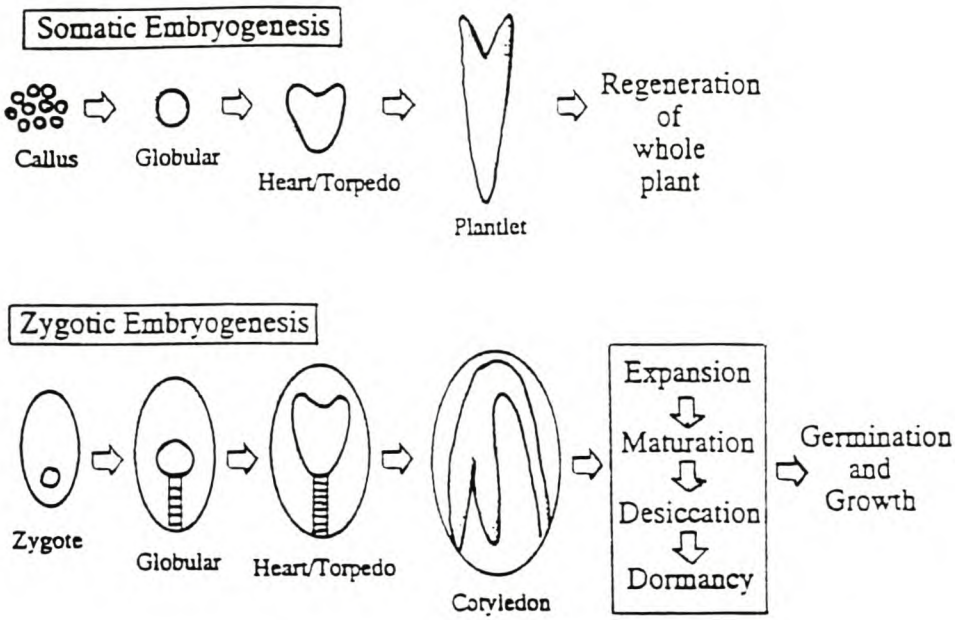


Figure 2.2 The stages in development of a somatic embryo (from Zimmerman, 1993).

It has been hypothesised (Sharp, 1980; Evans *et al.*, 1981) that direct embryogenesis in culture proceeds from cells which were already determined for embryogenic development prior to explanting i.e. they are pre-embryogenically determined cells, requiring only growth regulators or favourable conditions to allow release into cell division and embryogenesis. By contrast, indirect embryogenesis, from induced embryogenic determined cells, requires redetermination of differentiated cells, callus proliferation and the development of the embryogenically determined state. However, once induction of embryogenic determined cells has been achieved, there appears to be no fundamental differences between direct and indirect somatic embryogenesis (Williams and Maheswaran, 1986).

A noteworthy feature in some literature on somatic embryogenesis is the frequency with which recurrent or secondary cycles of direct embryoid formation have been reported (Williams and Maheswaran, 1986; Raemakers *et al.*, 1995; Vasic *et al.*, 2001). It appears that primary somatic embryoids, whether they are derived indirectly from callus cultures, or directly from organised

tissues, are themselves likely to show further cycles of direct somatic embryogenesis (Merkle *et al.*, 1990; Chapman *et al.*, 2000) (Figure 2.3). This is an important feature as embryogenic callus cultures are frequently the target cells in a DNA delivery system in the graminaceous monocots, and the transformed cells within a callus mass need to be able to produce somatic embryos and eventually plants.

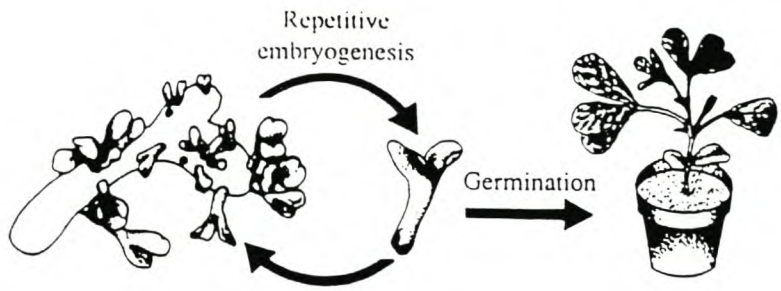


Figure 2.3 Secondary embryogenesis (from Merkle *et al.*, 1990).

The term embryogenic cell is restricted to those cells that have completed the transition from a somatic state to one in which no further exogenously applied stimuli such as the application of growth regulators are necessary to produce the somatic embryo (De Jong *et al.*, 1993). The cells that are in the transitional state, and have started to become embryogenic but still require exogenously applied stimuli, are defined as competent cells (Toonen *et al.*, 1994). However, many authors use the term 'embryogenic' loosely, and it is often used to refer to cells that have the capacity to regenerate. It is in this context that the word has been used in this thesis.

Although all the cells in a plant are derived from a fertilised egg cell and therefore contain identical genetic information, callus derived from somatic cells varies in its competence to express totipotency. This is especially true of cereal cell cultures (Wernicke and Milkovits, 1987; Williams and Maheswaran, 1986; Vasil, 1987,1988). Because of this, the callus which originates from some plants or some kinds of explants may not be responsive to techniques and media which frequently result in morphogenesis in other systems (Vasil, 1987).

2.4.3 Factors affecting embryogenesis

2.4.3.1 Selection of explants

In most herbaceous broad-leafed plants, it is possible to initiate morphogenically competent cells from explants derived from many different tissues. However, in monocotyledons there is a narrower range of suitable organs, the most common sources being: zygotic embryos in forage and turf grasses (Vasil and Vasil, 1982; Gonzales and Franks, 1987); immature embryos (or scutellar tissue) in maize (Green and Phillips, 1975; Armstrong and Green, 1985; Vasil *et al.*, 1984; Vasil *et al.*, 1985), oat (Torbet *et al.*, 1998), sorghum (MacKinnon *et al.*, 1986), wheat (He *et al.*, 1990; Barro *et al.*, 1998), rye (Ward and Jordan, 2001) and rice (Hartke and Lorz, 1989); young leaf tissue in barley (Pasternak *et al.*, 1999), orchardgrass (Hanning and Conger, 1986), sugarcane (Ho and Vasil, 1983; Irvine *et al.*, 1983; Chen *et al.*, 1988a; Guiderdoni and Demarly, 1988; Taylor *et al.*, 1992b; Brisibe *et al.*, 1993, 1994; Fitch and Moore, 1993), sorghum (Wernicke and Brettell, 1980; Wernicke *et al.*, 1982) and wheat (Wernicke and Milkovits, 1984); and immature inflorescences in wheat (Benkirane *et al.*, 2000) and sugarcane (Gallo-Meagher and Irvine, 1996; de los Blanco *et al.*, 1997).

In addition to the effect of the source of the explant on somatic embryogenesis, the developmental and physiological stage of the explant is also important in the establishment of totipotent cultures. There seems to be a brief period during which embryos, inflorescences and leaves have the competence to form embryogenic cultures (Ho and Vasil, 1983). At this stage selected cells in the explants are meristematic, only partially differentiated and not fully committed to specialised functions (Guiderdoni and Demarly, 1988). Only non-morphogenic cells are obtained after this stage.

In sugarcane there appears to be a close relationship between the state of differentiation of the excised region and the subsequent production of callus, with nodular embryogenic calli being obtained from the bases of fast-growing leaves, while more mature parts of older leaves produce only non-embryogenic calli (Guiderdoni and Demarly, 1988) (Figure 2.4). A similar response has been observed in wheat (Wernicke and Milkovits, 1984). The age of the plant does not seem to be an important factor, as young leaves obtained from mature sugarcane plants have been used successfully for the initiation of callus cultures (Ho and Vasil, 1983), and this appears to hold for most of the Gramineae (Irvine *et al.*, 1983; Brisibe *et al.*, 1993; Fitch and Moore, 1993; Pasternak *et al.*, 1999).

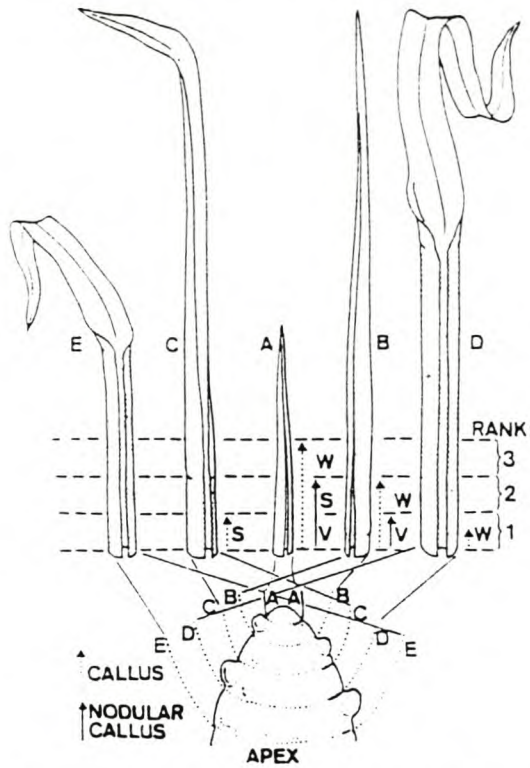


Figure 2.4 Schematic representation of the sugarcane immature leaf roll area with shoot apex, showing cultured leaf segments which are able to form callus and nodular callus. The quantitative intensity of the proliferation of the inititated calli are indicated by the letters W=weak, S=strong, V=very strong. (A-E=leaves A-E). (from Guiderdoni and Demarly, 1988).

Furthermore, somatic embryo production may be affected by genotype (Duncan *et al.*, 1985; Christou *et al.*, 1992; Lacock and Botha, 2000; Popelka *et al.*, 2001). In a study on embryogenic callus production from different cultivars of wheat using comparable explants, variation was observed in the amounts and appearance of embryogenic callus, but despite this all cultivars responded to transformation by microprojectile bombardment, and yielded fertile plants (Koprek *et al.*, 1996). Similarly in sugarcane, different cultivars produced different amounts of embryogenic callus (Taylor *et al.*, 1992b; Snyman *et al.*, 1996). However, Vasil (1987) is of the view that the physiological and developmental state of the explant has a greater impact on morphogenetic competence than genotype.

2.4.3.2 The role of auxins in embryogenesis

Tissues of the Gramineae have been cultured *in vitro* on a range of media, including B5 (Gamborg *et al.*, 1968), SH (Schenk and Hildebrandt, 1972), MS (Murashige and Skoog, 1962) and that of Skoog and Miller (1957). These media contain high levels of macro- and micro-elements, vitamins, carbon and nitrogen sources, as well as growth hormones to promote cell proliferation. The synthetic auxin 2,4-D is the most commonly used growth regulator for the

induction and maintenance of embryogenic callus in graminaceous plants, and concentrations in the range of 0.5-3 mg/l have reportedly been used (Vasil, 1987; Bhaskaran and Smith, 1989; Zheng and Konzak, 2000). Other synthetic auxins used in culture include dicamba (2-methoxy-3,6-dichlorobenzoic acid) and CPA (4-chlorophenoxyacetic acid), which have been used in maize cultures (Duncan *et al.*, 1985), and picloram (4-amino-3,5,6-trichoropicolinic acid) in sugarcane cultures (Fitch and Moore, 1990; Brisibe *et al.*, 1994).

2,4-D is the most frequently used hormone for the induction of somatic embryogenic cultures of sugarcane (Heinz and Mee, 1969; Nadar *et al.*, 1978; Ho and Vasil, 1983; Lee, 1987; Chen *et al.*, 1988a; Taylor *et al.*, 1992b; Fitch and Moore, 1993; Aftab and Iqbal, 1999), although some comparative studies have been carried out using dicamba and picloram (Irvine *et al.*, 1983; Fitch and Moore, 1990; de los Blanco *et al.*, 1997).

A two-stage production of somatic embryos is common among systems using 2,4-D as the auxin (Sharp *et al.*, 1980). This involves transferring the somatic embryos produced on 2,4-D-containing medium to that without to promote the germination of embryoids (Irvine *et al.*, 1983). In liquid culture media employed for suspension cultures, concentration levels of 2,4-D are critical, since at low concentrations (0.5-1.5 mg/l) root production occurs, and at high concentrations (3-4 mg/l) calli do not break up and proliferate (Ahloowalia, 1984; Vasil, 1987, 1988; Bregitzer *et al.*, 1995).

The ratio of auxin to other hormones is also important for morphogenesis in certain systems, e.g. in sorghum, where a high auxin: cytokinin ratio in the callus induction medium resulted in root-forming callus, while a high cytokinin: auxin ratio produced shoot-forming callus (Bhaskaran and Smith, 1989). The role of cytokinins in conferring competence to regenerate the Gramineae is not clear. Initiation of embryogenic callus from shoot meristem cultures of sorghum required 2,4-D and low levels of kinetin (Bhaskaran and Smith, 1989), but cytokinin alone inhibited embryo formation in sorghum callus cultures (Wernicke and Brettell, 1980). In barley, which has been exceptionally recalcitrant *in vitro*, the addition of BAP and copper to auxin in the callus induction medium resulted in shinier, more compact callus which was more regenerable (Cho *et al.*, 1998; Jiang *et al.*, 1998). In sugarcane, 2,4-D is required for somatic embryo formation, and regeneration takes place on auxin-free medium (Bower *et al.*, 1996; Taylor *et al.*, 1992b). However, in rice, callus cultures require the addition of a cytokinin in the regeneration medium for shoot formation (Oard *et al.*, 1990; Christou, 1995).

Callus development is usually initiated by placing the explant on to a solid or liquid medium into which auxin has been incorporated at a relatively high level. Because more than one kind of callus may arise from a single explant (Taylor *et al.*, 1992b; Jaligot *et al.*, 2000), successful propagation can depend on being able to recognise and subculture only the type(s) which will eventually give rise to shoots or somatic embryos. Once morphogenic callus has been isolated, propagation is carried out either by callus subdivision or by the preparation of cell suspensions. Unfortunately embryogenesis in both callus and suspensions cultures is seldom synchronous so that embryoids at different stages of development are usually present (Ammirato, 1987; Ibaraki *et al.*, 2000).

2.4.4 Genetic stability in culture and in regenerated plants (somaclonal variation)

Larkin and Scowcroft (1981) proposed the term somaclone to describe the plants originating from any type of tissue culture. Genetic variation found to occur between somaclones was then called somaclonal variation. Several types of genetic changes are associated with somaclonal variation (reviewed by Phillips *et al.*, 1994; Rani and Raina, 2000), notably numerical and structural chromosomal changes (Choi *et al.*, 2000a, 2000b; Cho *et al.*, 2001), DNA mutations and transpositional changes, chromosome number changes (Karp, 1995), alteration in DNA methylation (Shimron-Abarbanell and Breiman, 1991; Jaligot *et al.*, 2000) and hypervariable sequences (Linacero *et al.*, 2000).

Although much research has focused on the potential of somaclonal variation for the production of agronomically useful mutants (Heinz *et al.*, 1977; Larkin and Scowcroft, 1981; Semal and Lepoivre, 1990; van den Bulk, 1991; Maluszynski *et al.*, 1995; Ahloowalia and Maluszynski, 2001; Jain, 2001), variation can pose a major problem in genetic manipulation systems such as transformation, where specific genetic changes are desired in otherwise unaltered genomes. It is therefore imperative to establish culture and regeneration procedures that reduce or eliminate variation.

Plants regenerated through somatic embryogenesis are usually morphologically and cytologically normal (Litz and Grey, 1995). However, plant tissue culture can promote mutational phenomena induced by stress imposed by *in vitro* conditions (Morrish *et al.*, 1990; Phillips *et al.*, 1994; Cho *et al.*, 1998). Genetically aberrant plants are more likely to occur where embryogenesis is initiated in callus or suspension cultures after a period of unorganised growth or when embryogenic structures are maintained for several months (Larkin and

Scowcroft, 1981). There is some evidence that plants regenerated via an embryogenic culture route are likely to be characterised by greater genetic stability than plants regenerated via organogenesis (Morrish *et al.*, 1990; Shimron and Breiman, 1991; Shenoy and Vasil, 1992; Rani and Raina, 2000).

There are contradictory reports about somaclonal variation in cereals, ranging from considerable variation in wheat (Larkin and Scowcroft, 1981) and rice (Muller *et al.*, 1990), to little or no variation in barley (Breiman *et al.*, 1987; Karp *et al.*, 1987) and *Panicum* (Hanna *et al.*, 1984). One of the earliest reports of somaclonal variation was by Heinz and Mee (1971), who were working with *in vitro* derived plantlets of sugarcane. Among the regenerated plants there were changes in morphology, such as the presence or absence of hairs, differences in isozyme profiles, as well as variation in crop parameters such as sugarcane stalk diameter, stalk length and weight, and cane and sugar yield. Several additional studies have been carried out on sugarcane which suggest that callus culture particularly, results in regenerated plants displaying phenotypic variation (Hanna *et al.*, 1984; Irvine, 1984; Irvine *et al.*, 1991; Joyce *et al.* 1998). By using phenotypic marker genes, both indirect callus culture and direct organogenesis resulted in chimaeral disruption, epigenetic effects and mutational variation (Irvine *et al.*, 1991). Irvine and Benda (1985) recognised the need to avoid possible genetic change caused by regenerating plants via a callus stage, and in a study to rid sugarcane of SCMV, regenerated plantlets directly from leaf tissue with a minimum of callus formation. However, another study in which sugarcane callus was exposed to concentrations of up to 5 mg/l 2,4-D for a period of five months, RAPD (random amplified polymorphic DNA) analysis using six primers showed no genetic variation (Sweby *et al.*, 1994).

There are several molecular techniques used to detect DNA changes. Among them RAPD's have been employed in sugarcane (Sweby *et al.*, 1994), rye (Linacero *et al.*, 2000) and oil palm (Jaligot *et al.*, 2000) to study DNA polymorphisms among and between populations and cultivars, and to detect variation originating through tissue culture. In a recent study which compared three techniques to detect somaclonal variation in begonia, it was found that RAPD's were not sensitive enough to detect variation in plants where there was clearly a phenotypic aberration (Boumann and De Klerk, 2001). A refinement of these earlier technologies has shown that more sensitive methods can detect DNA methylation polymorphisms and sporadic loss of DNA in *in vitro*-derived sugarcane plants (Harrison *et al.*, 2001).

A lack of uniformity amongst the plants regenerated from callus has been described in a wide range of plant species (Larkin and Scowcroft, 1981; Rani and Raina, 2000). Some regenerated plants may be grossly abnormal. Others may show useful changes in general plant morphology, yield attributes, flower colour or disease resistance (Semal and Lepoivre, 1990; van den Bulk, 1991; Karp, 1995). Possible sources of genetic variability include point mutations, changes in chromosome number and structure, cryptic changes associated with chromosome rearrangements, transposable elements, somatic crossing over and DNA amplification and deletion (Karp, 1995). For breeding purposes, variability can be further increased by deliberate mutagenic treatments (Maluszynski *et al.*, 1995). However, somaclonal variation can probably be affected by a number of factors, including species, genotype, explant type, length of time in culture and duration of the subculture period (Rani and Raina, 2000).

Recent studies which have investigated somaclonal variation in transgenic plants, have shown that the transformation procedure, such as microprojectile bombardment of rice (Arencibia *et al.*, 1998a), barley (Bregitzer *et al.*, 1998b) and wheat (Cannell *et al.*, 1999), and electroporation of rice (Arencibia *et al.*, 1998a) and sugarcane (Arencibia *et al.*, 1999), resulted in negligible changes. However, when transgenic barley plants were compared to non-transformed controls (Choi *et al.*, 2000b), 58% of transgenic plants had chromosomal abnormalities (aneuploids and structural changes). This was attributed to the transformation process, the osmoticum treatment, bombardment or selection stages. Similarly in transgenic rice, phenotypic abnormalities such as dwarfism, over-tillering, sterility and purple coloured seeds were observed (Jiang *et al.*, 2000). For the application of gene transfer technology, identifying and reducing the stresses associated with the transformation and associated tissue culture process will lead to the generation of transgenic plants which are genetically and agronomically identical to the parent plants.

2.5 DNA DELIVERY SYSTEMS

Over the last decade there has been tremendous progress in the genetic transformation of plants, which has now become an established tool for the insertion of specific genes. There are verified methods for stable introduction of novel genes into the nuclear genome of over 200 diverse plant species. This review attempts to examine several methods used to deliver DNA to isolated plant cells or tissues in cereals and grasses.

The first successful transformation of higher plants was reported for tobacco in 1980

(Hernalsteens *et al.*, 1980). Since then, numerous higher plants have been transformed and a number of reliable techniques and protocols for transformation have been developed (reviewed by Potrykus, 1991; Jahne *et al.*, 1994; Birch, 1997; Hansen and Wright, 1999; Kempken, 2001; Repellin *et al.*, 2001). Because of the difficulties involved in transformation and regeneration of monocots using protoplasts, a variety of approaches have been tested to introduce foreign DNA into intact plant cells. These include macroinjection (de la Pena *et al.*, 1987) and microinjection (Toyoda *et al.*, 1990; Simmonds *et al.*, 1992; Lusardi *et al.*, 1994), pollen and embryo DNA imbibition (Topfer *et al.*, 1990), electroporation of intact tissues (Dekeyser *et al.*, 1990; D'Halluin *et al.*, 1992; Sorokin *et al.*, 2000; He *et al.*, 2001), laser microbeam injection (Weber *et al.*, 1990), pollen tube pathway (Luo and Wu, 1988), agroinfection (Grimsley *et al.*, 1987), shaking cells with small silicon carbide fibres (whiskers) in a DNA solution (Kaeppler *et al.*, 1992; Frame *et al.*, 1994; Thompson *et al.*, 1995; Dalton *et al.*, 1998; Petolino *et al.*, 2000) and microprojectile bombardment (see section 2.5.1). Of all of these approaches, the most reproducible and successful for the recovery of transformed plants from a wide range of species has proved to be microprojectile bombardment.

As well as being used to transform callus cultures, biolistic transformation also enables the transformation of chloroplasts of higher plants (Daniell *et al.*, 1998) which may be useful in avoiding pollen-based horizontal gene transfer from transgenic plants to weedy relatives.

2.5.1 Particle bombardment mediated transformation

2.5.1.1 History

The introduction of the 'particle gun' or high velocity microprojectile technology was a significant breakthrough in plant transformation. Since the initial description of the process (Klein *et al.*, 1987; Sanford *et al.*, 1987), impressive breakthroughs leading to the engineering of all major crop species using this technology have been carried out (reviewed by Birch and Franks, 1991; Klein *et al.*, 1992; Christou, 1993a, 1993b, 1995; Vain *et al.*, 1993a; Sanford, 2000). DNA is propelled through the cell wall into intact cells or tissues using small metal (tungsten or gold) particles (0.5-5 μm diameter) as carriers, which are accelerated to a high speed. The acceleration can be provide by gun-powder (Klein *et al.*, 1987), by gases such as helium, nitrogen or CO_2 (Finer *et al.*, 1992; Vain *et al.*, 1993a; Brown *et al.*, 1994) or by electrical discharge (Christou *et al.*, 1991; McCabe and Christou, 1993).

The advantage of particle bombardment is that DNA can be introduced into virtually any tissue from any cultivar (Birch, 1997; Iser *et al.*, 1999). Success depends upon the ability of the target tissue to proliferate and give rise to a plant (Vasil, 1988; Koprek *et al.*, 1996). The procedure is particularly useful for obtaining the transformation of specific tissue or for graminaceous monocots, which are difficult to transform with other techniques (Vasil, 1987).

Cobombardment is a simple process in which genes carried on separate plasmids, are mixed prior to transfer by particle bombardment. In most cases, the gene of interest plus a selectable marker are inserted into plants. However two recent studies in soybean (Hadi *et al.*, 1996) and rice (Chen *et al.*, 1998a) have shown that as many as 12 genes can be transferred simultaneously.

2.5.1.2 Physical parameters affecting particle bombardment

While there are numerous particle accelerators now in use, the helium-driven apparatus is the most widely used and is safer and cleaner than the gunpowder-driven designs (Sanford *et al.*, 1993; Vain *et al.*, 1993a; Kemper *et al.*, 1996; Harwood *et al.*, 2000). It also produces higher velocities over a wider target area combined with better dispersal of particles, resulting in a more uniform field of transformation (Sanford *et al.*, 1993).

The acoustic shock that is generated in the acceleration of microparticles can kill cells, especially those that are multiply traumatised by being penetrated by the microprojectiles, and which may also be subjected to a selective medium (Sanford *et al.*, 1993). The use of baffling screens over the target tissue (Finer *et al.*, 1992; Russell *et al.*, 1992) may reduce shock wave intensity.

The choice of microprojectile may influence the success of bombardment (Russell *et al.*, 1992; Kemper *et al.*, 1996; Folling and Olesen, 2001). Both gold and tungsten have been used with success in transformation systems. Gold is frequently preferred because it is more uniform in size, biologically inert, non-toxic and does not degrade DNA bonds (Christou, 1990; Russell *et al.*, 1992; Parveez *et al.*, 1997). However, no significant difference between number of stable transformants was obtained in sugarcane using gold and tungsten microcarriers (Gallo-Meagher and Irvine, 1996). Coating the particles with DNA in the presence of $CaCl_2$ and spermidine is also a critical step and several similar procedures are in use (Sanford *et al.*, 1993; Parveez *et al.*, 1997; Rasco-Gaunt *et al.*, 1999b).

2.5.1.3 Target tissue

An efficient and reproducible *in vitro* regeneration technique permitting the production of whole plants from transformed cells is an integral part of successful genetic engineering of plants (Potrykus, 1990; Vasil, 1987, 1990; Birch, 1997; Hansen and Wright, 1999). In the event that there are several tissue culture systems meeting this requirement for a species of interest, the choice of system can be made based on features affecting convenience or efficiency, including ready availability of explants and minimal time in tissue culture (Fisk and Dandekar, 1993; Futterer and Potrykus, 1995; Birch, 1997). Appropriate culture conditions for the production of target material (Takumi and Shimada, 1996; Cho *et al.*, 1998; Iser *et al.*, 1999; Pastori *et al.*, 2001) and proper treatment of the explant material (Bommineni and Jauhar, 1997; Barandiaran *et al.*, 1998), which may include exposure to an osmotic pretreatment (Vain *et al.*, 1993b; Nandadeva *et al.*, 1999), must also be considered in the process of particle bombardment.

A variety of different plant tissues have been used as targets for microprojectile bombardment (Fisk and Dandekar, 1993; Futterer and Potrykus, 1995). Selection of the appropriate target tissue is dependent on the nature of the research (Koprek *et al.*, 1996; Birch, 1997), e.g. in rapid gene expression studies, plasmid constructs can be introduced into various tissues and transient expression can be quickly analysed to assess promoter activity without the production of stably transformed plants (Iida *et al.*, 1995; Rooke *et al.*, 2000). The choice of the appropriate target tissue, physiological state of the plant material, and pre- and postbombardment treatments are critical to the successful regeneration of stable transformants.

Rapidly dividing, embryogenic cells have proven more suitable for successful transformation than slow growing, non-embryogenic tissues (Bommineni and Jauhar, 1997; Brisabe *et al.*, 2000). Most embryogenic cereal cell cultures are initiated from scutella of intact mature zygotic embryos or isolated scutella (Bommineni and Jauhar, 1997). Embryogenic cell cultures (initiated from both somatic cells and immature/mature zygotic embryos) have been very useful for the production of stably transformed graminaceous plant cells (Vasil 1987). Particle bombardment of embryogenic suspension culture cells of maize (Klein *et al.*, 1988; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Oard *et al.*, 1990; Vain *et al.*, 1993b, 1996), barley (Mendel *et al.*, 1989; Ritala *et al.*, 1993; Stiff *et al.*, 1995), wheat (Vasil *et al.*, 1991, 1992), rice (Wang *et al.*, 2000), turfgrass (Xiao and Ha, 1997) and sugarcane (Franks and Birch, 1991; Chowdhury and Vasil, 1992; Sun *et al.*, 1993); and embryogenic callus in turfgrass (Zhong *et*

al., 1993; Altpeter and Xu, 2000), maize (O'Kennedy *et al.*, 1998; Frame *et al.*, 2000), oat (Torbet *et al.*, 1998), wheat (Brisibe *et al.*, 2000), tall fescue (Cho *et al.*, 2000), sugarcane (Bower and Birch, 1992; Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996; Falco *et al.*, 2000) and rice (Oard *et al.*, 1990; Li *et al.*, 1993; Biswas *et al.*, 1998; Chen *et al.*, 1998b) were used to generate transgenic plants of these species.

Embryogenic cultures provide a reliable source of tissue for optimisation of bombardment conditions and can be used for year-round production of transgenic plants. An additional advantage of embryogenic cultures is that proliferation of transformed embryogenic cells under selective conditions results in the production of transformed embryogenic tissue, which subsequently gives rise to non-chimaeric plants (Birch and Franks, 1991). However, the maintenance of long-term embryogenic cultures can be labour intensive and time-consuming as the appropriate 'type' of embryogenic callus must be selectively subcultured (Redway *et al.*, 1990; Vasil *et al.*, 1991,1992; Bower *et al.*, 1996; Brisibe *et al.*, 2000). An additional complication is that long-term culture can result in abnormalities that may compromise the usefulness of the transgenic plant (Rhodes *et al.*, 1988; Cho *et al.*, 1998; Hansen and Wright, 1999; Sala *et al.*, 1999).

As a result of problems encountered with plants regenerated from long-term embryogenic cultures, transformation systems that involve rapid regeneration of primary tissue following particle bombardment have been developed (Becker *et al.*, 1994; Nehra *et al.*, 1994; Altpeter *et al.*, 1996). In wheat (Vasil *et al.*, 1992; Becker *et al.*, 1994; Altpeter *et al.*, 1996; Koprek *et al.*, 1996; Rasco-Gaunt *et al.*, 1999b), oat (Somers *et al.*, 1992) and maize (Gordon-Kamm *et al.*, 1990; Koziel *et al.*, 1993), immature embryos (or scutella tissue from these embryos) were either bombarded immediately after excision or cultured a few days prior to bombardment. Although this approach has eliminated some of the problems associated with long-term cultures, some of the young target tissues are not very responsive to cell culture manipulations and the continual production of high quality immature embryos as initial explant material can be difficult.

In an attempt to reduce the time spent in tissue culture, wheat leaf base segments, which have a high regenerative potential, were bombarded immediately or one day after harvesting (Gless *et al.*, 1998). This protocol reduced the time taken to produce transgenic plants and resulted in a high efficiency of transformation. Similarly, immature sugarcane leaf rolls were bombarded

and regenerated via somatic embryogenesis, reducing the time taken to produce transgenic plants (Snyman *et al.*, 2000, 2001).

Shoot apical meristems represent an attractive target material for bombardment, as the tissue culture component is reduced and plant regeneration is quicker from meristems than callus. Maize (Klein *et al.*, 1988; Lowe *et al.*, 1995; Zhong *et al.*, 1996), wheat (Sautter, 1993; Sautter *et al.*, 1995), sugarcane (Gambley *et al.*, 1993, 1994), oat and barley (Zhang *et al.*, 1999) meristems have been successfully used as transformation targets. Transformation of soybean, cotton, bean and peanut has been achieved by particle bombardment of meristematic cells of excised embryonic axes (Christou, 1995). However, the limiting factors remain the ability to mechanically prepare the explants, the transfer of the genes into regenerable cells, selection or screening for transformants at an efficiency sufficient for practical use in cultivar improvement, and chimaerism.

The disadvantage of using organised tissues as target cells is the frequency of obtaining plants with chimaeric gene expression (Gambley *et al.*, 1993; Bommineni and Jauhar, 1997). Chimaeras may prove a problem for the successful expression of agronomically important genes (Zhong *et al.*, 1996), although the work of Irvine *et al.* (1991) on sectoring natural sugarcane chimaeras into the constituent phenotypes indicates that it may be possible to produce fully transgenic plants by meristem transformation followed by conventional micropropagation, rather than by callus–based tissue culture (Gambley *et al.*, 1993). Both Lowe *et al.* (1995) and Zhong *et al.* (1996) were able to successfully recover transgenic maize plants using axillary bud culture from chimaeric tissues produced by bombardment of immature shoot meristems, and by re-culturing chimaeric tissue under stringent selection conditions.

Particle bombardment can be used to introduce DNA into any target tissue that is penetrable by DNA coated particles (Sanford *et al.*, 1993). For stable transformation studies, target tissue should be regenerable, but for transient expression studies, any tissue can be tested for the expression of a reporter gene. In addition to embryogenic cultures and shoot meristems, other tissues that have been subjected to particle bombardment include microspores (Jahne *et al.*, 1994), leaves (Klein *et al.*, 1988; Rooke *et al.*, 2000), inflorescences (Barcelo *et al.*, 1994), cereal aleurone cells (Kim *et al.*, 1992) and tassel primordia (Dupuis and Page, 1993).

2.5.1.4 Tissue treatment

There are limited reports on the preparation of target tissue to make it more receptive to microprojectile bombardment. The use of culture venting (Russell *et al.*, 1992), cell filtration (Finer *et al.*, 1992) and suspension culture cells at the proper phase of growth and density (Finer *et al.*, 1992) have been used for particle bombardment in different species. One treatment, however, that has greatly enhanced both transient and stable gene expression is the use of an osmoticum (Russell *et al.*, 1992; Vain *et al.*, 1993b). Osmotic conditioning can be both pre and post-bombardment and incorporates the use of mannitol and sorbitol at a relatively high concentration (approximately 0.4 M). The osmotica may act by causing plasmolysis of the target cells that lessens or eliminates extrusion of the protoplasm in cells that are penetrated by particles (Vain *et al.*, 1993b).

The proper pre-culture of explant material (i.e., the culturing of an explant for a few days prior to bombardment, rather than using freshly isolated explants) is also beneficial for successful transformation (Altpeter *et al.*, 1996; Kemper *et al.*, 1996).

2.5.2 *Agrobacterium*-mediated gene transfer

In the early 1980's, the era of plant transformation was initiated when the production of transgenic plants via *Agrobacterium tumefaciens*-mediated gene delivery was reported (Hernalsteens *et al.*, 1980; Horsch *et al.*, 1985; Hohn *et al.*, 1989). Although initial successes were limited to the Solanaceae, tobacco in particular, this dramatically changed over the next 20 years, and it is now possible to transform a wide range of plants, although graminaceous monocots were considered to be outside the host range amenable to transformation by *Agrobacterium* until recently (reviewed by Potrykus, 1991; Smith and Hood, 1995; Gelvin, 2000).

A. tumefaciens-mediated transformation has significant advantages over naked DNA delivery, such as the introduction of a few copies of genes into the plant genome (Spencer *et al.*, 1992; Cooley *et al.*, 1995), high co-expression of introduced genes and easy manipulation *in vitro*. However, the *A. tumefaciens*-mediated gene transfer system has been limited largely to dicotyledonous plants (Songstad *et al.*, 1995). In order to transform previously 'recalcitrant' plants using *A. tumefaciens*, it is necessary to manipulate various factors such as the addition of inducing compounds such as acetosyringone (Chan *et al.*, 1993), identification of critical target cells for successful bacterial attachment (Graves *et al.*, 1988; Grimsley *et al.*, 1987; Gould *et*

al., 1991; Amoah *et al.*, 2001), the *A. tumefaciens* strain (Ritchie *et al.*, 1993) and the use of appropriate promoters (Smith and Hood, 1995).

Several reports presented early attempts to transform the Gramineae with *A. tumefaciens* (Raineri *et al.*, 1990; Mooney *et al.*, 1991; Shen *et al.*, 1993), including *A. tumefaciens*-mediated infection of plants with viral genomes, termed 'agroinfection' (Grimsley *et al.*, 1987; Grimsley, 1990). Chan *et al.* (1993) first reported the production of transgenic rice plants by inoculating immature embryos with an *A. tumefaciens* strain and proved the transformation by molecular analyses. Subsequent to this, many reports have demonstrated unequivocally that *Agrobacterium* is a suitable vehicle for gene transfer in a range of plants including rice (Hiei *et al.*, 1994, 1997; Rashid *et al.*, 1994; Dong *et al.*, 1996; Toki, 1997; Azhakanandam *et al.*, 2000), maize (Gould *et al.*, 1991; Escudero *et al.*, 1996; Ishida *et al.*, 1996; Kemper *et al.*, 1996), barley (Tingay *et al.*, 1997), wheat (Cheng *et al.*, 1997) and sugarcane (Arencibia *et al.*, 1998b; Elliot *et al.*, 1998; Enriquez-Obregon *et al.*, 1998).

2.5.3 Transformation of protoplasts

Direct gene transfer to protoplasts is one of several methods which have been developed for the generation of transgenic plants and for transient gene expression studies (Potrykus, 1991). Protoplasts are isolated from plant tissue either by a mechanical or enzymatic process to remove the cell wall. This results in the production of a suspension containing millions of individual cells and therefore offers the advantage of single cell targets for transformation.

This method is based on the efficient uptake of plasmid DNA from the surrounding medium into protoplasts, and is promoted by polyethylene glycol (Lorz *et al.*, 1985) in barley (Funatsuki *et al.*, 1995), rice (Datta *et al.*, 1992b) and sugarcane (Chen *et al.*, 1987), and by electric pulses or electroporation (Shillito *et al.*, 1985) in tall fescue (Ha, 2000), rice (Toriyama *et al.*, 1988; Zhang *et al.*, 1988), wheat (He *et al.*, 1994), sorghum (Battraw and Hall, 1991), bentgrass (Asano *et al.*, 1991), maize (Fromm *et al.*, 1986; Sukhapinda *et al.*, 1993) and sugarcane (Rathus and Birch, 1992; Arencibia *et al.*, 2000).

Although the regeneration of transformed protoplasts to a microcalli stage was achieved for a number of plants including maize (Rhodes *et al.*, 1988; Lyznick *et al.*, 1989; Sukhapinda *et al.*, 1993), barley (Mordhorst and Lorz, 1992), rice (Datta *et al.*, 1992a; Gupta and Pattanayak, 1993; Alam *et al.*, 1995) and sugarcane (Srinivasan and Vasil, 1986; Chen *et al.*, 1988b;

Rathus and Birch, 1992), regeneration and the production of plants is notoriously difficult, and no general protocol applies.

2.6 APPLICATION OF MARKER GENES IN TRANSFORMATION TECHNOLOGY

Foreign genes can be introduced to plants in a number of ways, but it is only by investigating the expression of the introduced DNA that the success of the transformation and the efficiency of particular promoter constructs can be judged (Futterer and Potrykus, 1995). To avoid the lengthy process of selection and bulking up of tissue for analysis of integrated constructs, it is possible to study transient expression of reporter or screenable genes by immediate analysis (Birch, 1997).

2.6.1 Use of transient expression (reporter genes) to follow transformation parameters

The presence of DNA in plant cells can be demonstrated within 24 hours after gene transfer by means of transient assays (Werr and Lorz, 1986). These assays are based on the expression of genes, called reporter genes, that are expressed in plant cells without integration into the genome (Lindsey and Jones, 1990). For each transformation protocol, transient expression experiments are a preliminary step used to identify conditions that will allow efficient DNA delivery and measurement of the quantity of DNA introduced into the target cells. With so many variables and no evidence of combinations that are broadly applicable across plant species, this simple test provides some guidance for answering several initial questions. As the conversion rate from transient expression to stable integration in the plant genome is low (Birch and Franks, 1991; Potrykus, 1991; Sautter, 1993; Bower *et al.*, 1996; Harwood *et al.*, 2000) and is estimated to be 0.35-4% (Klein *et al.*, 1988; Finer *et al.*, 1992; Bower *et al.*, 1996), it is worthwhile achieving a high frequency of transiently expressing cells following DNA delivery.

Transient expression of the inserted gene occurs in the nucleus, but chromosomal integration is unlikely to occur, nor is it a prerequisite for expression to take place (Lindsey and Jones, 1990). Expression normally peaks after 48 hours, after which time the DNA is degraded (Werr and Lorz, 1986). In addition, expression is not confounded by influences exerted by chromosomal sequences adjacent to the sites of integrated genes, or so-called position effects (Dekeyser *et al.*, 1990).

Transient expression systems require a sensitive method for the detection of transformed cells/tissue (Hauptmann *et al.*, 1988). Specific methods are available to monitor the expression of several reporter genes such as the widely used β-glucuronidase or GUS (Jefferson, 1987), the gene product of which is an enzyme which can be assayed for fluorometrically or by histochemical staining in tissue extracts. In addition, luciferase genes (Ow *et al.*, 1986; Koncz *et al.*, 1987; Fromm *et al.*, 1990), chloramphenicol acetyltransferase (CAT) (Hauptmann *et al.*, 1987) and genes involved in anthocyanin biosynthesis (Ludwig *et al.*, 1990) may be used to detect transient expression in a nondestructive manner. More recently, the gene for green fluorescent protein (GFP) has become an important *in vivo* reporter in plants (Haseloff *et al.*, 1997; Kaeppler *et al.*, 2001; Stewart, 2001).

2.6.1.1 The GUS system

The *E. coli uidA* gene which encodes β-glucuronidase (GUS; Jefferson, 1987) has become the most widely used reporter gene in plant transformation. The gene product is a hydrolytic enzyme which catalyses the cleavage of a variety of β-glucuronides, many of which are available commercially as spectrophotometric, fluorimetric and histochemical substrates (such as X-Gluc; 5-bromo-4-chloro-3-indoloyl-b-D-glucuronide) (Jefferson, 1987). The GUS gene has been cloned and sequenced, and encodes an enzyme which can be used in the construction and analysis of gene fusions (Jefferson, 1987; Jefferson *et al.*, 1987).

The principal advantages of the GUS system include the ease of the assay, its sensitivity and the fact the gene product is detectable with histochemical assays that localise gene activity in specific cell types (Tor *et al.*, 1992). However, problems with the GUS assay have been encountered because of endogenous GUS activity in plant tissues (Kosugi *et al.*, 1990; Hodal *et al.*, 1992; Tor *et al.*, 1992). Modifications to the assay to overcome this activity include the addition of methanol to the incubation buffer (Kosugi *et al.*, 1990). In addition, plant pigment may mask the sites of GUS activity and the use of a clearing agent (2:1:1 mixture of chloral hydrate, lactic acid and phenol) was found to enhance visualisation after incubation in X-Gluc (Beeckman and Engler, 1994). Another disadvantage is that the histochemical GUS assay involves destructive sampling of the tissue, although an attempt has been made to sample in a non-lethal way (Martin *et al.*, 1992; Kirchner *et al.*, 1993). Also varying levels of GUS expression have been reported during different developmental stages of somatic embryogenesis in alfalfa (Tian *et al.*, 2000).

Despite the abovementioned problems, the GUS assay has been used to detect transient expression in a variety of tissues and cells in the development of transformation protocols and promoter studies in several grasses (Taylor *et al.*, 1993; Vain *et al.*, 1996), maize (Omirulleh *et al.*, 1993; Songstad *et al.*, 1993; Menossi *et al.*, 2000), oat (Higgs *et al.*, 1993), wheat (Wang *et al.*, 1988; Lonsdale *et al.*, 1990; Chibbar *et al.*, 1991; Iglesias *et al.*, 1994; Rooke *et al.*, 2000), rice (Wang *et al.*, 1988; Dekeyser *et al.*, 1990; Meijer *et al.*, 1991), and sugarcane (Bower and Birch, 1992; Gallo-Meagher and Irvine , 1993; Gambley *et al.*, 1993; Sun *et al.*, 1993).

2.6.1.2 Anthocyanin pigment biosynthesis

A non-lethal reporter system that does not require the application of selectable agents or external substrates for the detection of transgenic cells uses genes that regulate the anthocyanin biosynthetic pathway in maize (Ludwig *et al.*, 1990). Much of the work involving transformation with anthocyanin markers has been performed with maize (Ludwig *et al.*, 1990; Bowen, 1992; Songstad *et al.*, 1993). Subsequently it has been found that pigments can be induced in cells of other monocots such as sugarcane (Bower *et al.*, 1996; Snyman *et al.*, 1996) and wheat (Iglesias *et al.*, 1994; McKinnon *et al.*, 1996; Chawla *et al.*, 1999).

In maize, the anthocyanin biosynthetic pathway is regulated by two families of transcriptional activators, R and C proteins (Bowen, 1992). Coexpression of these genes is necessary for pigment formation, but whether R or C proteins are encoded by endogenous genes or an introduced template is immaterial for expression (Bowen, 1992). The induction of anthocyanin pigmentation has been effective in transient assays to establish gene delivery protocols (Iglesias *et al.*, 1994; Bower *et al.*, 1996; Snyman *et al.*, 1996) and to determine the optimal time and tissue type for bombardment (McKinnon *et al.*, 1996). However, it has been inefficient when used to screen for transgenic lines as overexpression of anthocyanin may cause a termination of cell division in pigmented cells (Chawla *et al.*, 1999).

2.6.2 Use of selection genes and selection media for elimination of untransformed individuals during long-term development of stable transformants

Tailoring a system for efficient transgenic plant production requires careful choice of an appropriate selectable marker (Park *et al.*, 1998). The rationale for using selectable marker genes for transformation is that the expression of the gene confers upon the cell or tissue the ability to grow in the presence of some selective agent under conditions which kill (or prevent the division of) all untransformed cells (Fisk and Dandekar, 1993). In general, the gene of

interest is co-delivered with a selectable marker to identify and encourage the growth of recipient cells (Bower *et al.*, 1996). Selectable markers confer resistance to chemical agents such as antibiotics or herbicides that inhibit various cellular functions (Wilmink and Dons, 1993). These include the gentomycin acetyltransferase gene, bacterial and plant EPSP synthase genes, the bacterial phosphinothricin acetyltransferase gene, the *Arabidopsis*, maize and tobacco acetolactate synthase genes, the bacterial neomycin phosphotransferase genes and the bacterial hygromycin B phosphotransferase gene. These genes, when expressed under the direction of plant promoters, have been shown to be effective in plant tissue (reviewed by Lindsey and Jones, 1990; Morrish *et al.*, 1993; Wilmink and Dons, 1993; Futterer and Potrykus, 1995).

One of the major problems encountered when using selective agents is protection of untransformed tissue by the detoxifying activity of surrounding transformed cells, or cross-protection (Park *et al.*, 1998). Such cross-protection allows regeneration of untransformed cells, leading to many nontransformed escapes. To overcome the 'rescuing' ability of transformed cells, the most effective concentration of the selective agent must be determined in tissue culture experiments for each crop and for each type of explant (Park *et al.*, 1998).

2.6.2.1 Antibiotic resistance: the *nptII* gene

Representatives of a major class of antibiotics, the aminoglycosides and the corresponding resistance genes are widely used for plant transformation studies. The best known member of the aminoglycoside antibiotics is kanamycin, which is widely applied as a selective agent in transformation experiments. All known aminoglycoside antibiotics inhibit protein synthesis in prokaryotic cells. Kanamycin, gentamycin and its derivative geneticin (G418), neomycin and paromomycin bind to the 30S ribosomal subunit thus inhibiting the initiation of translation, and consequently protein synthesis (Nap *et al.*, 1992). The most widely adopted, stable-integration marker is the neomycin phosphotransferase gene (*nptII*) (Beck *et al.*, 1982), which is discussed below.

Resistance to the aminoglycoside antibiotics is obtained by the *aphA2* gene from Tn5 of *E.coli* (Bevan *et al.*, 1983). This gene codes for the enzyme aminoglycoside 3' phosphotransferase (APH(3')II), also referred to as neomycin phosphotransferase (NPTII), which phosphorylates a specific hydroxyl group of the antibiotic molecule (Herrera-Estrella *et al.*, 1983a, 1983b).

Although kanamycin has been used successfully for selection of dicotyledons in transformation procedures, research in monocots demonstrated that kanamycin was less effective as a selective agent due to interference with regeneration, particularly in rice (Zhang *et al.*, 1988; Battraw and Hall, 1992), and/or endogenous resistance to kanamycin (Wilmink and Dons, 1993; Futterer and Potrykus, 1995). Hauptmann *et al.* (1988) addressed this factor with particular reference to grass species, by using the antibiotic geneticin. Since then geneticin has been included in the selection of transgenic barley (Ritala *et al.*, 1993), oat (Somers *et al.*, 1992) and sugarcane (Bower and Birch, 1992; Bower *et al.*, 1996; Falco *et al.*, 2000; Snyman *et al.*, 2000, 2001).

Identifying transformed plant lines carrying the *nptII* gene may require more definitive tests than the ability of the plant to grow on an antibiotic. Methods for detecting the presence of the *nptII* gene include the gel overlay method (Reiss *et al.*, 1984) and the dot-blot assay (McDonnell *et al.*, 1987; Platt and Yang, 1987; Staebell *et al.*, 1990), both of which utilise [^{32}P]-γ-ATP, are relatively time consuming and fairly cumbersome. The first reported use of anti-NPTII antibodies was with transformed tobacco and rape plants in immunoprecipitation (Baszczyniski, 1990) and immunoaffinity (Henderson *et al.*, 1990) assays. These assays provided a clear response to the presence of the NPTII enzyme. However, variability between the assays was noted and the use of [^{32}P]-γ-ATP was still required. Several groups subsequently utilised a commercially available ELISA kit (5 Prime→3 Prime Inc, Boulder, Colarado) for detecting the presence of the NPTII protein in cell extracts (Nagel *et al.*, 1992; McKenzie *et al.*, 2000). This system is faster than the previously described detection methods and allows semi-quantitation of NPTII levels in transformants. However, problems have been reported in terms of background variation as well as increased background when the plants were glasshouse grown (Nagel *et al.*, 1992).

Other antibiotics such as hygromycin have been used as selective agents in tall fescue (Dalton *et al.*, 1995), maize (Walters *et al.*, 1992), rice (Datta *et al.*, 1992b), creeping bentgrass (Xiao and Ha, 1997) and orchard grass (Horn *et al.*, 1988). Streptothricin has been used in tobacco, carrot and *Arabidopsis* (Jelenska *et al.*, 2000), and paromomycin was the selective agent used to regenerate transgenic turfgrass (Altpeter and Xu, 2000) and oat (Torbet *et al.*, 1998).

Recently the use of antibiotics as selective agents has caused widespread public concern because of inadequate knowledge of the agents' impact on the environment and human health.

To avoid the use of these agents, alternative selection systems have been proposed and developed (Joersbo and Okkels, 1996; Joersbo *et al.*, 1998; Negrotto *et al.*, 2000; Wang *et al.*, 2000a; Endo *et al.*, 2001; Haldrup *et al.*, 2001; Reed *et al.*, 2001). Transformation was achieved using the following selective agents: mannose in sugar beet, maize, wheat and barley, and xylose in potato, tobacco and tomato. These selection systems allow for separation of non-transformed from transformed tissue by arresting the growth of the non-transformed cells through carbohydrate starvation.

In addition, genes conferring resistance to herbicidal compounds are potential alternatives for selection in plant transformation (Wilmink and Dons, 1993). Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act (Botterman and Leemans, 1988).

2.6.2.2 Herbicide resistance: the *bar* gene

The bialaphos resistance gene (*bar*), which encodes the enzyme phosphinothrycin acetyl transferase (PAT), is widely used as a selectable marker for cereal transformation (Christou *et al.*, 1991; Castillo *et al.*, 1994; Altpeter *et al.*, 1996; Brettschneider *et al.*, 1997). In transformation studies, different selective agents based on PPT can be used: gluphosinate or its commercial formulation, Basta, or the tripeptide, bialaphos and its commercial formulation, Herbiace (Dennehey *et al.*, 1994).

The selection pressure applied has to be chosen carefully. A concentration too low bears the risk of undesirable numbers of escapes developing, and too high a concentration results in the loss of transformants expressing moderate levels of the foreign gene (Hansch *et al.*, 1998). In addition, regenerative capacity may be reduced (Bower *et al.*, 1996), transformants containing a higher gene copy number could be recovered preferentially (Stiff *et al.*, 1995), and the albinism problem in barley may be exacerbated (Wan and Lemaux, 1994) if the selection procedure is too stringent.

The mode of action of PPT *in vitro* is not clear, because although the accumulation of ammonia occurs, the mode of action of PPT is also influenced by culture conditions and the metabolic status of the cells, with carbon source in the media and the light regime influencing resistance (De Block *et al.*, 1995). This reinforces the need to develop a selection procedure for each

transformation system.

Phosphinothricin-resistant plants have been selected by either addition of the compound to the growth medium or by spraying plantlets (Futterer and Portykus, 1995; Wilmink and Dons, 1993). In the media, concentrations of 1-5 mg/l PPT is adequate to select for transformed cells in wheat (Weeks *et al.*, 1993; Altpeter *et al.*, 1996; Uze *et al.*, 1999; Sorokin *et al.*, 2000), maize (Gordon-Kamm *et al.*, 1990; Spencer *et al.*, 1990; Vain *et al.*, 1993b; Dennehey *et al.*, 1994; Frame *et al.*, 2000), turfgrass (Hartman *et al.*, 1994; Asano *et al.*, 1998) and sugarcane (Chowdhury and Vasil, 1992; Sun *et al.*, 1993; Gallo-Meagher and Irvine, 1996; Arencibia *et al.*, 1998b). Higher concentrations of 10-50 mg/l were optimal to discriminate between transformed and untransformed rice calli (Dekeyser *et al.*, 1989; Christou *et al.*, 1991).

Several authors (Dekeyser *et al.*, 1989; Dennehey *et al.*, 1994; Altpeter *et al.*, 1996; Frame *et al.*, 2000) have shown that for effective selection, it was important to omit several amino acids from the medium (glutamic acid, proline and arginine), as they allowed growth of untransformed cells in the presence of PPT. This seems contradictory to other reports (Tachibana *et al.*, 1986), but shows that the precise selection scheme should be optimised for each species and starting material. In addition, concentrations of PPT that allow 100% effective selection can interfere with the regeneration capacity of the tissue and this has been reported in wheat (Nehra *et al.*, 1994), barley (Wan and Lemaux, 1994) and sugarcane (Bower *et al.*, 1996). Suboptimal amounts are therefore used which also allow growth of some untransformed material, and in such cases, it is important to continue selection throughout plant regeneration (Nehra *et al.*, 1994).

There are a number of assays available to screen for PPT resistance in plants including the chlorophenol-red test (Kramer *et al.*, 1993; Hansch *et al.*, 1998), bromocresol-purple test (De Block *et al.*, 1995), silica gel thin layer chromatography (Spencer *et al.*, 1990), radioactive thin layer chromotography or PAT assays (De Block *et al.*, 1989), and herbicide application to leaves or whole plants (Gordon-Kamm *et al.*, 1990). Modifications of the detection methods in wheat and oilseed rape have been made (Rasco-Gaunt *et al.*, 1999a; Pfeilstetter *et al.*, 2000) to allow more rapid and sensitive evaluation of tolerance to PPT.

2.7 FACTORS AFFECTING TRANSGENE EXPRESSION

2.7.1 Gene silencing

Predictable and reproducible levels of expression of transgenes in engineered plants is one of the main goals of plant molecular biology. The transgene must be able to function under various environmental conditions in different genetic backgrounds and be stable over generations of breeding (Birch, 1997; Zhong, 2001). However, because there is random integration of the foreign DNA, transgene expression remains largely unpredictable (Spencer *et al.*, 1992; Register *et al.*, 1994; Meyer, 1995) and there is great variation in expression levels and inheritance patterns between independently transformed plants (Mannerlof and Tenning, 1997). In nearly all the cases where this phenomenon has been investigated at the molecular level, loss of expression does not correlate with the loss of the transgene, but rather with its inactivation (Finnegan and McElroy, 1994). It is becoming clear from published reports that there are several variations on the theme of gene silencing in transgenic plants (reviewed by Finnegan and McElroy, 1994; Matzke and Matzke, 1995; Meyer, 1995; Kumpatla *et al.*, 1998; Stam *et al.*, 1998; Covey and Al-Kaff, 2000; Fagard and Vaucheret, 2000; Iyer *et al.*, 2000).

Several explanations have been envisaged for variations in expression between transformants containing the same construct, such as the relative location of the integration of the foreign DNA in the genome - so called position effects (Matzke and Matzke, 1990), the chromatin structure (Meyer, 1999), the methylation state (Hobbs et al, 1993; Ingelbrecht *et al.*, 1994; Kohli *et al.*, 1999; Kumar and Fladung, 2000), post-transcriptional regulation (Meyer, 1995) and copy numbers (Linn *et al.*, 1990; Hobbs *et al.*, 1993; Bellucci *et al.*, 1999).

Gene silencing in transgenic plants, referred to as homology dependent gene silencing, can be due to either decreased transcription of the affected sequences or to post-transcriptional mechanisms, and may affect endogenous and/or transgenes (Ingelbrecht *et al.*, 1994; Johansen and Carrington, 2001). Both transcriptional inactivation and post-transcriptional silencing lead to reduced or undetectable levels of transcripts (Matzke and Matzke, 1995). The most common way of differentiating between the two possibilities is to perform transcription run-on experiments with isolated nuclei. If a run-on transcript is synthesised but no corresponding RNA is visible on a northern blot, then silencing is post-transcriptional (Baulcombe, 1996; Metzlaff *et al.*, 1997). This occurs when the promoter is active, but the messenger RNA does not accumulate (Stam *et al.*, 1998). Post-transcriptional gene silencing appears to act on any RNA that is homologous to the activating transgene (English *et al.*, 1996) and has an

interesting application in the protection of transgenic crops from viral attack. If transcriptional inactivation is involved, however, neither run-on transcripts nor steady-state RNA is detected on northern blots (Park *et al.*, 1996; Kohli *et al.*, 1999). A transcriptionally silenced transgene can transinactivate another (trans)gene with homology in the promoter region (Neuhuber *et al.*, 1994) and this is associated with increased promoter methylation (Linn *et al.*, 1990; Meyer, 1995; Park *et al.*, 1996).

Recent evidence has shown that post-transcriptional gene silencing is one mechanism by which pathogen-derived resistance is conferred (Baucombe, 1996; English *et al.*, 1996; Kasschau and Carrington, 1998; Covey and Al-Kaff, 2000). In the most studied system, tobacco plants are described that contain a nontranslatable tobacco etch virus (TEV) coat protein transgene. These plants are resistant to TEV infection (Dougherty *et al.*, 1994; Goodwin *et al.*, 1996). This resistance is manifested as two distinct phenotypes, immunity or recovery. Immune plants do not support virus replication, do not accumulate transgene mRNA, and show Mendelian inheritance of resistance. Recovery plants are initially susceptible to TEV, accumulating full-length transgene mRNA, but eventually they develop resistance, at which point there is little transgene mRNA (Doughery *et al.*, 1994).

In addition to complete silencing of transgenes, variation in expression levels among transformants is believed to be due to the fact that integrated transgenes cannot be regarded as independent transription units (Kohli *et al.*, 1999). Transgenes may integrate at different chromosomal locations (Kohli *et al.*, 1999). If integration occurs in a transcriptionally active area, the resulting expression may be influenced by proximal regulatory sequences (Topping *et al.*, 1991). Such transgene inactivation within the host genome is known as 'position effect' silencing (Matzke and Matzke, 1990).

The presence of multiple copies of a gene in transgenic plants is widely believed to trigger silencing (Matzke *et al.*, 1994). Consequently, it has been proposed that the presence of a transgene as a single copy is advantageous for expression. Additional evidence for this is provided by observations that a transgene homologous to an endogenous gene may suppress expression of both genes through co-suppression (Jorgensen *et al.*, 1996). However, several studies in rice suggest that expression levels (Chareonpornwattana *et al.*, 1999), the extent of methylation (Kumpatla *et al.*, 1997) and integrity of transgenes (Kohli *et al.*, 1999; Morino *et al.*, 1999) are major factors in the onset of silencing. Similar observations relating to transgene

copy number in sugarcane were reported by Hansom *et al.* (1999), where silencing was found to be promoter-dependent.

Very few studies have been carried out on the stability of transgenes in vegetatively propagated crops. The first such study was conducted with transgenic sugarcane, and the phenotypic expression of the *bar* gene remained unchanged over three generations (Gallo-Meagher and Irvine, 1996). However in a similar study in the forage grass, fescue, the expression of a GUS gene under the control of a rice actin promoter was unstable and often undetectable in the first three tiller generations, but had stabilised by the fifth generation (Bettany *et al.*, 1998). Loss of transgene expression was not due to deletion of the transgene, or gross rearrangement as demonstrated by Southern blot analyses, or methylation, and it is postulated that this variation may be due to some form of epigenetic effect (Bettany *et al.*, 1998).

In an attempt to overcome the effects of gene silencing, the use of matrix attachment regions may increase gene expression in transgenic plants (Holmes-Davis and Comai, 1998; Vain *et al.*, 1999; Allen *et al.*, 2000). Matrix attachment regions (MARs or scaffold attachment regions) are regions of DNA which have been isolated based on their ability to bind to the nuclear scaffold. MARs afforded improved stability of the transgene over two generations in rice and tobacco (Vain *et al.*, 1999). In addition it may be possible to make use of demethylation sequences (Lichtenstein *et al.*, 1994) or targeted integration systems (Albert *et al.*, 1995) to protect foreign gene sequences from silencing in transgenic plants.

2.7.2 Promoters

The availability of strong promoters, suited for driving transgene expression to high levels has been an important factor in the recent development of the technology for monocot transformation. The cauliflower mosaic virus (CaMV) 35S promoter is the most widely used constitutive promoter in dicot transformation and has also been tested in the early attempts of monocot transformation. It was found to be active in cereals and grasses, albeit at varying activity levels, with a tendency towards low expression (McElroy and Brettell, 1994; Futterer and Potrykus, 1995). The promoter was therefore modified by duplication of its own enhancer (Omirulleh *et al.*, 1993), by insertion of other transcriptional enhancers (Last *et al.*, 1991) or by combination with intron sequences (Tanaka *et al.*, 1990). Certain introns, like that from the maize ubiquitin promoter, can drive transient GUS expression in immature tritordeum in the absence of any other regulatory sequences (Salgueiro *et al.*, 2000).

Other constitutive promoters, capable of driving transgene expression up to ten times the levels achieved in monocots with the CaMV 35S promoter, such as the rice actin promoter (McElroy *et al.*, 1990), the pEmu recombinant promoter (Last *et al.*, 1991) and the maize ubiquitin, Ubi-1, promoter (Christensen *et al.*, 1996) have been identified and are widely used in the transformation of wheat (Vasil *et al.*, 1992; Weeks *et al.*, 1993; Becker *et al.*, 1994; Chamberlain *et al.*, 1994; Altpeter *et al.*, 1996; Rasco-Gaunt *et al.*, 1999b; Rooke *et al.*, 2000), maize (Kemper *et al.*, 1996; Zhong *et al.*, 1996; Frame *et al.*, 2000; Rooke *et al.*, 2000) and sugarcane (Gambley *et al.*, 1993; Bower *et al.*, 1996; Falco *et al.*, 2000). Recently, a rice ubiquitin promoter has been isolated and characterised (Wang *et al.*, 2000b), which may find application in other monocot transformation systems.

Tissue specific promoters have been used to drive expression in particular areas of the plant, such as the maize sucrose synthase–1 promoter suitable for phloem-specific expression (Yang and Russell, 1990), and the α-amylase promoter for driving expression in the aleurone of cereal grains (Christou, 1996). In addition, inducible promoters are available which induce gene transcription spatially, temporally and quantitatively in a number of cell types (Thornburg *et al.*, 1987; Conkling *et al.*, 1990; Gatz and Lenk, 1998).

The level of expression of a gene can often be enhanced by the addition of cis-acting sequences, also called enhancer elements, which increase the level of transcription from a promoter (Banerji *et al.*, 1981; Rathus *et al.*, 1993). In addition, the inclusion of an intron in the transcriptional region of the gene boosted expression levels in maize (McElroy *et al.*, 1990; Toki *et al.*, 1992), rice (Kyozuka *et al.*, 1994), bluegrass (Vain *et al.*, 1996) and sugarcane (Gallo-Meagher and Irvine, 1993; Bower *et al.*, 1996). However, the effect of an intron depends on the promoter with which it is combined because in some systems, introns which normally lead to enhanced expression in most cereals, can cause lower levels of gene expression (e.g. in sugarcane; Rathus *et al.*, 1993).

The choice of promoter used to drive transgene expression is critical, as demonstrated in a recent paper (Al-Kaff *et al.*, 2000) where genetically engineered herbicide tolerant oilseed rape lost their herbicide tolerance due to the silencing of the herbicide transgene because of homology in the 35S promoter region. This may have implications for the phenotype of economically useful transgenic crops through an effect on the 35S promoter, which is present in many transformed crops.

2.8 GENETIC STABILITY AND FIELD PERFORMANCE

For commercial applications, the ultimate test of a transgenic plant is its performance in the field. The field trial not only permits the determination of the genetic stability of the introduced trait and its inheritance in subsequent generations, but also allows the evaluation of competitive agronomic characteristics. In most instances transgenic plants have been produced by introducing traits into existing commercial cultivars. Field trials serve to determine whether any changes occurred as a result of the transformation process, as well as evaluating the introduced trait (de Greef *et al.*, 1989; van Rensburg, 1999; Buckmann *et al.*, 2000; Jiang *et al.*, 2000; Tu *et al.*, 2000a, 2000b; Leibbrandt and Snyman, 2001; Zhong, 2001).

Field studies on tissue culture-derived, non-transformed barley revealed a considerable number of mutations such as reduced plant height, yield and kernel plumpness (Bregitzer *et al.*, 1998b; Horvath *et al.*, 2001). These characteristics were also observed in transgenic barley and could be improved by cross breeding with cultivars of a different genotypic constitution (Horvath *et al.*, 2001).

Transgenic herbicide resistant sugarcane has shown stable gene expression over three ratoons (a ratoon is the regrowth of the sugarcane plant after harvesting) (Gallo-Meagher and Irvine, 1996; Snyman *et al.*, 1998). Extensive field testing of a Buster-resistant sugarcane line in South Africa established that stalk morphology, susceptibility to two diseases and yield of transformed and untransformed cane were not significantly different (Leibbrandt and Snyman, 2001). In another study, phenotypic variation in stalk height, diameter and tillering ability (tillering is the underground branching of sugarcane) was observed in both transgenic and untransformed sugarcane, suggesting that this was culture-induced variation (Sala *et al.*, 1999; Arencibia *et al.*, 1999, 2000).

CHAPTER 3
OPTIMISATION OF PROTOCOLS FOR TRANSFORMATION OF SUGARCANE VIA MICROPROJECTILE BOMBARDMENT

3.1 INTRODUCTION

Early work on sugarcane transformation involved the use of electroporation or PEG treatment of protoplasts (Chen *et al.*, 1987), but the recovery of plants from sugarcane protoplasts remains difficult (Chen *et al.*, 1987; Chowdhury and Vasil, 1992; Rathus and Birch, 1992) and has been restricted to a few cultivars (Arencibia *et al.*, 1995). Embryogenic suspension cultures have also been used as target material for microprojectile bombardment (Rathus and Birch, 1991; Chowdhury and Vasil, 1992), but no transgenic plants were regenerated.

Subsequently, transgenic sugarcane plants have been produced by microprojectile bombardment of embryogenic callus (Bower and Birch, 1992; Bower *et al.*, 1996; Gallo-Meagher, 1996; Snyman *et al.*, 1998; Falco *et al.*, 2000), although transformation efficiencies vary greatly, suggesting that further refinement of the approach is necessary in each laboratory. Limited success has been achieved using *Agrobacterium*-mediated DNA delivery to embryogenic callus (Arencibia *et al.*, 1998b; Elliot *et al.*, 1998), but this technique is not as widely employed as microprojectile bombardment.

Although most authors state the use of sugarcane 'embryogenic callus' as target material for bombardment, it appears as though the callus morphology is important, with Falco *et al.* (2000) stating specifically that they use 'nodular, compact and white calli' as target material. Bower and Birch (1992) observed that this type of callus shows the highest levels of transient expression and later studies (Bower *et al.*, 1996) utilising the same 'Type 3' callus (Taylor *et al.*, 1992b) recorded transformation efficiencies of up to 20 plants per bombardment, which are the highest reported for sugarcane.

The auxin 2,4-D has been widely used in the production of embryogenic callus cultures in sugarcane (Nadar *et al.*, 1978; Ho and Vasil, 1983; Chen *et al.*, 1988; Guiderdoni and Demarly, 1988; Taylor *et al.*, 1992b; Fitch and Moore, 1993). The concentrations range from 0.15-7 mg/l 2,4-D, with the lower concentrations resulting in faster production of embryoids, where the characteristic heart and torpedo stages of somatic embryo development are evident (Ho and

Vasil, 1983). Callus initiation from immature leaf roll discs occurs most frequently on media containing 3 mg/l 2,4-D (Heinz and Mee, 1969; Lee, 1987; Taylor *et al.*, 1992; Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996), but optimum embryoid formation occurs at concentrations of 0.5-1.5 mg/l 2,4-D, lower than that required for optimum growth of embryogenic callus (Ho and Vasil, 1983; Guiderdoni and Demarly, 1988). A concentration of 1 mg/l 2,4-D has been used to promote somatic embryo maturation prior to placement on regeneration medium which does not contain auxin (Guiderdoni and Demarly, 1988; Gallo-Meagher and Irvine, 1996).

The aims of the work presented in this chapter were to maximise production of Type 3 embryogenic calli from different cultivars and to establish bombardment parameters using a locally constructed Particle Inflow Gun from the design of Finer *et al.* (1992). Use was made of 2 different transient expression systems, involving the GUS and anthocyanin genes, respectively, to determine bombardment parameters and efficacy of four promoter constructs. In addition, regeneration and selection regimes were established.

3.2 MATERIALS AND METHODS

3.2.1. Explant material and embryogenic callus initiation

Callus was initiated from immature leaf rolls of mature, field-grown sugarcane cultivars NCo310, NCo376, N8, N11, N12, N16, N18, N19, N20, N22, N23, N24 and N27. Surface contaminants were removed by rinsing the leaf roll in tap water and a commercial dish-washing detergent, followed by swabbing with 70% (v/v) ethanol. The outermost mature leaves were aseptically removed and the young, innermost leaves sliced into sections approximately 3 mm thick. Leaf roll discs (21 per stalk, taken from 10-15 cm directly above the top-most node) were placed on a solid callus-induction medium (referred to as MS3 medium) which consisted of MS basal salts and vitamins (Murashige and Skoog, 1962; Sigma, St Louis) supplemented with casein hydrolysate (1 g/l), sucrose (30 g/l), 2,4-D (3 mg/l) and agargel (5 g/l) (Sigma, St Louis), pH 5.8. Cultures were placed in the dark at 26±1°C and were transferred to fresh medium every two weeks.

3.2.2 Microscopy

Small pieces of callus were squashed onto a microscope slide, stained with safranin and viewed by light microscopy.

3.2.3 Plasmid constructs

Several plasmid constructs were used for bombardment (Table 3.1; Appendix 1).

Table 3.1 Plasmid constructs used for microprojectile bombardment of embryogenic sugarcane callus. All contained the ampicillin resistance gene for selection in bacterial culture.

Plasmid name	*Size (kB)*	*Gene of interest*	*Selectable marker*	*Promoters*	*Source and/or reference*
pDP687	9.05	Anthocyanin	-	Double CaMV 35S	DuPont (formerly Pioneer Hi Bred International Inc.)
pAHC27	6.84	GUS	-	Maize ubiquitin	Aventis (formerly Plant Genetic Systems)
pBI221	3	GUS	-	CaMV 35S	Clonetech Laboratories
pAct-1D	5.2	GUS	-	Rice actin	McElroy *et al.* (1990)
pAHC25	9.88	*bar* and GUS	*bar*	Maize ubiquitin	Aventis
pEmuGN	6.5	GUS	-	Emu	Last *et al.* (1991)

3.2.4 Plasmid generation and purification

Plasmids were maintained and grown in *E. coli* (JM83) on Luria agar or broth (bacto-tryptone (10 g/l), yeast extract (5 g/l), NaCl (10 g/l) and ampicillin (100 mg/l) at pH7.5). Plasmids were purified using either a large-scale alkaline lysis methos (Armitage *et al.*, 1988) or a Nucleobond AX100 kit (Macherey-Nagel, Germany).

3.2.5 Assessment of DNA concentration, purity and integrity

The concentration of DNA was determined either spectrophotometrically (DU 7500, Beckman, Fullerton CA) or flourimetrically (Hoefer, San Francisco CA). Using the spectrophotometer, the concentration of purified DNA was determined from A260 nm and purity from A260: A280 nm and 280 nm (Maniatis *et al.*, 1982). Pure double stranded DNA should have a ratio of 1.8.

Restriction analysis was performed on plasmid DNA, and samples were run on an agarose gel (1%), and stained with ethidium bromide (0.5 µg/ml) to verify integrity.

3.2.6 DNA precipitation onto tungsten particles

Tungsten (M17; Bio-Rad Laboratories, CA) or gold particles (1 µm; Bio-Rad Laboratories) were sterilised with 70% ethanol and resuspended in sterile distilled water. While vortexing (moderate setting), this suspension (25 µl; 100 mg/ml) was mixed with the plasmid DNA

solution (20 µl; 5 µg), $CaCl_2$ (25 µl) and spermidine (10 µl; 0.1 M; free base). The mixture was vortexed for a further 1 min and placed on ice for 5 min before being pulse-centrifuged. The supernatant was removed and the coated particles were resuspended in ethanol (30 µl; 100% (v/v)). The microcarrier suspension was vortexed briefly prior to each bombardment.

3.2.7 Microprojectile bombardment

The Particle Inflow Gun (PIG) (Finer *et al.*, 1992) is based on helium propulsion of particles in a vacuum chamber. The PIG was constructed locally (Figure 3.1) and was used for all DNA deliveries. Type 3 embryogenic callus pieces (2 g callus, occupying an area of 1.5 cm^2) were placed on a grid in the vacuum chamber, 6 cm below the unit containing the DNA-coated tungsten or gold particles. A layer of nylon mesh (200 μm^2) was placed over the calli. For bombardment, 2 µl of the particle suspension was placed in the centre of a 1 mm^2 metal grid in a disassembled 13 mm Swinney filter holder (Millepore, Germany). The filter unit was reassembled and screwed into an adapter above the calli. The chamber was evacuated to a pressure of 10 kPa and the particles were discharged when the helium (600-2000 kPa) was released by a solenoid linked to a timer relay (0.05 s).

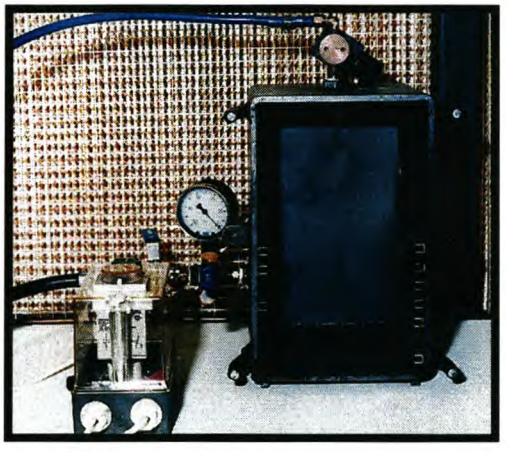


Figure 3.1 The PIG chamber is based on the design by Finer *et al.* (1992) and was constructed locally.

3.2.8 Transient gene expression assays

3.2.8.1 Anthocyananin assay

Callus was bombarded with pDP687 and kept in the dark for 48 hours, after which transient expression of the anthocyanin gene was assessed by enumeration of red foci using a stereomicroscope (Zeiss, Germany).

3.2.8.2 GUS assay

The histochemical GUS assay was performed by submersing bombarded calli in microfuge tubes containing 1 ml of GUS substrate containing 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (2 mM; X-Gluc, Sigma), potassium ferrocyanide ($K_3[Fe(CN)_6]$; 0.1 M), potassium ferricyanide ($K_4Fe(CN)_6.3H_2O$; 0.1 M) and Na_2EDTA (0.1 M) in a sodium phosphate buffer (0.2 M). Samples were vacuum infiltrated for 2 mins, before adding Triton X 100 (0.2% v/v). Samples were incubated at 37°C overnight, in the dark, and the presence of the enzyme was visualised under a stereomicroscope by the appearance of a blue stain.

3.2.9 Geneticin kill curve

Calli from cultivars NCo310, NCo376, N12, N19 and N27 were generated on MS3 for a period of four weeks, and were cycled between MS1 (as for MS3, but with 1 mg/l 2,4-D) and MS3 for a further four weeks, after which they were placed onto MS 3 media containing a range of concentrations of geneticin (0, 0.15, 1.5, 5, 15, 30, 45 and 60 mg/l geneticin; Roche Biochemicals, South Africa). Callus was subcultured fortnightly. After eight weeks, calli were transferred to regeneration medium containing the same concentrations of geneticin, and plant numbers were counted over the following eight weeks.

3.2.10 Selection of stably transformed calli

Bombarded calli were placed on MS3 medium for four days, before being transferred to a selection medium containing the antibiotic geneticin (G418, Roche; 45 mg active ingredient /l) (Bower *et al.*, 1996) for a further 12 weeks in the dark at 26°C, with fortnightly subculturing.

3.2.11 Regeneration of plantlets

Calli were transferred to MS regeneration medium, without 2,4-D, containing a range of additives such as kinetin (0.5 mg/l), coconut milk (10% v/v; Sigma, St Loius), benzyl-amino purine (BAP; 0.5 mg/l), and with the agargel replaced by gelrite (2 g/l; Saarchem, SA). Cultures were maintained in a 16 h light/8 h dark photoperiod at 26±1°C and were subcultured fortnightly.

Regeneration of stably transformed calli was carried out on MS medium with kinetin (0.5 mg/l) and G418 (45 mg/l) (referred to as RM) for 12 weeks. The selection pressure was maintained until plantlets were 10 cm high and had a well-developed root system. If young (2 cm) plantlets rooted poorly, they were transferred to MS medium containing 3-indolebutyric acid (IBA; 0.5

mg/l) for 2 weeks, before being placed in Magenta jars (Sigma, St Louis) on medium without kinetin, half-strength MS salts and vitamins, and a reduced sucrose content (10 g/l).

3.2.12 Hardening-off in the glasshouse

When plantlets in Magenta jars were approximately 10 cm tall, they were transferred to the glasshouse in moist potting soil (Grovida, South Africa) in 72-well polystyrene speedling trays and covered with plastic wrap for 1 week (Figure 3.2). Once the plastic was removed, the plants were watered for 1 min every 2 hours with a fine mist during natural daylight hours for a period of two weeks. Thereafter they were transferred to plastic pots (first to 3 l, and then to 20 l pots), watered for 10 minutes twice a day and fertilised once a week (Hygrotech hydroponic seedling nutrient mixture).

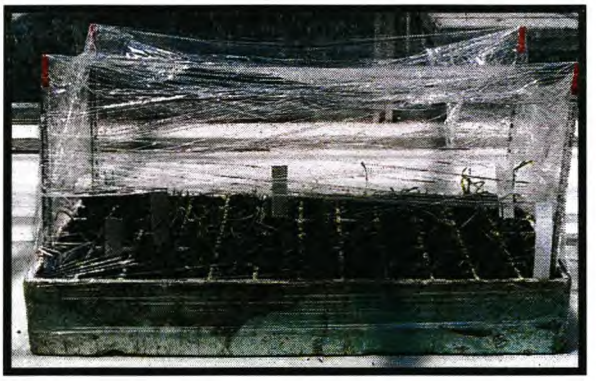


Figure 3.2 *In vitro*-derived sugarcane plantlets were hardened-off in speedling trays in the glasshouse. Plantlets suitable for hardening-off were obtained after a total of six-seven months in culture. The plastic wrap was removed after 1 week and plants were watered by mist sprayers for an additional two weeks before being transferred to a twice daily 10 min watering regime.

3.3 RESULTS

3.3.1 Confirmation of plasmid integrity

Each time plasmids were purified, a restriction analysis was performed to ensure that the restriction patterns matched the expected fragment sizes (plasmid maps shown in Appendix 1).

3.3.2 Establishment of culture conditions for production of Type 3 callus

3.3.2.1 Effect of cultivar on embryogenic callus production on conventional medium with 3 mg/l 2,4-D

A simple comparison of the amounts of embryogenic callus produced by several cultivars was made on conventionally used medium, MS3. Leaf roll discs from cultivars NCo310, NCo376,

N12, N19 and N27 were placed on callus induction medium in the dark and were subcultured fortnightly. Callus mass was measured after eight weeks. A mixture of yellow (Type 4; as classified by Taylor *et al.* (1992)) and white, nodular embryogenic callus (Type 3) (Figure 3.3) was observed in all cultivars, interspersed with small amounts of shiny non-embryogenic callus (Type 2) (Table 3.2). The mass of callus produced varied between the cultivars (Figure 3.4), but there were no significant differences. NCo310 produced the largest mass of callus (12.17 g/stalk ± 3.4, n=6), followed by NCo376, N12, N27 and N19, producing 98, 96, 85 and 80% of the callus mass in NCo310, respectively.

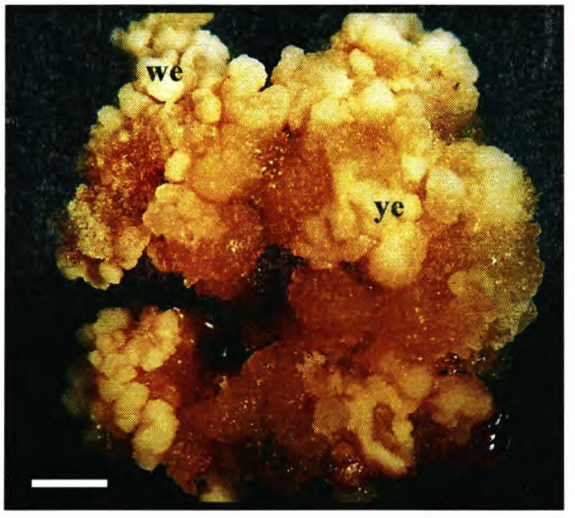


Figure 3.3 Embryogenic sugarcane callus. Callus from cultivar NCo310 generated on MS3 consists of nodular, white embryogenic (we) structures (Type 3 callus), interpersed with smaller regions of yellow embryogenic (ye) (Type 4 callus) and non-embryogenic regions (Type 2) after eight weeks in culture. Scale bar =2 mm.

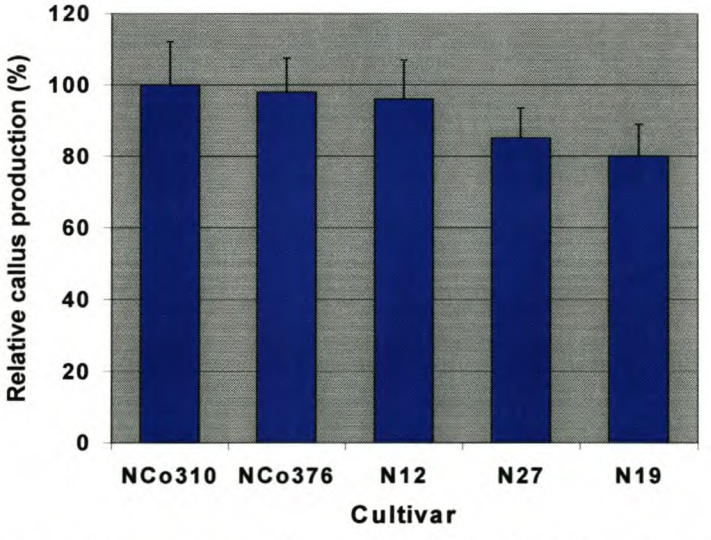


Figure 3.4 Comparative growth of embryogenic callus produced by cultivars NCo310, NCo376, N12, N19 and N27. Callus was massed after eight weeks in culture on MS3. Masses are expressed as a percentage of NCo310 (12.17 g ± 3.4 callus per stalk placed in culture) (n=6).

Table 3.2 A description of three sugarcane callus morphologies observed in all cultivars in this study according to the classification proposed by Taylor *et al.* (1992a).

Parameter	***Type 2***	***Type 3***	***Type 4***
Colour	Yellow-grey	White	Cream or yellow
Growth rate	Slow(er)	Fast	Fast
Texture	Wet/shiny, sticky	Dry, nodular, compact	Wet, grainy, friable
Overall characteristics	A shiny, wet matrix, no aggregates visible	Homogenous mixture of early somatic embryos, giving a compact, nodular appearance	Homogenous mixture of smooth-surfaced pre-embryogenic aggregates
			

3.3.2.2 Enhanced production of Type 3 callus on a lower 2,4-D concentration

Although embryogenic callus from sugarcane is most frequently produced on medium containing 3 mg/l 2,4-D, a culturing regime involving both a lower concentration of 2,4-D (1 mg/l) and cycling between low (1 mg/l 2,4-D; MS1) and high (MS3) 2,4-D-containing media was reported to result in superior callus growth and subsequent regeneration (Chen *et al.*, 1988). In addition, the use of white, nodular and highly embryogenic callus as target material for bombardment has been emphasised (Bower *et al.*, 1996; Snyman *et al.*, 1996; Falco *et al.*, 2000). In an attempt to increase the proportion of NCo310 Type 3 callus produced relative to the other callus types, selective subculturing of Type 3 callus on MS1 medium was investigated. It was necessary to use MS3 medium for callus induction and proliferation during the first four weeks of culture, after which time the production of Type 3 callus on MS1 was investigated over an additional eight week period. The difference in the relative amounts of Type 3 callus produced was first observed on a purely visual basis (Figure 3.5), where MS1 was used for selective subculturing of white, nodular embryogenic callus, after a four week period of callus initiation on MS3.

Microscopic examination of Type 3 callus revealed heart and torpedo-shaped somatic embryos (Figure 3.6A), whereas no definite structures were evident in the other callus types (Figure

3.6B). This has important implications for bombardment, as it is necessary to be able to bombard and subsequently regenerate plantlets from the most responsive target material.

In order to see whether culturing callus on MS1 could support the production of white, nodular embryogenic callus in several cultivars, the response of 12 cultivars was investigated for their ability to produce Type 3 callus on MS1 with fortnightly subculture intervals. At each subculture, calli were scored for the percentage of white callus occupied by the total callus volume. Callus was visually assessed every two weeks using the following scoring system: 1 = 0-25%; 2 = 25-50%; 3 = 50-75%; 4 = 75-100% Type 3 callus as a percentage of total callus volume (Figure 3.7). After each assessment, white, embryogenic callus was selectively subcultured, placed onto fresh medium and given a score of 75-100%. A decrease in the proportion of white callus (i.e. when the rating decreased from the initial score of 75-100%) reflected a combination of both a reversion of embryogenic callus to non-embryogenic callus, and growth of new callus that is non-embryogenic.

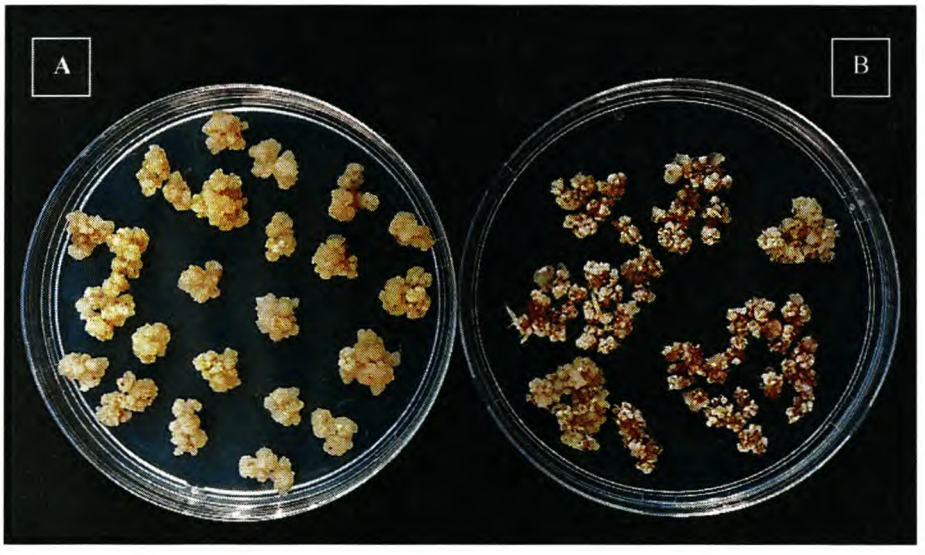


Figure 3.5 A visual comparison of the effect of 2,4-D concentration on callus production. NCo310 callus was initiated on MS3 and subcultured on this medium for four weeks, after which it was either maintained on MS3 (A) or transferred to MS1 (B) for a further four weeks.

A. The callus in Petri-dish A was initiated and maintained on MS3 for a period of eight weeks with fortnightly subculturing. It is predominantly yellow in colour, with small sections of white, nodular embryogenic callus.
B. Petri-dish B consists of a higher proportion of white, nodular and compact white embryogenic callus which was produced by transferring 4 week-old calli, initiated and maintained on MS3, to MS1 for a further four weeks with fortnightly subculturing.

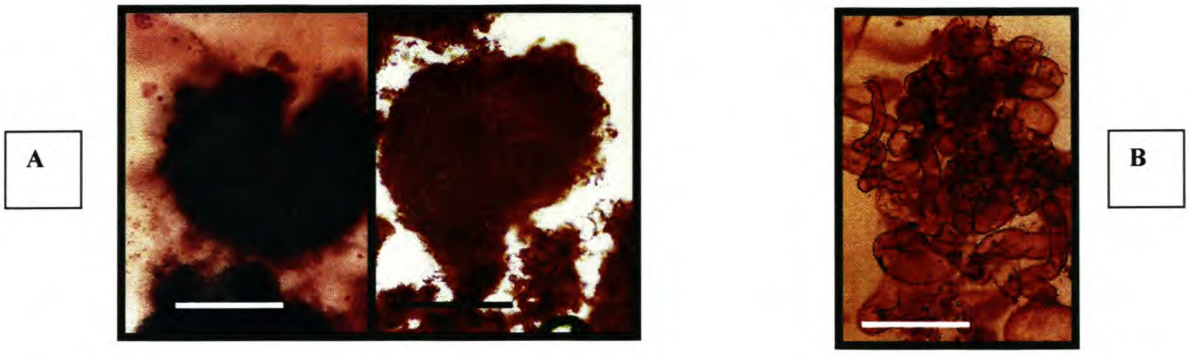


Figure 3.6 Microscopic examination of 2 types of sugarcane callus. The callus was squashed on a microscope slide, and stained with safranin. Bar = 1mm.

A. Type 3 callus consisting of heart and torpedo shaped somatic embryos. The NCo310 callus was grown on MS3 for four weeks, followed by culture on MS1 for a further four weeks.
B. Non-embryogenic callus consists of large cells with no obvious structure.

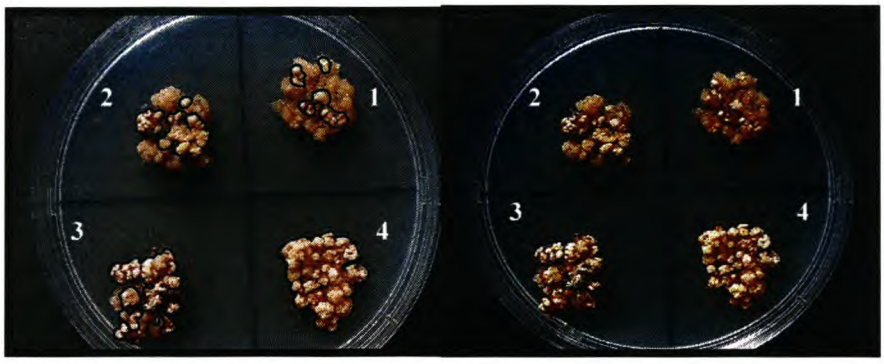


Figure 3.7 Visual rating system for assessing the proportions of white callus in relation to the total callus volume. Quadrant 1 represents 0-25%; 2= 25-50%; 3 = 50-75% and 4 =75-100% white, nodular callus as a percentage of total callus volume. The areas demarcated in black on the left are Type 3 callus.

Cultivars N11, N21, N22, N23 and N24 produced little or no Type 3 callus when transferred to MS1 and were discarded, suggesting that not all cultivars respond in the same manner. Cultivars NCo310, NCo376, N18, N16 and N20 produced the highest proportion of white callus after 2 weeks, with little change in any of these cultivars occurring during the first four weeks (Figure 3.8). The callus produced by NCo310 and NCo376 received a score of 3-4 for eight successive weeks (Figure 3.8), indicating that the new callus produced was white, nodular, Type 3 callus. There was some reversion of Type 3 callus to non-embryogenic callus eight weeks after transfer to MS1, indicating that embryogenic callus does lose 'form' and should not be maintained in culture for extended periods of time, particularly if it is produced

specifically for particle bombardment. Cultivar N18 performed well for the first four weeks in culture, after which there was a dramatic reduction in the proportion of Type 3 callus but in most other cultivars tested, callus was generally homogenous and appeared suitable for transformation purposes for six weeks.

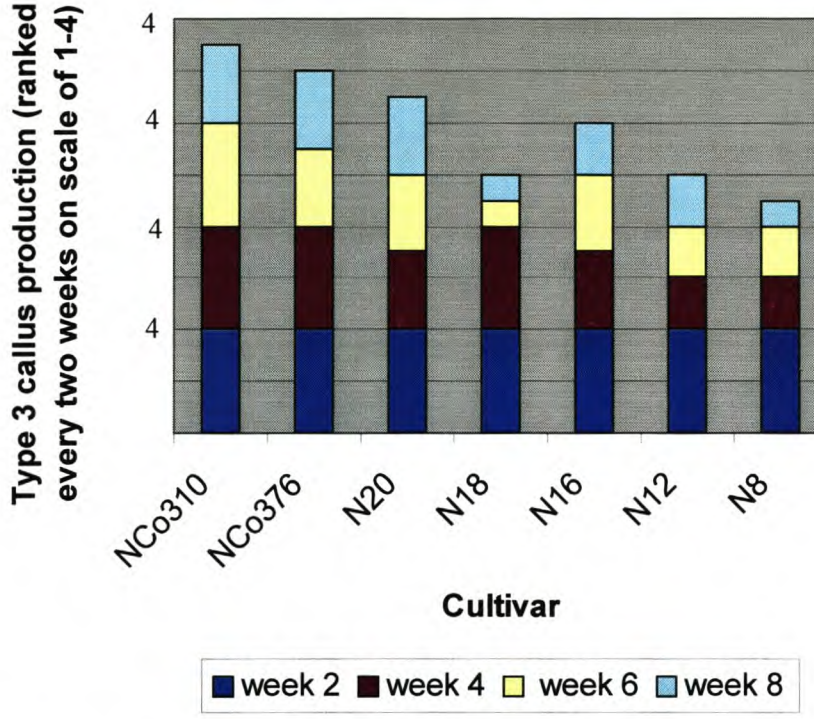


Figure 3.8 Relative proportions of Type 3 callus production on MS1 in several cultivars. Callus was generated on MS3 for 4 weeks prior to transfer to MS1. Every two weeks, the proportion of Type 3 callus was rated on a scale of 1-4, where 1 = 0-25%; 2 = 25-50%; 3 = 50-75%; 4 = 75-100% (Type 3 callus as a percentage of total callus volume). Callus was subcultured fortnightly and only Type 3 callus was transferred to fresh medium. (Scores presented are the mean of the ratings of three independent observers).

3.3.2.3 Effect of cycling between high and low concentrations of 2,4-D on embryogenic callus production

Having established the benefit of using MS1 medium to improve the production of Type 3 callus, the usefulness of another finding of Chen *et al.* (1988) needed to be established: that the cycling callus between MS3 and MS1 resulted in improved callus quality and regeneration potential. Callus from cultivars NCo376, N12 and N19 was initiated and maintained on MS3 for four weeks. Callus was massed after a further four weeks in culture on three regimes: 1) cycling between MS1 and MS3, 2) maintained on MS1 and 3) maintained on MS3. The increase in callus mass per cultivar was compared for each culturing regime and is presented in Figure 3.9. Although none of the differences are significant, cycling callus between MS1 and

MS3 resulted in the highest increase in callus mass. In addition, the callus in this regime maintained its white, nodular appearance, whereas new callus arising on MS3 was yellow in colour, reducing the overall proportion of Type 3 callus. Callus cultured on MS1 maintained its white, nodular structure, and the new callus produced was similar. For the purposes of generating Type 3 callusfor use in bombardment experiments, cycling between MS1 and MS3 appeared to be the most suitable.

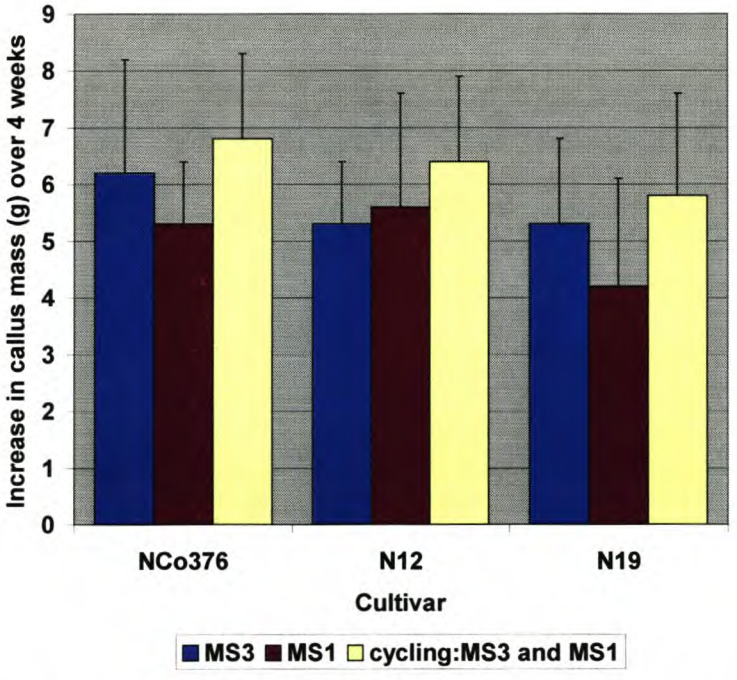


Figure 3.9 Comparative increase in callus mass of cultivars NCo376, N12 and N19 in 3 culturing regimes. Calli were initiated and maintained on MS3 for four weeks, after which identical masses were transferred to either MS1, MS3 or cycled between MS3 and MS1, with fortnightly subculturing, for a further four weeks. The increase in mass over these four weeks is the mass that is presented in this Figure. Bars represent standard errors (n=5).

3.3.3 Optimisation of helium pressure and microcarrier type

Several helium pressures (600, 1000, 1500 and 2000 kPa) were used for bombardment in order to determine which was most favourable, using the GUS transient expression assay. Embryogenic NCo310 callus was bombarded with plasmid pAHC27 and the GUS histochemical assay was carried out 48 hours later (Figure 3.10A). No expression was visualied in Type 2 callus. GUS transient expression rates increased with pressure of the helium pulse used to accelerate microprojectiles, up to 1000 kPa, after which expression levels decreased (Figure 3.10B). There was no significant difference in expression levels between 1000 and 1500 kPa, but it was decided to use the lower pressure to minimise damage to cells caused by bombardment.

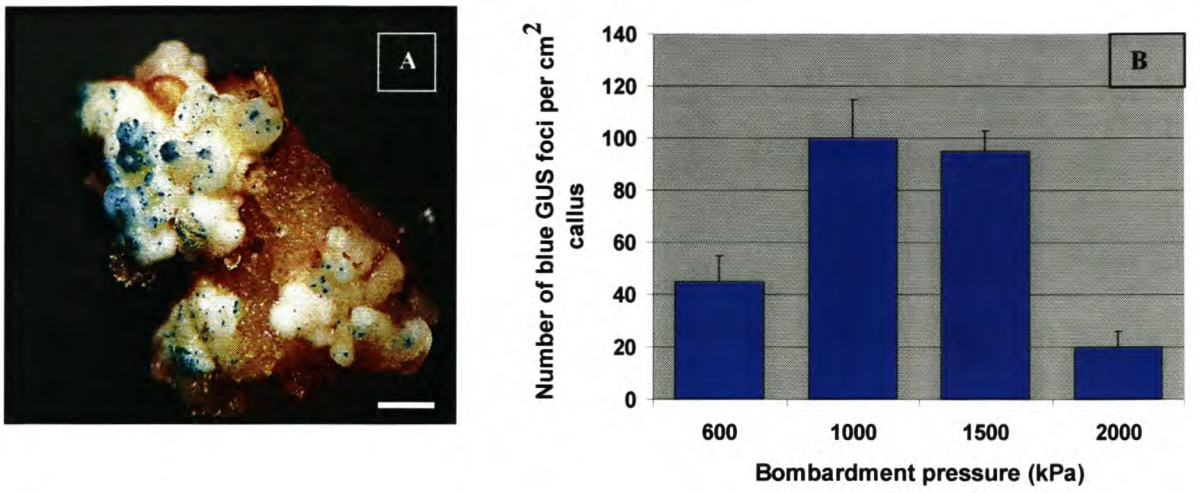


Figure 3.10 Effect of helium pressure for particle bombardment on transient expression.

A. Transient GUS expression is visible on Type 3 callus. No or very few foci are observed on Type 2 callus. The GUS histochemical assay was carried out 48 hours after bombardment, whereby the X-Gluc substrate was vacuum infiltrated into calli. Blue foci appeared after an overnight incubation at 37 °C in the substrate. Scale bar = 1 mm.

B. The number of GUS foci per cm^2 of callus was recorded 48 hours after bombardment at four helium pressures. SE are presented as vertical bars (n=3).

A comparison between gold and tungsten microcarriers was made. NCo310 callus was bombarded with pAHC27 and the GUS histochemical assay was carried out 48 hours after DNA delivery. Substitution of tungsten with gold particles did not significantly alter transient expression rates (tungsten 170 ± 32, gold 152 ± 27 GUS foci per cm^2 callus; mean ± SE of 4 replicates).

The pre-treatment of callus on an osmoticum medium (mannitol (0.2 M) and sorbitol (0.2 M); Vain *et al.* (1993b)) for 4 hours pre- and post-bombardment was another parameter investigated during the course of optimising bombardment conditions. No increase in transient expression levels using the GUS gene system was observed in callus cultured on osmoticum medium (results not shown). Consequently, osmoticum was not used in the standard bombardment protocol.

3.3.4 Cultivar effect on transient anthocyanin expression

Although the GUS assay is commonly used for assaying transient gene expression, the use of the anthocyanin pigment production system is a simpler and more inexpensive way of monitoring gene expression. Unlike the GUS assay, the anthocyanin assay is non-destructive and therefore regeneration studies could be performed on bombarded calli. Embryogenic Type

3 calli from cultivars NCo310, NCo376, N8, N12, N16, N18 and N20 were bombarded with the plasmid pDP687, containing the transcriptional activators for anthocyanin pigment production. Expression was evaluated by counting red foci 48 h after bombardment (Figure 3.11A). The highest transient anthocyanin levels were observed in cultivars NCo376 and NCo310 (Figure 3.11B). These showed expression levels approximately three times greater than the next best variety N8. N20 on the other hand, showed virtually no anthocyanin expression, indicating a difference in genotypic response to the DNA transfer procedure.

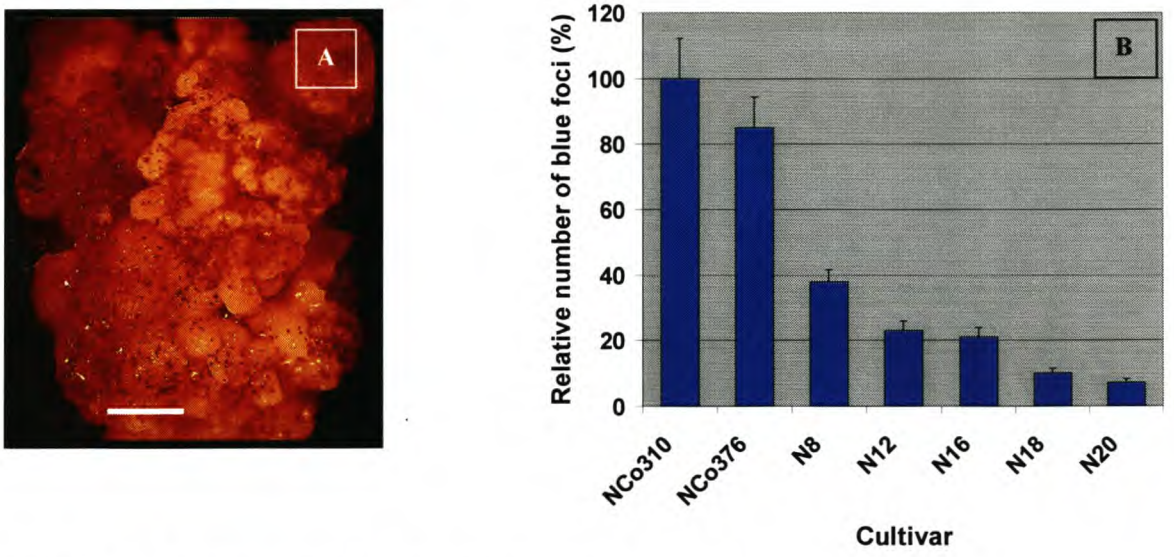


Figure 3.11 Effect of sugarcane genotype on transient expression.

A. Sugarcane callus transiently expressing the anthocyanin gene. The red foci were visible 48 h after bombardment with plasmid pDP687. Scale bar = 1 mm.

B. Relative number of anthocyanin foci after microprojectile bombardment in selected South African cultivars. For comparative purposes, foci are expressed as a percentage of the result observed in NCo310, where an average number of 850 (± 12.2) foci per 2 g callus per blast were recorded. SE are presented as vertical bars (n=3).

3.3.5 Effect of cultivar, bombardment and medium on regeneration

3.3.5.1 Cultivar differences and bombardment effects on regeneration efficiency

In order to assess whether there was a genotypic-specific response to regeneration and whether bombardment had any effect on regeneration, the number of plantlets produced per gram of both bombarded and non-bombarded embryogenic callus was assessed in several cultivars. Callus was bombarded with the anthocyanin construct and maintained in the dark for a period of 12 weeks, after which it was transferred to conventionally-used regeneration medium (Chen *et al.*, 1988; Bower *et al.*, 1996). Results are shown in Table 3.3. Although regeneration of non-bombarded embryogenic callus from varieties N8, N12, N16, N18, N20, NCo376 and

NCo310 has been observed in our laboratory previously, in this anthocyanin-transient expression study, regeneration was observed only in NCo310 from both bombarded and non-bombarded calli, and from non-bombarded N18 callus. It is apparent that the bombardment protocol had a negative affect on regeneration in cultivar N18, but not in NCo310.

When non-bombarded callus was allowed to regenerate, NCo310 showed the highest incidence of plantlet production (38 plants per g callus), while 30 plants per g callus were observed in N18 (Table 3.3). Although microprojectile bombardment did not appear to affect regeneration potential in NCo310, where 35 plants per g callus were produced, in N18 there was no regeneration (Table 3.3). Albino plantlets (Figure 3.12) were common in NCo310 but absent in N18. Half of the bombarded NCo310 plantlets were albinos, compared with 6% albinos observed in non-bombarded callus.

Table 3.3 Total number of plantlets per g callus produced from bombarded and non-bombarded sugarcane embryogenic calli. (Values are the mean of three replicates. SD were larger than 10% of the mean.)

	Number of plants per g callus	
	NCo310	N18
Total no. of plants from bombarded callus	35	0
Albino plants from bombarded callus	17	0
Ptotal no. of pants from non-bombarded callus	38	30
Albino plants from non-bombarded callus	2	0

The poor regeneration obtained in this experiment may have been due to regeneration medium not being optimal for all South African cultivars. As it is imperative to be able to regenerate potentially transformed plants, a comparison of several regeneration media was made.

3.3.5.2 Comparison of different regeneration media formulations

Due to the poor regeneration efficiencies observed in the previous section (3.3.5.1), the regeneration of non-bombarded NCo376 callus on five different regeneration media was compared to maximise plantlet production. Embryogenic callus was cultured for four weeks on MS3 and for a further eight weeks was cycled between MS1 and MS3 before being transferred to regeneration medium. Results presented in Figure 3.13 show that regeneration efficiency could be affected by slight changes in the composition of the medium. The medium containing kinetin supported the most plants, 134 per g callus (Figure 3.13). This was followed by the regeneration medium without hormones (115), that containing coconut milk (110), gelrite (98) and BAP (34).

Figure 3.12 Albino plantlet production in NCo310. Albinos were observed at a frequency of 6% in NCo310 in routine plantlet regeneration after culturing on MS3 for four weeks, followed by cycling between MS1 and MS3 for a further eight weeks before being placed onto regeneration medium in a light/dark photoperiod.

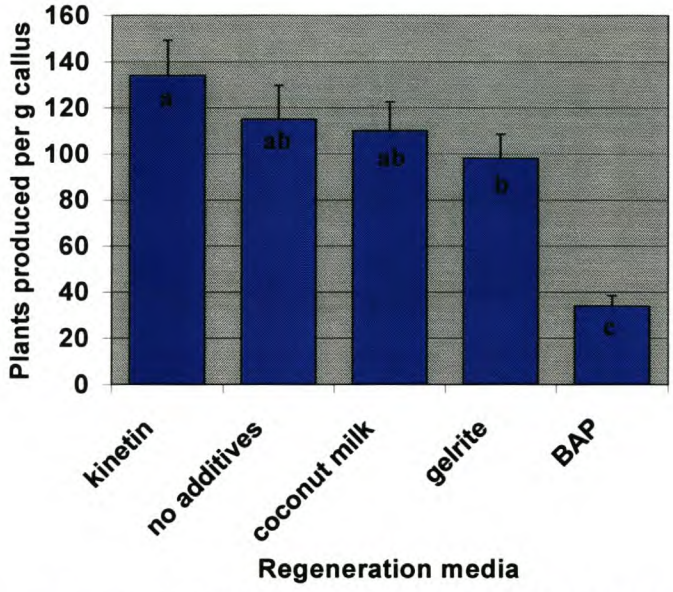


Figure 3.13 A comparison of the number of NCo376 plantlets regenerated per g callus on five different regeneration media. Callus was cultured on MS3 for four weeks, and cycled between MS1 and MS3 for a further eight weeks prior to transfer to regeneration medium in a 16 hour light/8 h dark photoperiod. Regeneration media consisted of MS with kinetin (0.5 mg/l), no additives, coconut milk (10% (v/v)), gelrite (2% (w/v)) and BAP (0.5 mg/l). Bars represent standard errors (n=6). Different alphabetical letters represent statistical differences (ANOVA SED=16).

3.3.6 Comparison of promoter strengths using transient GUS expression

In order to choose a promoter to drive stable gene expression, four promoters, namely Emu, rice actin, CaMV 35S and maize ubiquitin, driving the GUS gene in constructs pEmuGN, pActD-1, pBI221 and pAHC27, respectively, were introduced by microprojectile

bombardment to embryogenic NCo310 callus. GUS activity was monitored after 48 h by counting blue foci. Results are presented in Figure 3.14. The highest number of blue foci were observed with the maize ubiqutin promoter (275 foci per g callus), followed by the Emu (160) and CaMV (100) promoters. No significant GUS activity was detectable with the rice actin promoter.

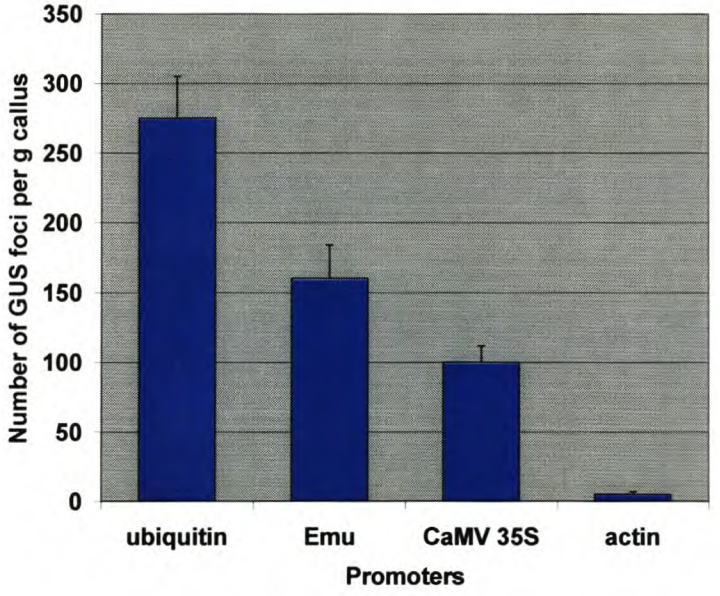


Figure 3.14 Assessment of promoter activity in NCo310 callus using the GUS assay. White, nodular callus was bombarded with the following plasmids (promoters): pAHC27 (maize ubiquitin), pBI221 (CaMV 35S), pAct-1D (rice actin) and pEmuGN (Emu) and GUS activity was assessed 48 h later. Bars represent standard errors (n=3).

3.3.7 Establishment of optimal geneticin concentration to use for selection

For stable expression studies, it was decided to use the selectable marker gene containing the *nptII* marker, which confers resistance to the antibiotic geneticin. The selection procedure described previously (Bower *et al.*, 1996) utilises 45 mg/l geneticin for an 'escape-free' selection regime. To determine the sensitivity of non-transformed sugarcane callus of South African cultivars to geneticin, growth rates of callus and numbers of plants regenerated were compared over a range of concentrations (0, 0.15, 1.5, 15, 30, 45 and 60 mg/l) of the antibiotic. Callus growth rates of several cultivars were measured by massing the callus at two-weekly intervals for eight weeks. The results are presented in Figure 3.15. The trend exhibited by all five cultivars was that callus mass decreased with increasing concentration of geneticin (Figure 3.15A). Negligible callus growth was observed in any of the cultivars after 8 weeks on medium containing geneticin at 30, 45 and 60 mg/l. Cultivars exhibited different sensitivities to geneticin, with NCo310 being the most susceptible to the selective agent (except at

concentration of 1.5 mg/l) (Figure 3.15A), and N27 the most resistant, up to a concentration of 5 mg/l, after which it responded in a similar fashion to the other cultivars.

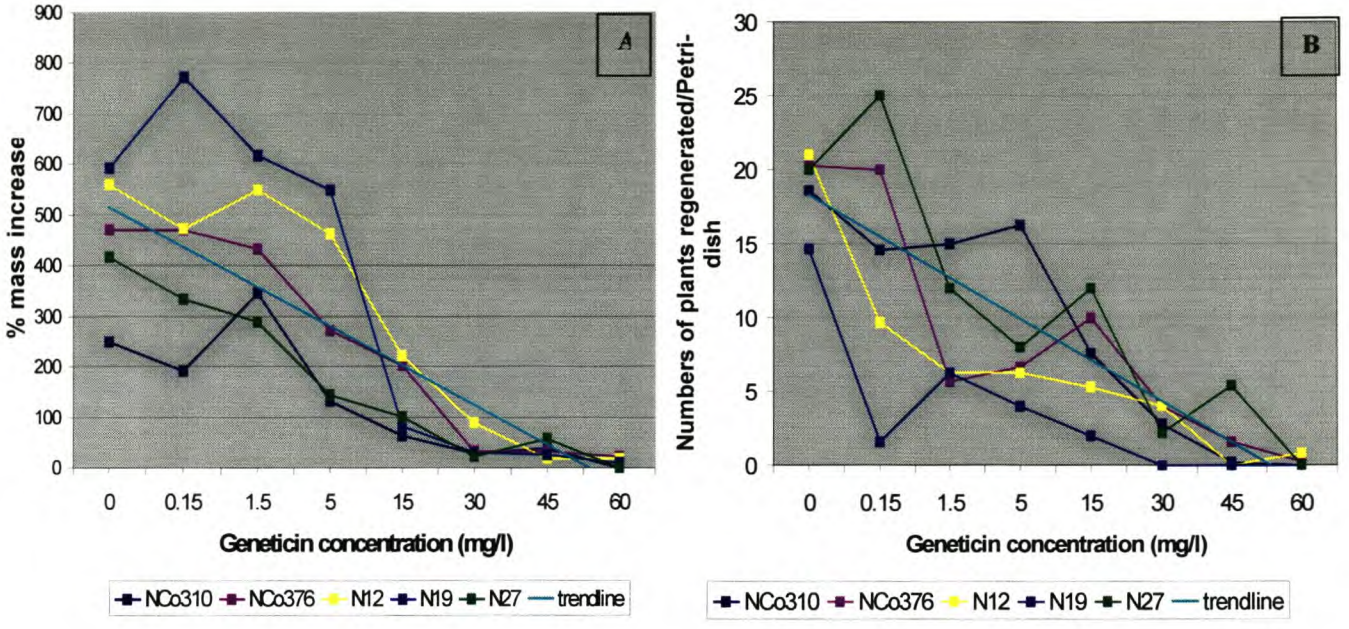


Figure 3.15 Effect of geneticin on callus growth and plant regeneration.

A. Eight-week old calli (nine similar sized pieces per Petri-dish) from cultivars NCo310, NCo376, N12, N19 and N27 were transferred to MS3 media containing different concentrations of geneticin (0, 0.15, 1.5, 5, 10, 15, 30, 45 and 60 mg/l) for eight weeks with fortnightly subculturing. The initial fresh mass was determined and calli were massed again after eight weeks of culture in the dark. The growth rate is defined as 100 times the ratio of fresh mass at week eight to the initial mass. The values presented are averages of calli on three-five petri-dishes. (n=3-5; SE not shown).

B. Eight-week old calli were cultured on geneticin-containing MS3 medium (0, 0.15, 1.5, 5, 15, 30, 45 and 60 mg/l) for a further eight weeks before being transferred to regeneration medium containing the same concentration of geneticin in a light (16 h)/dark (8 h) photoperiod. Plantlet numbers were counted eight weeks after placement on regeneration medium (n=3-5; SE not shown).

After eight weeks, callus was transferred to regeneration medium containing geneticin and plantlet numbers were counted over the next eight weeks, as shown in Figure 3.15B. Plantlet regeneration was inhibited at the higher concentrations of 30, 45 and 60 mg/l geneticin. N27 appeared to be the most tolerant to the antibiotic in terms of numbers of plants regenerating, while the least number of plants was recorded for N12 (Figure 3.15B).

Based on callus growth and numbers of plants regenerated per concentration and cultivar on a range of geneticin concentrations, a concentration of 45 mg/l geneticin was chosen for selection of stably transformed callus and plants as it would result in the death of nontransformed cells.

3.4 DISCUSSION

In this chapter, culture conditions for the production of Type 3 callus were established for several South African sugarcane cultivars. Based on the observation by Chen *et al.* (1988), who reported an improvement in morphogenic capacity of embryogenic callus if cycled between media containing low and high 2,4-D concentrations, the relative proportions and regeneration potential of Type 3 callus was established for a range of commercial cultivars.

Due to their respective success in genetically engineering sugarcane (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996; Falco *et al.*, 2000), a similar bombardment protocol was used to transform South African cultivars. Promoter strengths were compared to verify that constructs intended for use in stable gene expression studies contained strong promoters. In addition, a procedure for plant regeneration from callus was established. The effect of the selection antibiotic geneticin on callus growth rates and plant regeneration was monitored for different cultivars, to establish which concentration would inhibit growth of untransformed cells, and could therefore be used for the selection of transformed cells.

3.4.1 Genotype dependency of callus production

South African cultivars NCo310, NCo376, N8, N11, N12, N18, N19, N21, N22, N23, N24 and N27 were compared for their ability to produce embryogenic callus. Cultivars NCo310, NCo376, N12, N16, N19, N20 and N27 produced large proportions of embryogenic callus (Figure 3.4, 3.8 and 3.9), especially Type 3 callus (Figure 3.8), which has been used as target material in several transformation programmes. Non-morphogenic callus was observed in cultivars N11, N21, N22, N23 and N24, indicating that callus production is genotype dependent. In this study, cultivar differences in both callus production and regeneration (Table 3.3) were evident. Cultivar NCo310 was the most responsive cultivar under several *in vitro* culture regimes (Figures 3.4 and 3.8, Table 3.3) and resulted in the highest transient expression levels (Figure 3.11). Although it is no longer commercially grown in South Africa, it was chosen for use in model transformation studies. It was also the cultivar chosen by Gallo-Meagher and Irvine (1996) for the development of a sugarcane transformation system in Texas, USA.

Genotypic differences in response to *in vitro* culture have been reported for sugarcane by other laboratories (Fitch and Moore, 1993; Taylor *et al.*, 1992b). Due to its favourable response in

culture (Taylor *et al.*, 1992b), a single Australian cultivar, Q117, was used for establishing a transformation protocol (Bower *et al.*, 1996). Similarly NCo310 was used by Gallo-Meagher and Irvine (1996).

Cultivar specific responses are not limited to sugarcane. In a study on six barley cultivars, a wide range of both embryogenic callus formation and somatic embryogenesis was observed (Koprek *et al.*, 1996). In order to transform barley, a protocol had to be developed for each cultivar, based on their tissue culture response (Wan and Lemaux, 1994; Koprek *et al.*, 1996). Similarly in wheat (Iser *et al.*, 1999) and maize (Lowe *et al.*, 1995), callus induction and regeneration were genotype-dependant, as was transformation frequency. As the success of any transformation programme depends on the routine production of transgenic plants, it would appear as though more intensive studies need to be conducted on regeneration and transformation of agronomically important cultivars where a genotype-dependant response is evident.

3.4.2 Hormonal regulation of callus production

For this study, callus initiation was carried out on medium containing 3 mg/l 2,4-D, as this concentration is commonly used by other groups working on *in vitro* culture of sugarcane (Heinz and Mee, 1969; Nadar *et al.*, 1978; Lee, 1987; Franks and Birch, 1991). However, a range of 2,4-D concentrations has been used for callus initiation and maintenance, from 1.5 mg/l (Ho and Vasil, 1983) to 7 mg/l 2,4-D (Guiderdoni and Demarly, 1988).

The concept of using lower 2,4-D concentrations to produce organised sugarcane embryos (Ho and Vasil, 1983; Chen *et al.*, 1988) prompted the use in this study of medium containing 1 mg/l 2,4-D (MS1). Ranking the proportions of Type 3 callus produced in different cultivars on MS1 showed that over 50% of the callus produced by NCo310, NCo376 and N20 was Type 3 after eight weeks (Figure 3.8). The proportion of Type 3 callus in N18, however, decreased after four weeks of culture on MS1, suggesting that close visual monitoring of callus cultures and the length of time in culture are important factors.

Cycling between high (3 mg/l) and low (1 mg/l) 2,4-D concentrations has been shown to maintain the morphogenic competence of sugarcane calli (Chen *et al.*, 1988). Further investigations involving cycling NCo376, N12 and N19 callus between MS1 and MS3 resulted in marginally higher callus mass compared to culturing on either 1 or 3 mg/l 2,4-D (Figure

3.9). In addition, visual observations indicated that a high proportion of the callus was Type 3. This cycling regime was therefore chosen for routine production of Type 3 callus for bombardment.

3.4.3 Importance of Type 3 callus production

Different callus types were observed when leaf roll discs were cultured on MS3 (Figure 3.3). Sugarcane callus was initially classified into four types by Taylor *et al.* (1992) and a thorough description of three of the four callus morphologies observed in this study is given in Table 3.2. The reason for producing high quality Type 3 callus was that this type of callus is identified as the most suitable target material for microprojectile bombardment (Bower and Birch, 1992; Bower *et al.*, 1996; Falco *et al.*, 2000). The ability to recognise and visually select embryogenic callus at each subculture has been previously reported (Chen *et al.*, 1988; Taylor *et al.*, 1992).

Microscopic examination of Type 3 callus in this study has shown that it comprises embryos at the heart and torpedo stage of development (Figure 3.6A). When Type 3 callus is used as the target material for bombardment, embryoids at the heart or torpedo stage are the recipient cells of the novel DNA. This implies that once cells within an organised embryo receive the DNA, they will have to divide and multiply to form pro-embryos and eventually mature embryos in a process known as secondary embryogenesis (Merkle *et al.*, 1990; Chapman *et al.*, 2000) in order to give rise to transgenic plants.

A recent paper reported a dramatic variation in GUS expression levels among different developmental stages of somatic embryogenesis of alfalfa, with the highest levels being observed in mature somatic embryos (Tian *et al.*, 2000). They suggested that for optimum transformation results, specific stages of the embryogenic process should be targeted. This finding seems to correlate with our observations in sugarcane, where GUS transient expression levels were higher on Type 3 nodular callus, which is highly embryogenic in nature, consisting of heart- and torpedo-stage somatic embryos than Type 2 callus, which is non-morphogenic (Figure 3.10).

3.4.4 Significance of microcarrier type and bombardment pressure conditions

Physical parameters for bombardment such as the helium pressure and type of microcarrier were established using Type 3 callus generated from NCo310. The pressure of 1000 kPa

(Figure 3.10) which resulted in the highest levels of GUS transient expression, is similar to the 1200 kPa used by Bower *et al.* (1996) in their optimisation studies.

In a comparison of two microcarriers, gold and tungsten, both were found to successfully deliver DNA to sugarcane cells and based on transient GUS assays, similar transient expression levels were observed for both. In other reports comparing tungsten and gold particles in sugarcane transformation, no difference in transient expression levels was evident (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996). Consequently, tungsten was used for all gene deliveries as a more cost-effective option.

However, optimising protocols for transient expression may have been overemphasised with respect to establishing a stable transformation protocol (Christou, 1995). This was demonstrated in a recent study by Nandadeva *et al.* (1999) dealing with bombardment of rice suspension culture cells, where parameters optimised for transient expression did not result in improvements in integrative/stable expression. Although one has to use transient expression as a means of determining initial parameters, in the long-term, stable transformation frequencies should be determined independently.

3.4.5 Geneticin selection regime

A range of concentrations of geneticin were used to monitor both callus growth and plant regeneration of three cultivars. Callus growth rates were severely retarded by increasing concentrations of geneticin (Figure 3.15A), and nominal increase in callus mass was observed in all cultivars at concentrations above 15 mg/l. Similarly plant regeneration was inhibited and negligible regeneration occurred after a concentration of 30 mg/l (Figure 3.15B). It was therefore decide to employ a concentration of 45 mg/l for selection of transformed cells, as no nontransformed cell growth or regeneration would occur at this concentration. This was also the selection regime used by Bower *et al.* (1996), where as many as 20 independently transformed plants were reported per bombardment.

3.4.6 Regeneration efficiencies

Regeneration frequencies were compared in bombarded (with anthocyanin construct) and non-bombarded callus. No regeneration was observed in cultivars N8, N12, N16, N20 and NCo376, despite the fact that plants have been regenerated from non-bombarded calli from these cultivars in the past. However, two cultivars did regenerate, NCo310 with almost equal

efficiency in both bombarded and non-bombarded samples, and N18, where regeneration only occurred from non-bombarded material (Table 3.3). This may be explained by the proposal of Bower *et al.* (1996) that the presence of the anthocyanin gene has a deleterious effect on the development of plants due to the extent of anthocyanin accumulation, or to high levels of the R and C transactivating factors affecting transcriptional control of other genes. In this study, it is likely to have been the latter, as the plants that regenerated were not red in colour.

Albino plants were observed in NCo310, but not in N18 (Tale 3.3). Half of the plantlets regenerating from bombarded callus in NCo310 were albinos, compared to 6% albinos observed in non-bombarded callus. As albino plantlets in sugarcane have been recorded almost exclusively in regenerating long-term callus cultures (Fitch and Moore, 1993), the high proportion of albino plantlets observed in bombarded callus after short culture periods in this study cannot be explained. Initially it was thought that DNA was being inserted into the portion of the genome that encodes pigment production. However, it seems unlikely that microprojectile bombardment could result in DNA integration in a specific area of the genome at such a high frequency. In barley, transgenic cells of most genotypes frequently cannot be induced to differentiate into plants or only albino plants can be recovered (Lemaux *et al.*, 1999). At present, the only successful approach to barley transformation has been the utilisation of particular genotypes that are amenable to the transformation procedure (Jahne *et al.*, 1994; Wan and Lemaux, 1994) or the modification of existing tissue culture protocols (Bregitzer *et al.*, 1998a).

An essential component of any transformation system is the use of target material that is regenerable. Refinement of the regeneration medium by adding kinetin (0.5 mg/l) in this study resulted in an increased regeneration frequency from NCo310 callus (Figure 3.13). In most papers dealing with regeneration of sugarcane plants *in vitro*, plantlets form from mature somatic embryos when 2,4-D is removed from the medium (Heinz and Mee, 1969; Nadar *et al.*, 1978; Ho and Vasil, 1983; Lee, 1987; Chen *et al.*, 1988; Guiderdoni and Demarly, 1988; Bower *et al.*, 1992, 1996; Joyce *et al.*, 1998; Aftab and Iqbal, 1999; Falco *et al.*, 2000). Occasionally additives such as IBA may be added to aid rooting (Gallo-Meagher and Irvine, 1996), or NAA and kinetin (Irvine *et al.*, 1983; Lee, 1987; Grisham and Bourg, 1989) and more recently thidiazuron for improved shoot formation (Gallo-Meagher *et al.*, 2000) in callus cultures.

A comparison of promoter efficiency in driving transient GUS expression levels on NCo310 callus showed that the ubiquitin promoter resulted in highest expression levels (Figure 3.14). This finding is in agreement with earlier work on investigation of promoter efficacy in sugarcane (Gallo-Meagher and Irvine, 1993), where GUS expression with the ubiquitin promoter surpassed levels obtained with Emu, actin and CaMV 35S promoters. Their results differed from the ones obtained in this study in that although they observed similar levels of expression with Emu and actin, and very low levels with 35S, no GUS expression was observed in this study with the actin promoter and moderate levels with CaMV 35S and Emu. They did, however, use leaf segments as target material for bombardment and this may have influenced the expression levels. In a separate study (Rathus *et al.*, 1993), the Emu promoter surpassed GUS expression levels obtained using the CaMV 35S and nopaline synthase promoters. In this study, the ubiquitin promoter was used to drive stable expression of the *pat* gene, and the Emu promoter was used in the *nptII* plasmid construct. The latter plasmid construct had been successfully used in the Australian transformation programme, and it was available for immediate use in this study.

3.5 CONCLUSIONS

In vitro culture and bombardment conditions were optimised in this chapter with a view to stable transformation of sugarcane, which is reported in Chapter 4. Observation for optimal *in vitro* conditions are as follows:

- selective subculturing and cycling of callus on MS media containing 1 and 3 mg/l 2,4-D favoured optimal Type 3 callus production,
- regeneration efficiencies were improved by the addition of kinetin (0.5 mg/l) to the medium,
- the effect of different concentrations of geneticin on callus growth rates and plant regeneration indicated that a concentration of 45 mg/l would not support the growth of untransformed cells during selection of transformants, and
- cultivar NCo310 performed well in culture, and was therefore chosen as a model system for stable gene expression, dealt with in the following chapter.

CHAPTER 4

TRANSFORMATION OF SUGARCANE BY MICROPROJECTILE BOMBARDMENT FOLLOWED BY REGENERATION VIA INDIRECT EMBRYOGENESIS

4.1 INTRODUCTION

The use of embryogenic callus as target material for microprojectile bombardment in genetic engineering has been reported by several laboratories for the production of transgenic sugarcane (Bower and Birch, 1992; Gallo-Meagher and Irvine, 1996; Snyman *et al.*, 1996; Ingelbrecht *et al.*, 1999; Falco *et al.*, 2000). However the transformation efficiency ranges widely from one (Gallo-Meagher and Irvine, 1996; Falco *et al.*, 2000) to 20 (Bower *et al.*, 1996) transgenic plants per bombardment. This may be due to the cultivars used, choice of which is based on the geographical situation; the selection regime employed, either geneticin (Bower and Birch, 1992; Bower *et al.*, 1996; Snyman *et al.*, 1998; Falco *et al.*, 2000) or PPT (Sun *et al.*, 1993; Gallo-Meagher and Irvine, 1996); the regeneration procedure, indirect embryogenesis (Bower *et al.*, 1996; Joyce *et al.*, 1998; Falco *et al.*, 2000), indirect organogenesis (Ingelbrecht *et al.*, 1999) or direct organogenesis (Gambley *et al.*, 1993), and factors such as the design of the gene gun and the plasmid constructs used (Franks and Birch, 1991; Birch, 1997). Optimisation of the majority of these factors are described in the previous chapter.

Once transgenic plants have been generated, the next step is to verify the presence of the transgene. Several review articles have dealt with this issue (Potrykus, 1991; Futterer and Potrykus, 1995; Birch, 1997) This analysis may be confounded by multiple gene copies and silencing. Proof of transformation can best be shown by molecular techniques such as Southern DNA hybridisation, and phenotypic expression of the introduced gene.

It was decided to use herbicide resistance as a model trait for assessing transformation efficiencies in a genetic engineering programme. The advantages of using herbicide resistance are that the trait is conferred by a single gene and assessment of the phenotype is simple. The *pat* gene, driven by the maize ubiquitin promoter, confers resistance to glufosinate ammonium (the active ingredient of herbicides such as Basta, Ignite and Buster), and was an ideal choice for use as a transgene since the plasmid construct was made available for research and development by Aventis, France.

In this study it was postulated that the *pat* herbicide resistance transgene would allow efficient tracking of transgene expression in sugarcane. In addition, estimates of transformation efficiency could be made on the basis of both phenotypic and genotypic evaluations. NCo310 was chosen as the recipient cultivar because it responded well in *in vitro* culture, and it was also the cultivar transformed by Gallo-Meagher and Irvine (1996). Further, it was hypothesised that the overall efficiency with which South African sugarcane cultivar NCo310 could be transformed would be within the range reported elsewhere (1- 20 transgenic plants per bombardment reported by Falco *et al.* (2000) and Bower *et al.* (1996)).

The aims were as follows: to calculate transformation efficiency; to establish PCR conditions for early detection of the presence of the introduced *pat* (herbicide resistance) and *nptII* (selectable marker) genes in putative transgenic plants; to determine *pat* gene integration patterns in the genome and transcriptional expression of the gene; and to characterise phenotypic expression of the *pat* gene in hardened-off plants in the glasshouse.

4.2 MATERIALS AND METHODS

4.2.1 Plasmid constructs

Plasmid pUbiAck (Aventis, France) contains the synthetic herbicide resistance gene, *pat*, which confers resistance to glufosinate ammonium, under the control of the maize ubiquitin promoter (Figure 4.1). Plasmid pEmuKN (D Last, Australia) contains the *nptII* gene lined behind the Emu promoter (Figure 4.2). Plasmids were maintained and generated in *E. coli* JM109.

4.2.2 Plasmid purification and assessment of concentration and purity

Plasmid DNA was purified using the Nucleobond AX100 kit (Macherey-Nagel, Germany). The concentration was assessed as described in 3.2.5.

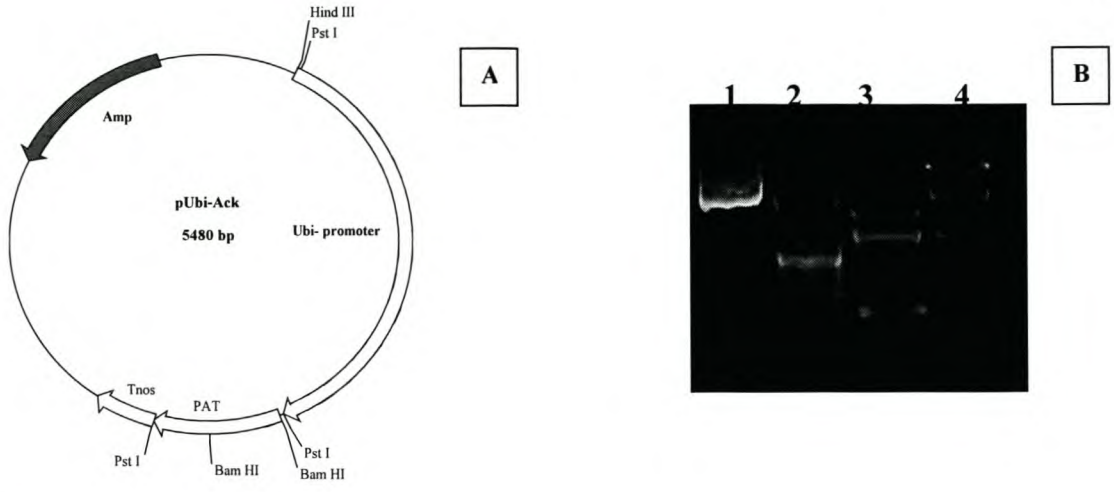


Figure 4.1 Plasmid map and restriction analysis of pUbiAck.

A. pUbiAck contains the *pat* gene which confers resistance to the herbicide Buster, driven by the maize ubiquitin promoter. The ampicillin resistance gene and *Agrobacterium* nopaline synthase terminator gene were incorporated in the construct.

B. Restriction enzyme Hind*III* linearises pUbiAck, and *Pst*I restricts it three times (resulting in fragments 2905, 1990 and 585 bp in size), as shown in lanes 2 and 3, respectively. Lane 1: undigested plasmid. Lane 4: molecular weight marker (Lambda DNA restricted with Eco*RI* and Hind*III*). The gel is stained with ethidium bromide and bands are visualised under UV light.

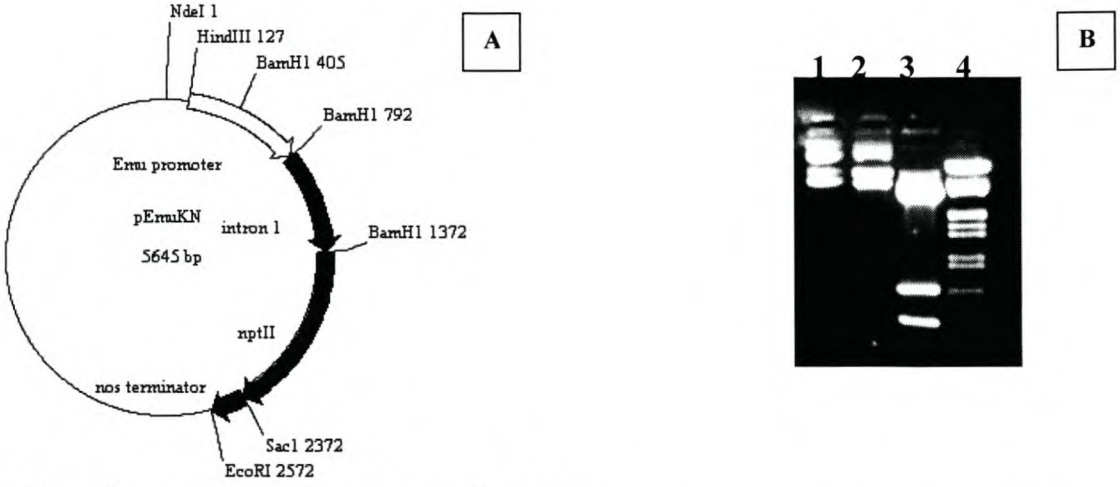


Figure 4.2 Plasmid map and restriction analysis of pEmuKN.

A. pEmuKN contains the *nptII* selectable marker gene which confers resistance to the aminoglycoside antibiotics, and is driven by the Emu monocot specific promoter (Last *et al.*, 1991) and maize *Adh1* intron 1.

B. Restriction enzyme *EcoR1* linearises pEmuKN (partial digest seen in lane 2) and *BamHI* restricts it 3 times (resulting in fragments of 5164, 560 and 481 bp in size, as seen in lane 3). Lane 1 shows unresticted pEmuKN. Lane 4 is a molecular weight marker (Lambda DNA restricted with *EcoRI* and *Hind III*). The gel is stained with ethidium bromide and bands are visualised under UV light.

4.2.3 Sequence determination of the *pat* gene

The *pat* gene was sequenced (Sanger dideoxy-mediated chain termination method (Sanger *et al.*, 1977)) and a partial sequence was obtained using the T7 and T3 primers. This sequence was compared with GenBank non-redundant protein database using the BLASTX algorithm

(Altschul *et al.*, 1990). Significant homology was found to the phosphinothricin acetyltransferase gene (E=1.3 X 10^{-21}). The full-length sequence was used to generate specific primers for PCR (Figure 4.3) (Primer Detective software (Palo Alto, CA)).

5'GTCGACATGTCTCCGGAGAGGAGACCAGTTGAGATTAGGCCAGCTACAGCAGCTGATATGGCCG
CGGTTTGTGATATCGTTAACCATTACATTGAGACGTCTACAGTGAACTTTAGGACAGAGCCACAAAC
ACCACAAGAGTGGATTGATGATCTAGAGAGGTTGCAAGATAGATACCCTTGGTTGGTTGCTGAGGTT
GAGGGTGTTGTGGCTGGTATTGCTTACGATGGGCCCTGGAAGGCTAGGAACGCTTACGATTGGACAG
TTGAGAGTACTGTTTACGTGTCACATAGGCATCAAAGGTTGGGCCTAGGATCCACATTGTACACACA
TTTGCTTAAGTCTATGGAGGCGCAAGGTTTTAAGTCTGTGGTTGCTGTTATAGGCCTTCCAAACGATC
CATCTGTTAGGTTGCATGAGGCTTTGGGATACACAGCCGCGGGTACATTGCGCGCAGCTGGATACAA
GCATGGTGGATGGCATGATGTTGGTTTTTGGCAAAGGGATTTTGAGTTGCCAGCTCCTCCAAGGCC
AGTTAGGCCAGTTACCCAGATCTGAGTCGAC 3'

Figure 4.3 Full nucleotide sequence of the *pat* gene. A partial sequence was determined using T7 and T3 (reverse) primers, and was compared to the deduced amino acid sequence database by BLAST X. The full-length *pat* gene sequence (564 bp) is shown above and the design of specific PCR primers is indicated in red.

4.2.4 Microprojectile bombardment

Microprojectile was carried out as described in 3.2.7. For the generation of stable transformants, two plasmids, one containing the *pat* herbicide resistance gene and the other the *nptII* selectable marker, were co-bombarded into cells. The total concentration of DNA used to coat the tungsten particles was 5 µg (2.5 µg of each plasmid).

4.2.5 Selection and regeneration of transformed plants

Bombarded embryogenic callus was allowed to recover on MS3 (3.2.1) for four days prior to transferring to selection medium containing geneticin (45mg/l). Callus was subcultured fortnightly for 12 weeks on selection medium. At various stages of the subculturing process, small callus pieces were removed and stained (overnight, in the dark) with tetrazolium red (2,3,5 triphenyltetrazolium choride; Sigma (0.8% in 0.05 M sodium phosphate buffer)) to determine viability (Purvis *et al.*, 1964). Transgenic calli were transferred to a light/dark photoperiod on regeneration medium (plus 0.5 mg/l kinetin) with 45 mg/l geneticin 12 weeks after bombardment (3.2.11) or when they were approximately 0.5 cm^2 in size.

4.2.6 Plantlet DNA extraction for PCR analysis

DNA was extracted from leaf tissue of young plantlets using a cetyl triethylammonium bromide (CTAB) extraction buffer (50 mM Tris pH8; 10 mM EDTA; 1% (w/v) CTAB; 0.7 M NaCl) and a small-scale procedure modified from Draper and Scott (1988). Leaf tissue (50-100 mg) was frozen in liquid nitrogen and crushed with a pestle prior to the addition of 2X CTAB extraction buffer (500 µl). Samples were incubated at 55 °C for 30 min. Chloroform (300 µl) was added to the extract before being centrifuged at room temperature (5 min at 12 000 rpm). The aqueous phase was transferred to a clean microfuge tube, isopropanol was added (400 µl) and tubes were centrifuged (10 min at 12 000 rpm). DNA pellets were washed with 70% (v/v) ethanol, air-dried, and resuspended in TE (50 µl; 10 mM Tris; 1 mM EDTA, pH8). Assessment of DNA purity and quantity was carried out according to methods presented in 3.2.5

4.2.7 PCR for *nptII* and *pat* genes

A 430 bp portion of the *pat* gene was amplified using the primer pair 5'-AAC ATC ATG CCA TCC ATG C-3' (22 mer) and 5'GAG ACC AGT TGA GAT TAG GC-3' (20 mer). The 15 µl reaction mixture was composed of the following (final amounts /concentrations): DNA template (40 ng), $MgCl_2$ (4 mM), dNTP (0.2 mM), BSA (0.1 mg/ml), primer (0.2 µM of each), DNA polymerase Stoffel fragment (1 U; Perkin Elmer) and Stoffel PCR buffer (1X). Amplification conditions consisted of 2 initial cycles (94°C for 3 min; 60°C for 2 min; 72°C for 2 min) followed by 30 cycles (denaturation: 94°C for 2 min; annealing: 60°C for 2 min; elongation: 72°C for 3 min) in a thermal cycler (Hybaid, OmniGene). A final extension step was performed at 72°C for 7 min. PCR products were analysed on a 1% (w/v) agarose gel and visualised by staining with ethidium bromide.

An 800 bp portion of the *nptII* gene (sequence anlaysis performed on ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA)) was PCR ampified using the primer pair 5'-AGA GGC TAT TCG GCT ATG AC-3' (20 mer) and 5'-CCA TGA TAT TCG GCA AGC AG-3' (20 mer) (primers designed using Primer Detective software, Linetech, Palo Alto, CA). A 33 µl cocktail consisting of DNA (50 ng), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), primer (0.2 µM of each), AmpliTaq Gold (2.5 U per reaction; Applied Biosystems) and AmpliTaq Gold PCR buffer (1X) were used to set up each reaction. Amplification conditions consisted of a 'hot start' of 12 min at 92°C, followed by the same PCR protocol as for the *pat* gene.

4.2.8 Southern analysis

DNA was extracted from immature leaf rolls of young plantlets according to Dellaporta *et al.* (1985). Purified DNA (10 µg) for each sample was restricted with *Hind*III (Roche Molecular Biochemical, SA; 3 U/µg DNA), spermidine (4 mM) and restriction enzyme buffer B (Roche Molecular Biochemicals; 1x) in a volume of 400 µl. Samples were digested at 37°C overnight. Contaminating RNA was removed by the addition of RNAse (1 µl; 20 U/mg) and incubated at 37°C for 30 mins. The restriction digests were phenol-cleaned by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). After centrifugation (12 000 rpm for 5 mins), the restricted products were precipitated by the addition of 1/20 of the volume of NaCl (5 M) and 2.5 x the volume of absolute ethanol. Samples were incubated at -80°C for 30 mins and centrifuged at 12 000 rpm for 45 min at 4°C. After aspiration of the ethanol, the DNA pellet was dried (Speed Vac, Savant) and then solubilised in TE buffer (15 µl).

Samples were electrophoresed in a 1 % (w/v) agarose gel and transferred to Hybond N+ membrane (Amersham, England) using a standard downward capillary blotting assembly with NaOH (0.4M). Membranes were UV cross-linked (1 200 J; Hoefer, San Francisco). The membrane was prehybridised at 65°C for at least 4 h in buffer (125 µl/cm^2) containing 2 x SSPE (NaCl (0.36 M), sodium phosphate (0.02 M), EDTA (0.002 M), pH 7.7), sheared, denatured salmon sperm DNA (0.1 mg/ml), 1 x Denhardt's solution and SDS (0.1% (v/v)). Hybridisation was carried out at 65°C overnight in the same buffer, containing the PCR generated 430 bp *pat* gene probe, radioactively labeled with [α-^{32}P] dCTP (6 000 Ci/mmol; Amersham) using a random primer labeling kit (Megaprime DNA labeling system; Amersham).

After hybdrisation, blots were washed rapidly in 2 x SSPE plus SDS (0.1 % (v/v)) at room temperature, followed by one wash for 15 min in 1 x SSPE plus SDS (0.1% (v/v)) at 65°C and one wash for 10 min in 0.1 x SSPE plus SDS (0.1 % (v/v)) at 65°C. Hybridising bands were detected by five days' exposure to autoradiography film (Hyperfilm MP; Amersham).

4.2.9 RT-PCR

Total RNA was extracted from leaf tissue (100 mg) using the SV total RNA isolation kit (Promega). The reverse transcription PCR (RT-PCR) reactions were carried out using the Titan one-tube RT-PCR system (Roche, SA) and the *pat* gene primers described in 4.2.3. Products were analysed by gel electrophoresis. To monitor genomic DNA contamination, a control

reaction for each sample was run using the extracted RNA as the template for a PCR reaction.

4.2.10 Phenotypic analysis by glasshouse spraying

Sugarcane plants which had been hardened-off four months previously were sprayed with Buster® herbicide (4 l/ha; active ingredient: 200 mg/l glufosinate ammonium; Aventis, France). A dose of 4 l/ha was chosen as it was lethal for untransformed control plants. The herbicide was applied using a hydraulically operated knapsack fitted with a Teejet nozzle (110°) spraying at a pressure of 100 kPa. Plants were rated for their susceptibility three weeks after spraying.

The transformation efficiency was calculated as the number of independent herbicide resistant lines per bombardment (± SE).

4.3 RESULTS

4.3.1 Selection and regeneration of callus bombarded with the *pat* herbicide resistance gene

Plasmids pUbiAck, containing the *pat* herbicide resistance gene under the maize ubiquitin promoter, and pEmuKN, encoding the Emu promoter driving the *nptII* selectable marker gene were cobombarded in to NCo310 callus. Callus was transferred to selection medium containing the selection antibiotic, geneticin, four days after bombardment and callus growth was monitored visually over the next 12 weeks. Small, white outgrowths were visible after 6-8 weeks (Figure 4.4A), which by 12 weeks were clearly embryogenic in nature (Figure 4.4B). By comparison, calli that were untransformed showed signs of necrosis after 2-4 weeks, and by 10 weeks were black. Although some calli did not turn black on the selection medium, they did not produce any visible new growth, took on a shiny appearance and did not regenerate. These calli were stained with the vital stain, tetrazolium red, to determine whether they were alive. Calli which did not show signs of necrosis, but which did not increase in size during the 12 week period, did not take up the stain, indicating that the cells were dead, whereas calli which had increased in size and were therefore viable, stained red in colour (Figure 4.4C).

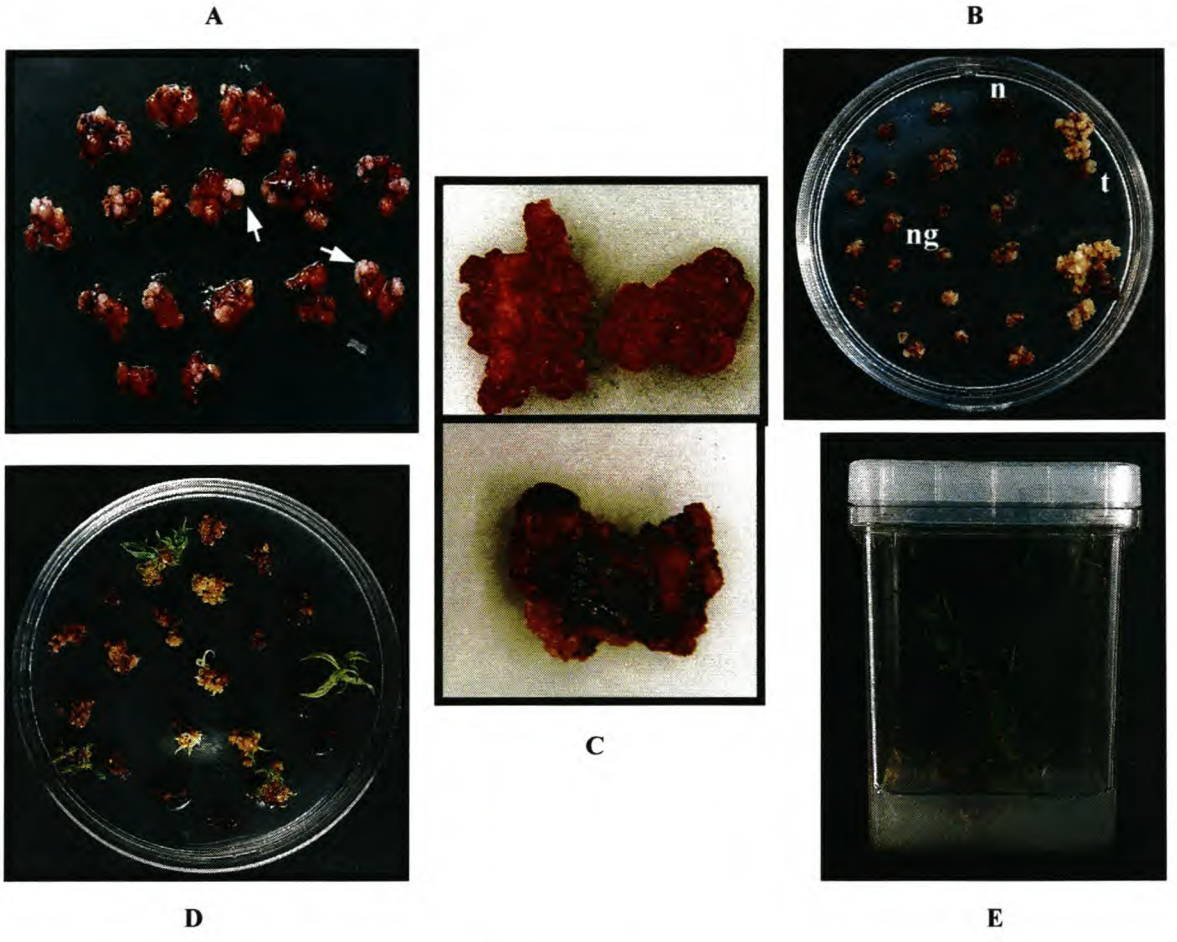


Figure 4.4 Calli and plantlets at different stages of selection and regeneration.

A. Type 3 calli, six weeks after bombardment. Calli were placed on selection medium (MS3 plus 45 mg/l G418) 4 days after bombardment, and were subcultured every two weeks. The arrows indicate small white outgrowths from otherwise necrotic calli. These outgrowths, which are transformed and able to grow on geneticin-containing medium, form within six weeks of culture under selection pressure. Secondary embryos will be formed 12 weeks after transfer to selection medium and will eventually give rise to transgenic plants when transferred to regeneration medium.

B. Bombarded sugarcane calli, 12 weeks after DNA delivery. Note the black calli which are non-transfomed (n), the cream-coloured calli which have showed no growth (ng), and the fast-growing transformed (t) embryogenic calli.

C. Bombarded calli that were subcultured on selection medium for a 12 week period. The tetrazolium red-stained calli are viable, whereas the calli that had not undergone any visible growth and that excluded the stain were dead.

D. Regeneration of transgenic plants. Bombarded calli were transferred to regeneration medium (MS with 0.5 mg/l kinetin) after 12 weeks. The transgenic calli which were highly nodular and embryogenic in nature, turned green within two weeks, and plantlets were visible four weeks after placement in a light/dark photoperiod.

E. Sugarcane plantlet in a Magenta jar. Plantlets were transferred to Magenta jars (1/2 strength MS and sucrose (5 g/l), and were hardened off once plants reached the top of the jar and the roots were well established.

After 12 weeks on selection medium, viable calli were transferred to regeneration medium (containing kinetin and geneticin). Greening of the calli occurred within two weeks of transfer to regeneration medium, and plantlets emerged after 2-4 weeks (Figure 4.4D). Plantlets were transferred to Magenta jars once the production of roots was observed (Figure 4.4E).

From a total of 200 bombardments of NCo310 callus, seven plants regenerated on selection medium. These bombardments were carried out on 14 independent occasions or 'sittings', with 10 or 20 bombardments carried out per sitting. Regenerated plants were named numerically according to the sitting and bombardment they originated from. Each principal number designates an independent bombardment sitting (e.g. 1, 22 and 51). The number following the decimal point indicates the particular bombardment event in that sitting (e.g. 1.4 or 22.2), whilst a letter after this number refers to different lines from the same bombardment event (e.g. 1.7a, 1.7b and 1.7c).

4.3.2 Detection of the *pat* and *nptII* genes in stable transformants

A rapid molecular technique was necessary for establishing the presence of the introduced genes in regenerated plants surviving the selection procedure. PCR is a widely used, reliable technique for detection of specific DNA sequences, and was used for the detection of both the *pat* and *nptII* genes in young plants in Magenta jars prior to hardening-off in the glasshouse. The presence or absence of both the *pat* and *nptII* genes in leaf tissue was determined by PCR analysis and the results are shown in Figures 4.5A and 4.5B, respectively. Table 4.1 summarises the PCR results. Three plants from bombardment event 1.7 were regenerated and it was expected that that these would be identical, but until Southern analysis could confirm this, lines were designated 1.7a, 1.7b and 1.7c. One line (51.3) contained neither gene, despite being able to survive and regenerate on geneticin-containing medium, and is considered to be an 'escape'.

One line (1.4) did not contain the *pat* gene, although the *nptII* gene could be amplified (Table 4.1). This explains why it could grow on the selection medium. Co-transformation of the selectable marker gene with an unselected gene on a separate plasmid, occurred in five of the seven regenerated plants (71%), indicating that most antibiotic-resistant cells also contain the construct encoding the *pat* gene.

Table 4.1 Summary of PCR results for seven NCo310 plants regenerated on selection medium. Plantlets were regenerated on geneticin–containing medium 12 weeks after being bombarded with pUbiAck and pEmuKN. (+ represents a *pat* or *nptII* PCR amplicon; - indicates no PCR amplification)

	Line number						
Gene	1.4	1.7a	1.7b	1.7c	22.1	22.2	51.3
pat	-	+	+	+	+	+	-
nptII	+	+	+*	+	+	+	-

*result obtained in separate experiment

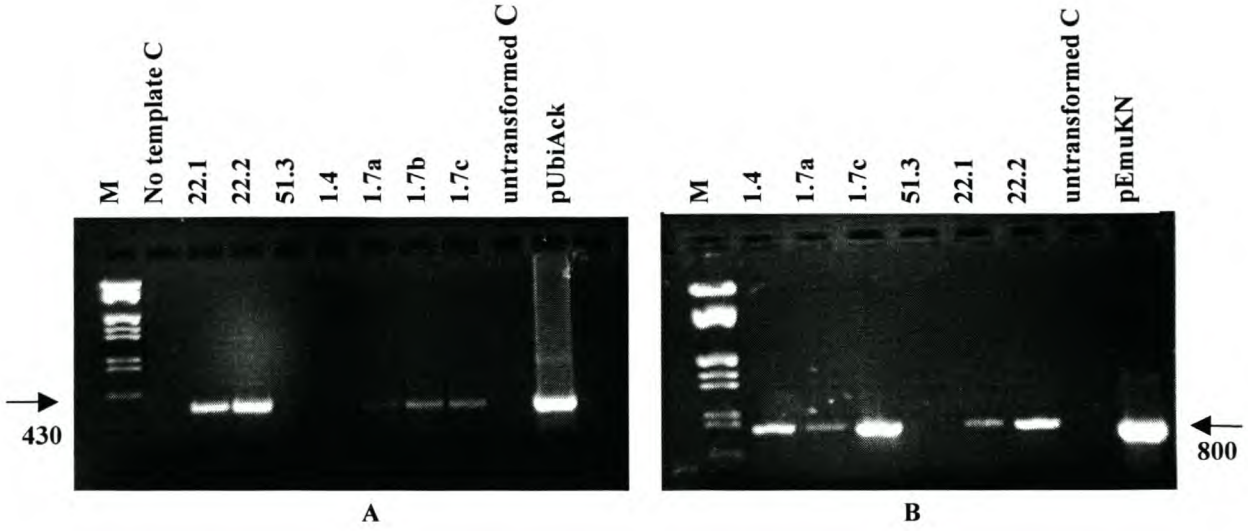


Figure 4.5 PCR amplification of *pat* and *nptII* genes from genomic DNA of plants regenerated under selection after cobombardment with the pUbiAck and pEmuKN plasmid constructs. DNA was extracted from leaves of plantlets in Magenta jars. PCR products were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining.

A. Amplification of the *pat* gene shown by the presence of a 430 bp band in lines 22.1, 22.2, 1.7a, 1.7b, 1.7c and in the positive control pUbiAck. No bands were observed in the no template control and untransformed NCo310 negative control or in lines 51.3 and 1.4. M is a molecular weight marker (Lambda DNA restricted with *EcoRI* and *Hind III*).

B. The *nptII* amplicon is 800 bp in size and was successfully PCR-amplified in lines 1.4, 1.7a, 1.7c, 22.1, 22.2 and in the positive control, pEmuKN. No amplification occurred in line 51.3 and in the untransformed NCo310 sample. M is the molecular weight marker as described above.

4.3.3 Genomic integration patterns

In order to assess copy number and patterns of integration of the transgene, Southern blot analysis of restricted genomic DNA was carried out on the five lines that had been shown by PCR to contain the *pat* gene. Genomic DNA was digested with *Hind*III and hybridised with a PCR generated portion (430 bp) of the *pat* gene. As *Hind*III cuts only once within pUbiAck (outside the coding region), the copy number of the *pat* gene can be estimated by scoring the number of hybridising bands on the Southern blot. All bands were scored once, irrespective of size and intensity. The hybridisation patterns of the transgenic lines appear in Figure 4.6. Line 22.1 has a single copy of the *pat* gene and 22.2 has nine copies of the gene. Lines 1.7a, 1.7b and 1.7c have identical banding patterns and a copy number of three. This is not surprising, as they originated from the same bombardment event, and probably from a single transformed cell.

4.3.4 Transcriptional expression of the *pat* transgene

Total RNA was extracted from leaves of transformants to determine whether they were capable of transcribing the *pat* gene to produce mRNA. RNA was reverse transcribed and the

resultant cDNA was PCR amplified using the protocol developed for detection of the *pat* gene. Figure 4.7 shows agarose analysis of the amplification products after conducting the RT-PCR reaction. The presence of a band in samples 22.2, 1.7a, 1.7b and 1.7c indicates that *pat* transcripts are being produced. As it is likely that 1.7a, b and c are identical lines, they will be considered as such, and further reference to them will be as 1.7. Although *pat* DNA had integrated in the genome of line 22.1, it did not produce *pat* gene transcripts.

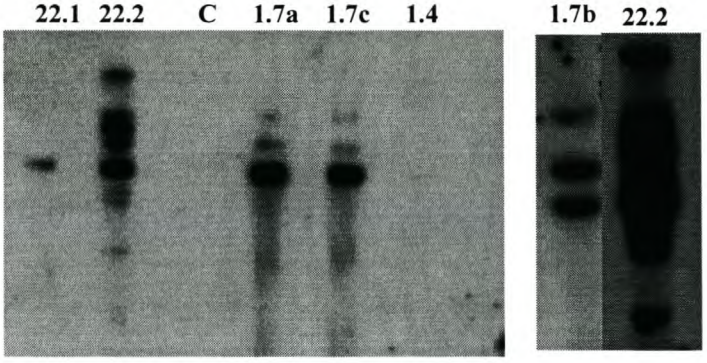


Figure 4.6 Southern blot analysis of genomic DNA from transgenic sugarcane plants, hybridised with a PCR-generated 430 bp radioactively labeled fragment of the *pat* gene. A 10 µg sample of total DNA restricted with *Hind*III, which cuts once within pUbiAck. The gel blot of *Hind*III-restricted genomic DNAshows the following lines: 22.1,22.2, 1.7a, 1.7c and 1.4. Untransformed NCo310 is in lane C. Hybridisation of line 1.7b was carried out on a separate blot, and line 22.2 was included for comparative purposes.

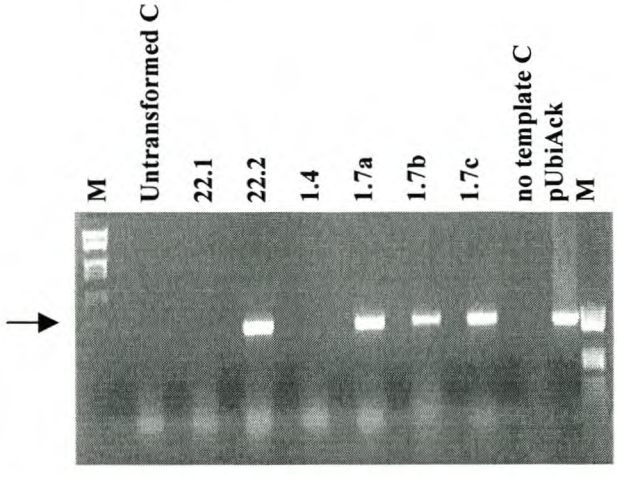


Figure 4.7 Gel electrophoresis of the RT-PCR products obtained with the Titan-one tube PCR system and *pat* specific primers. Lanes M: molecular weight markers. (Marker on left is Lambda DNA restricted with *Eco*RI and *Hind*III; marker on right is pBR322 restricted with *Hae*III). Lanes preceding each numbered lane are RNA samples that were subjected to PCR to confirm that there was no contaminating DNA. The arrow indicates the *pat* gene amplicon, 430 bp in size.

4.3.5 Phenotypic expression of herbicide resistance

Phenotypic expression of the *pat* herbicide resistance gene was tested in all plants that regenerated on selection medium, by herbicide application in the glasshouse. To clearly distinguish between herbicide-resistant and untransformed plants, Buster at a rate of 4 l/ha was used. Herbicide applied at 4 l/ha had no effect on resistant transgenic plants, but killed untransformed plants after three weeks. Untransformed control plants displayed phytotoxic symptoms one week after spraying. Yellowing of the leaves and large brown necrotic areas were observed (Figure 4.8A). By the third week, the plants were brown and necrotic and no further growth was observed (Figure 4.8B). Lines 22.2 and 1.7 were resistant and did not display any phytotoxic symptoms when sprayed with the herbicide (Figure 4.8B). However, line 22.1 died after herbicide application. In this genotype the presence of the *pat* gene could be detected by PCR and Southern blot analyses, but RT-PCR indicated that no transcription was taking place.

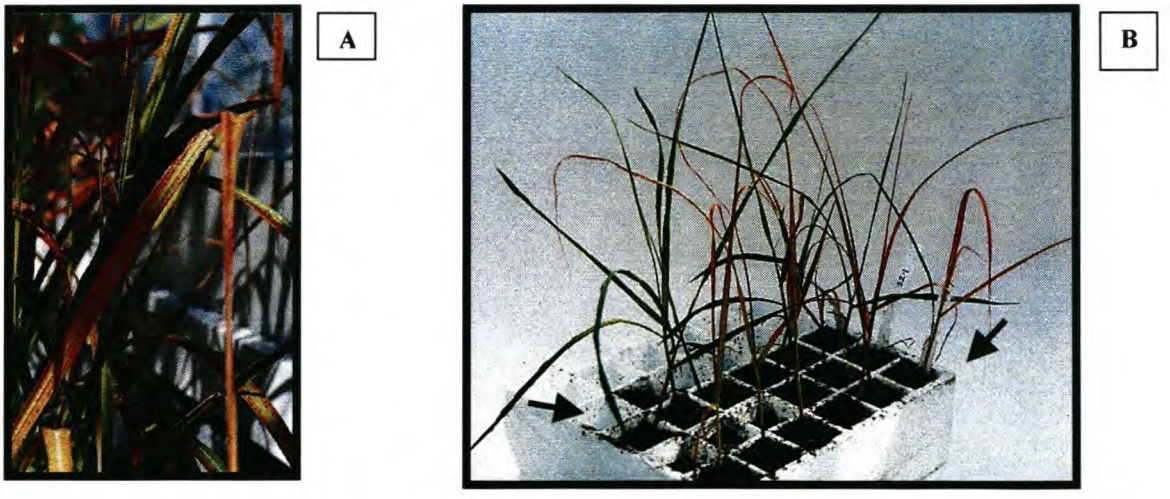


Figure 4.8 Phenotypic assessment of herbicide resistance on four-month old sugarcane plants in the glasshouse. Buster was sprayed onto plants at a rate of 4 l/ha.

A. Mild phytotoxic symptoms (yellowing of the leaves) were observed after one week, which became more severe after three weeks (browning and necrotic patches, as shown) on susceptible plants.
B. Untransformed plants and line 22.1 died after herbicide application (situated on the right diagonal of speedling tray; arrow at top). No damage was observed on plants from line 22.2 or 1.7 (situated on the left diagonal of the speedling tray; arrow at base). Damage was assessed three weeks after herbicide application.

Based on visual observations, the growth characteristics of transgenic plants in the glasshouse appeared to be similar to untransformed plants. Tillering and ratooning capabilities in pots appeared normal and there was no evidence of somaclonal variation.

According to phenotypic analysis, which showed that two lines from 200 bombardments were herbicide resistant, the transformation frequency is 1% (1 transformed plant per 100 bombardments (± 0.01)).

4.4 DISCUSSION

In this chapter it was demonstrated that transgenic plants resistant to the herbicide Buster could be produced, albeit at a low frequency of 1%. A summary of the molecular and phenotypic analysis for the seven regenerated plants is presented in Table 4.2. The presence of both *pat* and *nptII* genes could be detected at an early stage of plant development by PCR analysis and Southern blot hybridisation showed that copy number varied between one and nine. Line 22.1 contained the *pat* gene, but no transcription was observed, suggesting that some form of gene silencing was occurring.

Table 4.2 Summary of the phenotypic and molecular analysis of the seven plants regenerated on selection medium. – represents a negative response, while + indicates the presence of the transcript.

Line no.	*PCR*		*RT-PCR*	*Southern*	*Phenotype*
	nptII	pat	pat	pat	
Untransformed control	-	-	-	-	Susceptible
1.4	+	-	-	-	Susceptible
1.7 (3 plants)	+	+	+	+	Resistant
22.1	+	+	-	+	Susceptible
22.2	+	+	+	+	Resistant
51.3	-	-	-	-	Susceptible

4.4.1 Efficiency of transformation

Based on phenotypic analysis of expression, the transformation efficiency is low (1% or 0.01 plants per bombardment) which compared to the 20 transgenic plants per bombardment reported by Bower *et al.* (1996), is inefficient. The difference in efficiencies may be related to different cultivars used in the studies. However, in a paper where the identical cultivar, NCo310 was used, the transformation efficiency reported was 1.5 plants per bombardment (Gallo-Meagher and Irvine, 1996), over 100 times higher than that obtained in this study, indicating that there is the potential to generate higher numbers of transgenic plants.

One reason for the low transformation efficiency in this study may be that conditions which were optimised for transient expression, may not necessarily be the same as required for integrative expression. This lack of correlation between the two gene expression systems has

been the focus of recent papers dealing with wheat (Iser *et al.*, 2000) and tobacco (Tian *et al.*, 2000). Another reason may be related to the fact that most bombardment sittings, where 10-20 bombardments are carried out, yielded no plants. All of the transgenic plants came from two sittings, numbers one and 22. This may be due to the fact that callus is initiated from material which is field-grown and may therefore be variable and perhaps show seasonal effects. In addition, Sanford *et al.* (1992) reported that of all parameters tested in bombardment, microprojectile coating was one of the most important sources of variation affecting biolistic efficiency. His laboratory reported fluctuations in transformation efficiency from 1 microcentrifuge tube to the next, from day to day and month to month that could not be explained. It was suggested that the low transformation rates obtained in summer were related to humidity.

Low transformation efficiencies similar to that found in this study, have been reported in crops such as wheat (Gopalakrishnan *et al.*, 2000) and barley (Koprek *et al.*, 1996; Cho *et al.*, 1997, 1998). A factor which may lead to an improvement in transformation efficiencies is a reduction in length of culture times. In barley, the success of transformation has been limited by poor regeneration frequency (cultured barley tissues lose regenerative capacity during the time frame required to regenerate transgenic plants) and a high incidence of both albinism (Bregitzer *et al.*, 1995; Jiang *et al.*, 1998; Lemaux *et al.*, 1999) and chimaerism (Ritala *et al.*, 1994). The idea that a reduction in culture time may result in increased regeneration and potentially higher transformation rates in sugarcane will be furthered in Chapter 5.

4.4.2 Preliminary indication of the presence of the introduced gene

Molecular analysis of plants regenerated on selection medium was carried out at an early stage by PCR analysis of both co-bombarded genes. Although PCR is not routinely used as a definitive test of the success of transformation, it is useful as a preliminary assessment of the presence of a specific transgene. The presence of both *pat* and *nptII* gene amplicons in five putative transgenic plants was observed (Figure 4.5 and Table 4.1). Generally, analysis of a novel gene by PCR alone is insufficient evidence to support integrative transformation. Although although isolated papers report PCR results (Gambley *et al.*, 1993; Enriques-Obregon *et al.*, 1998), this method is not as informative as a Southern blot.

4.4.3 Co-bombardment frequency

PCR was useful in determining the co-bombardment frequency. Transformed NCo310 plants

containing both the *pat* and *nptII* genes were verified by PCR analysis (Figure 4.5 and Table 4.1), and this showed that 71% of the regenerated plants contained both genes. This is less than the co-transformation rate of 94% reported by Bower *et al.* (1996), but indicates that the majority of antibiotic resistant cells also contain the gene of interest.

4.4.4 Integration patterns and copy number of transgenic lines

Additional information from PCR-positive lines regarding integration patterns and copy numbers was obtained by Southern analysis (Figure 4.6). Most lines that contained the *pat* gene were found to contain several copies of the novel gene. Multiple copies of the introduced gene is a phenomenon which has been observed in sugarcane previously (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996; Falco *et al.*, 2000), and is not uncommon for transgenic plants derived from particle bombardment (Somers *et al.*, 1992; Weeks *et al.*, 1993). Lines 1.7a, 1.7b and 1.7c, which arose from a single bombardment, each contained three copies of the *pat* gene (Figure 4.6). The identical banding patterns displayed by these lines confirms that they arose from the same transformation event. Lines 22.1 and 22.2 contained one and nine gene copies, respectively. These findings are similar to what has previously been reported in transgenic sugarcane. Bower *et al.* (1996) reported an average number of five gene insertions.

4.4.5 Transcriptional expression of the *pat* gene and gene silencing

RT-PCR was carried out to see whether transcription of the *pat* gene was taking place (Figure 4.7). Positive results were obtained for lines 1.7 and 22.2. However, no transcript was present in line 22.1, suggesting that the gene is being silenced, or that the promoter is truncated. This observation was confirmed by phenotypic evaluation where 22.1 was susceptible when sprayed with Buster herbicide, while 22.2 and 1.7 were resistant (Figure 4.8). The negative phenotype of line 22.1 supports the hypothesis that the introduced gene is being silenced.

Silencing appears to be unpredictable, because 22.1, which contains one copy of the *pat* gene, was silenced, while 22.2, which contains nine copies was not. Silencing may be influenced by other factors such as position effect (Matzke and Matzke, 1990) and promoter homology (Finnegan and McElroy, 1994; Al-Kaff *et al.*, 2000). In sugarcane, Hansom *et al.* (1999) found that promoter homology, and not copy number, had the largest effect on gene silencing. All promoters that were tested, even those isolated from sugarcane, caused silencing in a proportion of transgenic plants, indicating the importance of work directed to the retention of promoter activity.

4.5 CONCLUSIONS

The use of the *pat* herbicide resistance trait allowed efficient tracking of the transgene in genetically modified sugarcane plants by molecular and phenotypic analyses. As this transformation protocol resulted in a transformation efficiency an order of magnitude lower than previously reported results, it was decided to investigate the possibility of producing transgenic plants via a novel regeneration route, which would result in reduced *in vitro* culture times. In addition, the effect of using a lower selection pressure was investigated. These modifications are discussed in Chapter 5.

CHAPTER 5

TOWARDS A NOVEL APPROACH FOR THE PRODUCTION OF TRANSGENIC SUGARCANE USING DIRECT MORPHOGENESIS

5.1 INTRODUCTION

Aside from varying efficiencies in gene transfer, the use of embryogenic callus as target material for sugarcane bombardment has other limitations. Establishing, developing and maintaining callus cultures is labour intensive and the recovery of plants ready for transfer to the glasshouse may take as long as 36 weeks (Bower *et al.*, 1996; Birch, 1997; Snyman *et al.*, 2000, 2001). A combination of long culture periods and the high levels of auxin required for callus induction and maintenance could significantly increase the risk of somaclonal variation, and morphological abnormalities have recently been observed in field-grown sugarcane plants in Australia (Grof, 2001; Grof and Campbell, 2001). To minimise the time spent generating embryogenic callus, one approach would be to employ a faster route of morphogenesis from leaf discs. There are several reports of sugarcane plants being micropropagated directly from leaf discs without an intervening callus stage, either via organogenesis (Irvine and Benda, 1985, 1987; Grisham and Bourg, 1989; Gambley *et al.*, 1993, 1994) or embryogenesis (Aftab and Iqbal, 1999; Snyman *et al.*, 2000, 2001).

One of the long-term aims of genetic engineering programmes is to reduce the tissue culture component to minimise the effects of somaclonal variation and to increase throughput (Birch, 1997). Examples of where this approach has been successful are oat (Gless *et al.*, 1998) and orchardgrass (Denchev *et al.*, 1997), where leaf base segments have been used for bombardment. Shoot apical meristems have been used as target material in sugarcane (Gambley *et al.*, 1993) and maize (Lowe *et al.*, 1995). However, the utilisation of organised tissues as target cells frequently leads to plants with chimaeric expression of foreign genes (Gambley *et al.*, 1994; Lowe *et al.*, 1995; Bommineni and Jahaur, 1997; Denchev *et al.*, 1997). This drawback may be overcome by placing partially transformed material back in to culture on selection medium for subsequent recovery of transformed sectors (Fromm *et al.*, 1990).

A combination of the use of leaf material as target material and regeneration via direct somatic embryogenesis has recently been reported for the production of transformed orchardgrass (Denchev *et al.*, 1997). Regeneration via direct embryogenesis from leaf tissue has been observed in sugarcane (Aftab and Iqbal, 1999). Consequently, it was decided to investigate

direct morphogenesis as a means of producing transgenic plants from immature leaf material more rapidly, and with a potentially reduced risk of somaclonal variants.

It was hypothesised that 1) a direct route of morphogenesis could be established by manipulating auxin levels, and 2) the bombardment of leaf roll discs at the start of the process would reduce the overall time spent in culture and result in a more rapid production of transgenic plants. The first aim of this study was to develop a protocol for direct somatic embryogenesis and regeneration. Once this had been established, the second aim was to compare the efficiency of transformation using two target materials, namely embryogenic callus (which would be regenerated via indirect somatic embryogenesis) and leaf rolls discs pre-cultured for a few days prior to bombardment. Two regeneration routes from bombarded leaf discs were compared, i.e. direct and indirect embryogenesis.

The model transgene system chosen for the experimental work involved the *pat* and *nptII* genes, encoding herbicide and antibiotic resistance, respectively, both driven by the maize ubiquitin promoter. In order to make a comparison of genotype response, three commercially grown sugarcane cultivars, N12, N19 and NCo376, were used as recipient genotypes. As the transformation efficiency in this study has been low (Chapter 4), it was decided to reduce the selection pressure in an attempt to improve regenerative capacity of bombarded material. Although it was recognised that this might lead to a higher proportion of escapes (Fromm *et al.*, 1990), the concentration of geneticin incorporated in the medium was decreased from 45 to 15 mg/l, as 75% of cells are inhibited at the latter concentration (3.3.7).

5.2 MATERIALS AND METHODS

5.2.1 Plasmid constructs: purification and verification

Plasmid pUbiAck (Aventis, France) which contains the *pat* synthetic herbicide resistance gene was described previously (4.2.1). Plasmid pUbiKN (E Mirkov, Texas, USA) contains the *nptII* gene cloned behind the ubiquitin promoter (Figure 5.1). Plasmids were maintained and generated in *E. coli* JM109. Plasmid DNA was purified using the Nucleobond AX100 kit (Macherey-Nagel, Germany). Concentration was assessed as described in 3.2.5.

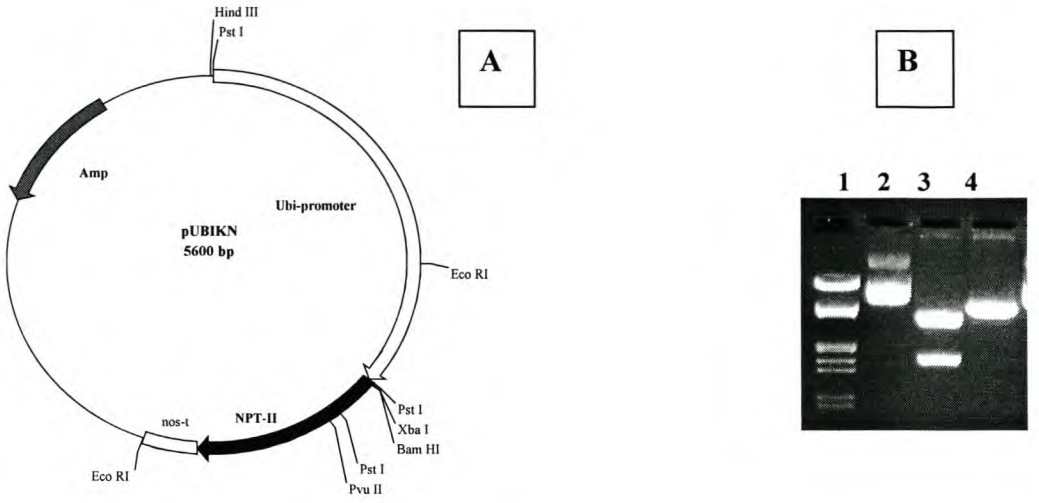


Figure 5.1 Plasmid map and restriction digest of pUbiKN.
A. Plasmid map showing restriction sites of plasmid pUbiKN
B. Gel photo showing restriction analysis of pUbiKN. Lane 1: molecular weight marker; lane 2: undigested pUbiKN with supercoiled, concatenated DNA; lane 3: pUbiKN restricted with *Eco*RI, yielding 2 fragments, 3 900 and 1 700 bp in size; lane 4: linearised pUbiKN with *Hind*III (5 600 bp).

5.2.2 Preparation of plant material

Immature leaf roll was removed from the apex of sugarcane stalks of field grown cultivars N12, N19 and NCo376. After surface sterilisation with ethanol (70%; w/w) as described in 3.2.1, the outermost mature leaves were aseptically removed and the immature leaf roll was sliced transversely into discs approximately 1.5 mm thick. Typically, 14 discs from each leaf roll were placed on to two Petri dishes containing commercially prepared MS basal salts and vitamins (Murashige and Skoog, 1962) (Highveld Biological, SA) supplemented with casein hydrolysate (1 g/l), sucrose (30 g/l), 2,4-D (0.15-3 mg/l) and agargel (Sigma; 5 g/l), pH 5.8. Cultures were maintained in the dark at 26° C.

5.2.3 Microscopy

Leaf roll discs from direct somatic embryo production experiments were sampled after two and four weeks, respectively, in culture and were fixed in formalin-acetic-alcohol (FAA) by vacuum infiltration, dehydrated in a butanol-ethanol alcohol series and embedded in Paraplast wax (Lancer, Ireland). Sections were cut at 5-10 µm on a rotary microtome (AO 820, American Optical, NY, USA), stained with safranin-fast green and mounted with DPX (Unilab, Saarchem, SA) (Purvis *et al.*, 1964). In addition, stereomicroscopy was used to observe and photograph leaf roll discs at several stages of somatic embryo production and plantlet regeneration.

5.2.4 Microprojectile bombardment

Two target materials were used for bombardment: leaf discs pre-cultured on MS medium for 10-14 days, and eight to 12 week old embryogenic calli produced on MS3 (3.2.1). The target material was cobombarded with plasmids pUbiAck and pUbiKN, containing the *pat* and *nptII* genes respectively, using the protocol described in 3.2.7.

5.2.5 Selection procedure and regeneration of plants

Following bombardment, explants were cultured in the dark for four days on the same medium used for initial culture, before being placed on a medium containing geneticin (G418) (15 mg/l active ingredient; Roche, South Africa) with fortnightly subculture intervals. Leaf discs undergoing direct embryogenesis were transferred to regeneration medium (RM; MS without 2,4-D, plus kinetin (0.5 mg/l)) after six to eight weeks. Leafs discs and embryogenic callus undergoing indirect embryogenesis were maintained on selection medium for 12 weeks, after which they were transferred to regeneration medium and were cultured in a 16 h (light)/8h (dark) photoperiod at 35 μm m^{-2} s^{-1}. All cultures were subcultured fortnightly.

Plantlets with 2 cm high shoots were transferred to half strength MS, sucrose (5 g/l) and geneticin (15 mg/l) in Magenta jars (Sigma, USA) until a well developed root system was observed and shoots were 10 cm in length. Hardening off was carried out in the glasshouse (3.2.12).

5.2.6 Plantlet DNA extraction and PCR analysis

DNA was extracted from leaf tissue of young plantlets (two to three months after transfer to the glasshouse) using a small-scale cetyl triethylammonium bromide (CTAB) extraction procedure and PCR amplified as described in 4.2.5 and 4.2.7, respectively. As a result of both faint and multiple bands commonly being observed in the PCR reactions where Stoffel polymerase was used, conventional Taq polymerase (Promega, USA) or AmpliTaq Gold (Applied Biosystems) was used.

5.2.7 RT-PCR

RT-PCR was carried out using leaf roll material from tillers of plants aged three to six months old, as described in 4.2.9.

5.2.8 Southern blots

Genomic DNA was extracted from leaf rolls of tillers from plants that were planted in 25 l drums and that were six to 12 months old. The DNA was extracted and the Southern blot protocol was followed as laid out in 4.2.8.

5.2.9 Glasshouse spraying

Plants which were three to four months old were sprayed with Buster (4 l/ha) as described in 4.2.10.

5.3 RESULTS

5.3.1 Establishment of the direct embryogenic pathway of morphogenesis

5.3.1.1 Optimisation of 2,4-D concentration for direct somatic embryo formation

Preliminary studies (results not shown) had shown that a reduction in 2,4-D concentration (to levels below 1 mg/l) resulted in the production of somatic embryos directly from leaf discs. Plantlets could be regenerated from these embryos in six to eight weeks. In order to determine which concentration of 2,4-D best supported regeneration via direct somatic embryogenesis, leaf roll discs were placed on MS medium containing 0.15, 0.3, 0.6, 1.2 and 3 mg/l 2,4-D. The effect of cultivar was also investigated: a comparison was made between N12, N19 and NCo376. The type of growth, amount of callus and numbers of plants regenerated were recorded. Neither embryo nor plant production was influenced by the distance of the leaf disc from the apical meristem (results not shown). Leaf discs were cultured in the dark for eight weeks after which time callus production was rated on a scale of 1 (negligible production of callus) to 5 (explant covered with callus) for each concentration of 2,4-D. Explants were transferred to RM in a light/dark photoperiod and numbers of plants per immature leaf roll were determined. Data was analysed statistically by ANOVA. The ratings are summarised in Table 5.1. Substantial callus formation was observed on the cut surfaces of explants cultured at 1.2 and 3 mg/l 2,4-D, for all cultivars. Leaf discs cultured on 0.6 mg/l 2,4-D produced a small amount of callus that rapidly became white, nodular and highly embryogenic. The lower 2,4-D concentrations of 0.15 and 0.3 mg/l resulted in the formation of somatic embryos directly on the leaf disc with no callus development. However, a high proportion of these discs were unresponsive: 71 and 57% on 0.15 and 0.3 mg/l 2,4-D, respectively, and became necrotic within eight weeks. Plantlet regeneration efficiencies were therefore compared to determine whether this concentration range would be viable in a transformation programme.

Table 5.1 A comparison of the relative amounts of embryogenic callus produced on leaf discs at a range of 2,4-D concentrations, for all cultivars. Leaf discs were rated for callus production using a scale of 1 (negligible amounts of callus produced) to 5 (entire explant covered with embryogenic callus). The percentage of leaf discs which did not respond in culture and which became necrotic were compared after eight weeks in culture.

2,4-D concentration (mg/l)	*% unresponsive leaf discs*	*Relative amounts of embryogenic callus production (rated 1-5)*	*Appearance of leaf discs (after eight weeks)*
0.15	71.4	1	A few somatic embryos formed directly on disc
0.3	57.1	1	Somatic embryos formed on approximately half the disc
0.6	48.5	2	Initially small amounts of callus produced, which rapidly became highly embryogenic
1.2	14.3	5	Entire explant covered in callus, with large areas comprising Type 3 callus
3	0	5	Entire explant covered with pre-embryogenic callus, with small areas of Type 3 callus

The numbers of plantlets that regenerated on different concentrations of 2,4-D were compared for the three cultivars used and are presented in Figure 5.2. Plant numbers regenerated per cultivar and at each 2,4-D concentration were analysed (ANOVA (SED=15.97)). The only difference in cultivar response was observed at 1.2 mg/l 2,4-D, where NCo376 regenerated significantly fewer plants than N12 and N19.

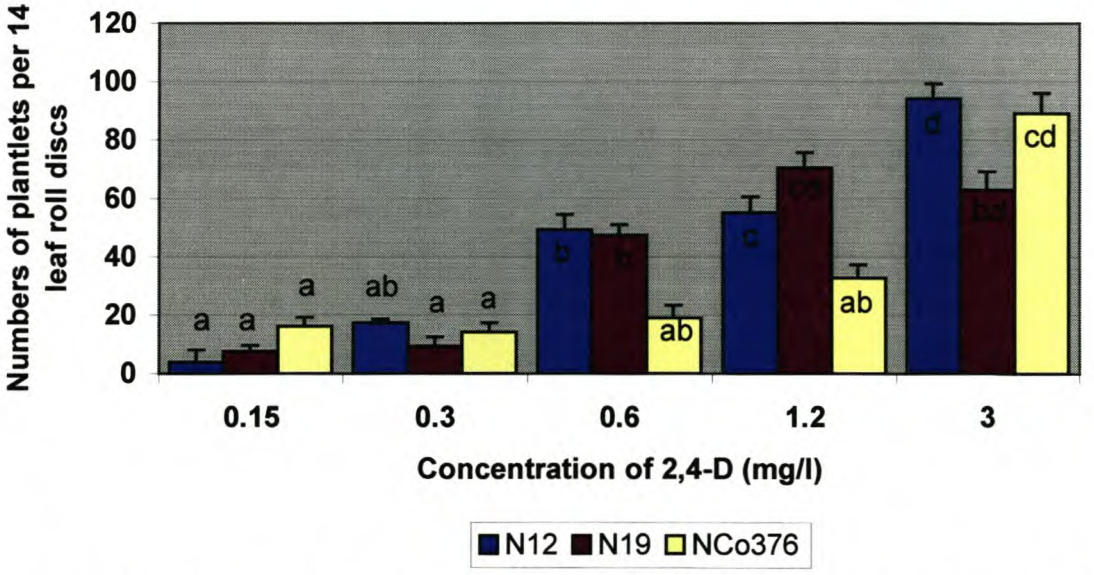


Figure 5.2 Relative efficiency of regeneration of cultivars N12, N19 and NCo376 on a range of 2,4-D concentrations. Leaf discs were cultured on 2,4-D-containing medium for eight weeks (with fortnightly subculturing), followed by a further eight weeks on regeneration medium (containing 0.5 mg/l kinetin, without 2,4-D), after which final plantlet regeneration numbers were established. Values presented are means ± SE, where n=9-15. Bars labeled with different alphabetical letters indicate significant differences (ANOVA SED=15.97).

As the generation of somatic embryos and plantlets directly from leaf discs occurred at a slightly higher level at 0.3 mg/l 2,4-D than at 0.15 mg/l, the former concentration was used in subsequent transformation experiments.

5.3.1.2 Regeneration via direct somatic embryogenesis

Direct somatic embryo formation from leaf roll discs has previously been observed on media containing 0.3 (Snyman *et al.*, 2000, 2001) and 2 mg/l 2,4-D (Aftab and Iqbal, 1999). In the first report, plant regeneration was observed after as little as six weeks in culture. The rapid regeneration and short time in culture make this route of morphogenesis attractive for use in a transformation programme. However, it was seen as important to investigate the morphogenic process. To this end, microscopic examination was carried out on N12 leaf discs cultured on MS medium containing 0.3 mg/l 2,4-D (MS0.3) after two and four weeks in culture in order to 1) assess the time-frame during which somatic embryos were produced on leaf discs, and 2) determine at which stage leaf roll discs should be bombarded. Microscopic examination of wax-embedded two week-old leaf disc sections revealed the presence of semi-organised outgrowths on the cut surface of the discs (Figure 5.3A and B), which by three to four weeks had a defined cellular structure and consisted of somatic embryos at the heart stage of development (Figure 5.4A), and appeared white, nodular and clearly embryonic (Figure 5.4B). Embryo germination and shoot regeneration were visible as early as two weeks after transfer to regeneration medium, usually at or near the cut surface of the outer leaves (Figure 5.5).

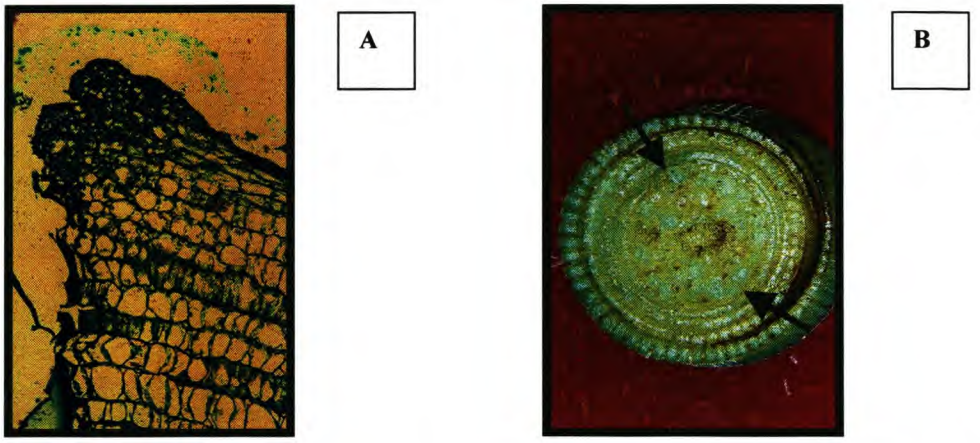


Figure 5.3 Leaf disc showing embryoid outgrowths two weeks after initiation. Leaf discs from cultivar N12 were cultured on MS medium containing 0.3 mg/l 2,4-D for two weeks prior to wax-embedding.

A. Longitudinal, radial section of wax-embedded leaf disc showing embryoid outgrowths arising directly from the explant (100 X).

B. Appearance of semi-organised, globular embryoids on a leaf disc as seen under a stereomicroscope (6.4 X). Arrows indicate the presence of the pro-embryos on the cut surface of the leaf disc.

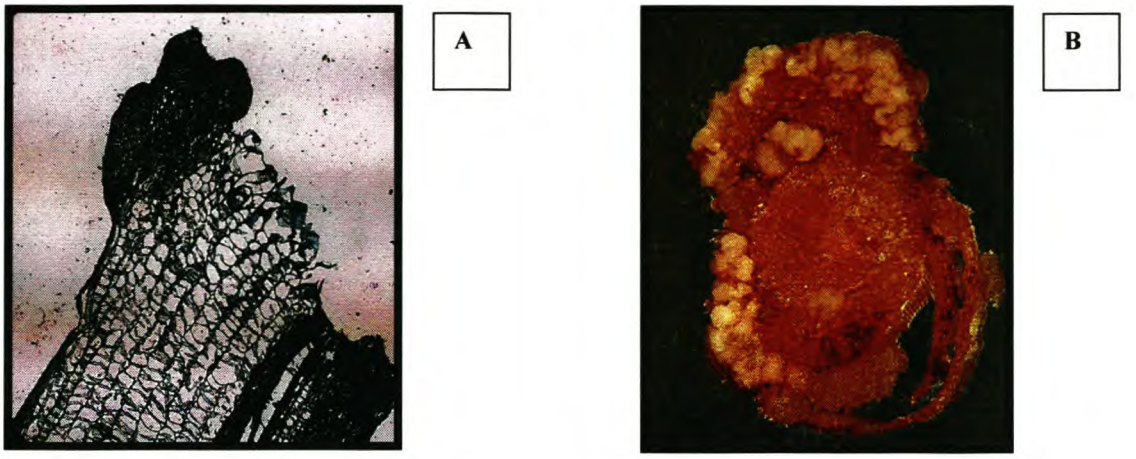


Figure 5.4 Immature leaf roll discs showing direct formation of somatic embryos after four weeks of culture on MS medium containing 0.3 mg/l 2,4-D.

A. Microscopic examination of wax-embedded longitudinal leaf section showing heart-shaped embryo emerging directly from leaf disc after four weeks in culture (40 X).
B. Somatic embryo development directly on leaf disc (6.4 X).

Figure 5.5 Plantlets regenerating directly from somatic embryos on leaf roll disc on regeneration medium (0.5 mg/l kinetin, without 2,4-D) after a total of 10 weeks in culture. Leaf discs from cultivar N12 were cultured on MS medium with 0.3 mg/l 2,4-D for six weeks prior to placement on regeneration medium.

Based on the observation that embryoids consisting of only a few cell layers were visible in wax-embedded sections after two weeks of culture, as opposed to somatic embryos at the heart stage after four weeks in culture, it was decided to culture leaf discs directed towards direct somatic embryogenesis for 10-14 days on MS0.3 prior to bombardment. A one week culture period was found necessary to eliminate microbially contaminated discs (results not shown). For this reason, it would not be practical to bombard leaf discs earlier than seven days. Similarly, leaf discs to be used for the production of plantlets via indirect embryogenesis were

cultured on MS medium with 3mg/l 2,4-D (MS3) for 10-14 days prior to bombardment.

5.3.1.3 Relative time scale and efficiency of regeneration via direct and indirect regeneration

The production of sugarcane plants via indirect embryogenesis from callus from the time of initiation to hardening-off normally takes 16-24 weeks (Ho and Vasil, 1983; Lee, 1987; Guiderdoni and Demarly, 1988). A comparison was therefore made of the relative times and efficiencies with which somatic embryos are produced and plants are regenerated, using the conventional indirect callus-based procedure and the novel approach of direct somatic embryogenesis. For the direct regeneration route, leaf discs were cultured on MS0.3 for a period of four weeks, until somatic embryos had formed, and then transferred to regeneration medium. Plantlets could be observed as early as six weeks after culture initiation. A comparison of time frames for plantlet production in the different routes is summarised in Table 5.2. Although plantlet regeneration efficiencies via indirect embryogenesis are six times higher than via direct embryogenesis, the culturing time is two to three times longer, and the exposure to high levels of auxin may be problematic in terms of somaclonal variation.

Table 5.2 Comparative times and efficiencies to regenerate plants via direct and indirect embryogenesis.

	Regeneration route	
	Indirect embryogenesis	*Direct embryogenesis*
Time taken to form somatic embryos (weeks)	8-12	2-4
Time taken to regenerate plants ready for hardening-off (weeks)	8-12	4-6
Total time spent in culture (weeks)	16-24	6-10
Total number of plants regenerated per leaf roll	82 ± 13.7	13.6 ± 3.2

5.3.2 Comparison of transformation efficiency using different target materials and regeneration routes

5.3.2.1 Comparison of regeneration efficiencies from different target materials and regeneration routes on selection medium after bombardment, for three cultivars

Plant regeneration via direct morphogenesis has the potential to reduce the time taken to regenerate a plant by three times compared to indirect embryogenesis. The use of leaf discs as target material, followed by direct embryogenesis, was compared to the more conventionally used embryogenic callus-based procedure in terms of regeneration efficiency after bombardment and selection. A third approach was compared, that of bombarding leaf discs followed by the conventional indirect morphogenic route for the production of plants. As the use of embryogenic callus as a target for microprojectile bombardment necessitates an initial

culturing phase of 12 weeks, the use of leaf discs as target material (followed by indirect morphogenesis), would substantially reduce the culture time in the transformation procedure. Both bombarded leaf discs and embryogeneic calli were placed on selection medium containing 15 mg/l geneticin four days after bombardment.

As it is desirable to have a transformation protocol that is independent of cultivar, comparative regeneration efficiencies for cultivars N12, N19 and NCo376 were assessed. The numbers of bombardments and plants regenerated per cultivar for the three regeneration routes are given in Table 5.3. Although N12 regenerated via direct embryogenesis and NCo376 regenerated from bombarded leaf discs via indirect embryogenesis, resulted in the most plants per bombardment (50 per 100 bombardments), no cultivar consistently performed better than any other across the three regeneration routes.

Table 5.3 Comparative numbers of bombardments and plants regenerated on selection at 15 mg/l geneticin for three cultivars. Three regeneration approaches were compared: 1) indirect morphogenesis from bombarded embryogenic callus, 2) indirect embryogenesis from bombarded leaf roll discs and 3) direct somatic embryogenesis from bombarded leaf roll discs. Mean values (± SE) followed by the same letter are not statistically different at P=0.05.

Regeneration approach (target material)	*No. bombardments per cultivar*	*No. plants surviving selection and which were hardened-off*	*Average no. plants regenerated per 100 bombardments ± SE*
Indirect (callus)			
N12	82	1	1 ± 1 a
N19	144	11	7 ± 2 b
NCo376	118	10	8 ± 2 b
Indirect (leaf disc)			
N12	64	17	27 ± 6 c
N19	46	5	10 ± 4 c
NCo376	10	5	50 ± 16 d
Direct (leaf disc)			
N12	54	30	55 ± 6 d
N19	46	1	2 ± 2 a
NCo376	10	0	0

5.3.2.2 Relative time scale for production of transgenic plants via three approaches

A comparison of the overall time it takes to produce a transgenic plant through the three approaches was compared. This comparison is summarised in a flow diagram (Figure 5.6). In the direct route, leaf disc explants used for the production of transformants were pre-cultured for one to two weeks on MS0.3, bombarded, and then cultured on the same medium but containing the selection antibiotic geneticin. Under these conditions, embryo formation and maturation (5.3.1.2) was faster than from callus and occurred directly on the discs four to eight

weeks after bombardment. Plantlets were established on the selection medium eight to 12 weeks after placement on regeneration medium, after which plantlets were transferred to the greenhouse.

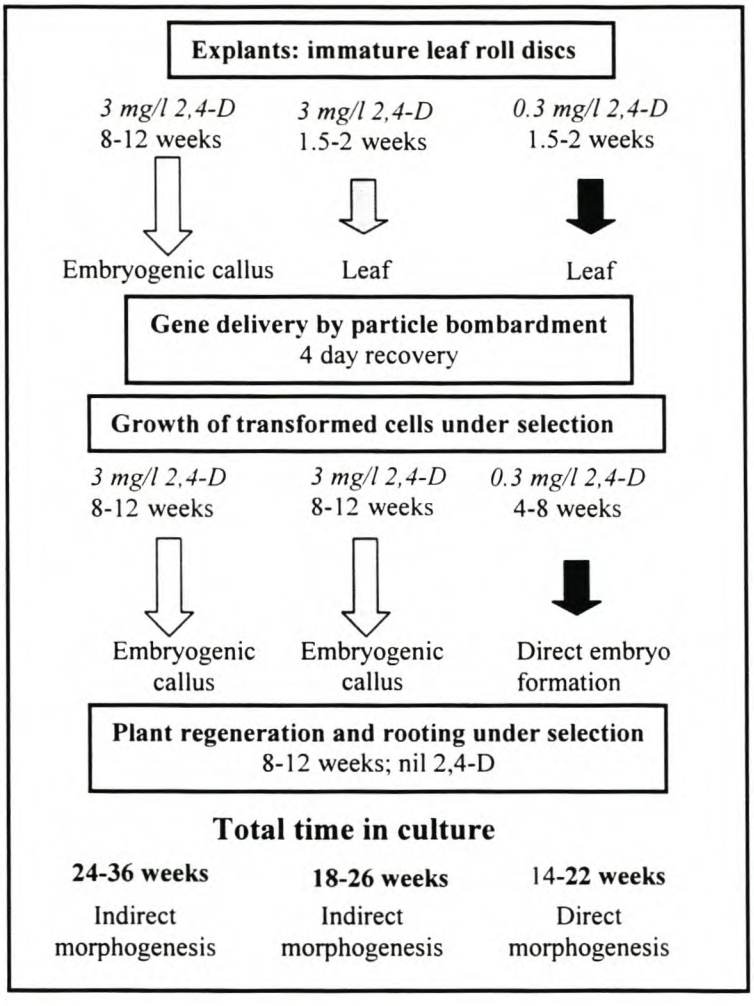


Figure 5.6 Relative time taken to produce transgenic sugarcane via direct and indirect morphogenic routes.

Leaf discs that were cultured on MS3 for one to two weeks prior to bombardment, formed callus over the next eight to 12 weeks on MS3, and plantlet regeneration was observed during the following eight to 12 weeks.

In contrast, leaf discs exposed to MS3 developed embryogenic callus suitable for bombardment eight to 12 weeks after culture initiation. Bombarded calli were then cultured on selection medium for a further eight to 12 weeks for embryo formation and maturation. Plants were produced four to six weeks later. A further four to six weeks were necessary to produce plants ready for hardening off, bringing the total to 24-36 weeks required for the production of a

transgenic plant, which is the same time-frame reported by Birch (1997).

In summary, the direct route of morphogenesis resulted in plantlet production in a shorter time (13-22 weeks) than the indirect route when leaf discs were bombarded (18-26 weeks) and when callus was bombarded (24-36 weeks). Furthermore, the time lapse from when plants were first observed in culture to when the last ones emerged was nine weeks in the direct, and eight to 12 weeks in the indirect route. This indicates that the process of embryogenesis and embryo germination were asynchronous.

5.3.2.3 Comparative transformation efficiencies measured by specific PCR amplification of the *pat* gene: direct versus indirect morphogenesis

A preliminary assessment of transformation efficiency of sugarcane plants regenerated directly and indirectly via somatic embryogenesis was performed using specific amplification of the *pat* gene. PCR analysis was carried out on DNA from young plants, extracted from leaf material. The replacement of Stoffel polymerase with Taq polymerase is a modification that resulted in improved visualisation of bands (less smearing and a reduction in the presence of faint and double bands) and the comparative results are shown in Figure 5.7.

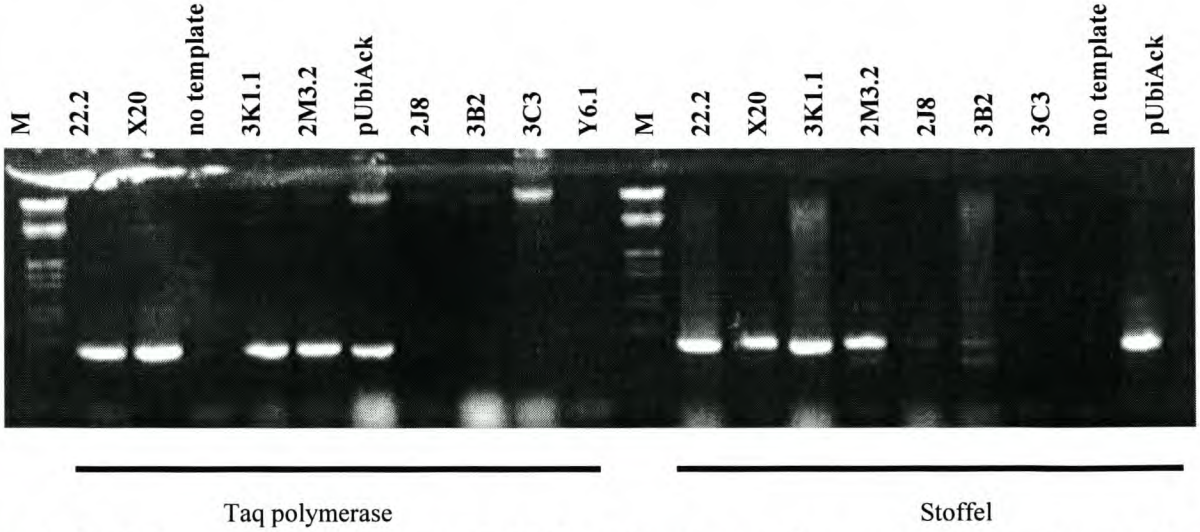


Figure 5.7 PCR amplification of the *pat* herbicide resistance gene from genomic DNA of young plants regenerated after cobombardment with pUbiAck and pUbiKN plasmids. DNA was extracted using the CTAB protocol from two to three month old glasshouse-grown plants. The *pat* amplicon was successfully PCR-amplified using both Taq and Stoffel polymerases. When using Taq, the PCR protocol was shortened and the 15 µl reaction mixture comprised the following: DNA template (40 ng), $MgCl_2$ (1.5 mM), dNTP (0.2 mM), primer (0.2 µM of each), DNA Taq polymerase (Promega; 1 U per reaction) and buffer (1X). Amplification conditions consisted of denaturation at 94°C for 2 min, followed by 30 cycles (denaturation: 94°C for 1 min; annealing: 60°C for 1 min; elongation: 72°C for 2 min) and a final extension step of 72°C for 5 min in a thermal cycler (GeneAmp 9700, Applied Biosystems). The molecular weight size markers are designated M (Lambda DNA digested with *EcoR*I and *Hind*III to give a size ladder of 564-21 226 bp). When the Stoffel fragment polymerase was used in the PCR amplification reactions, both smearing (in samples 22.2, 3K1.1 and 3B2) as well as faint and multiple bands (3B2) were observed.

PCR analysis (using Taq polymerase) for plants derived from the three treatments are presented in Table 5.4. When regeneration occurred via the indirect route, regardless of whether callus or leaf discs were bombarded, only 18-27 % of the plants contained the *pat* gene. This indicates that over 70% of the plants are escapes and do not contain the *pat* gene. When leaf discs were bombarded and plants regenerated via direct embryogenesis, half (45%) of the plants contained the *pat* amplicon. The high proportion of escapes in all routes is likely to be due to the low concentration of geneticin (15 mg/l) in the selection medium that allows untransformed plants to regenerate. The higher percentage of potentially transformed plants via the direct route may be due to a more efficient regeneration procedure.

Table 5.4 PCR detection of the *pat* gene in plants regenerated using three approaches for the production of transgenic sugarcane plants. Specific PCR amplification was carried out using Taq polymerase on DNA extracted from leaf material (from two to three month old plants) using the CTAB method.

	Regeneration approach (target material)		
	Indirect (callus)	*Indirect (leaf discs)*	*Direct (leaf discs)*
Total no. bombardments	344	120	110
Total no. plants hardened-off	22	27	31
Regeneration frequency (no. plants regenerated per 100 bombardments) ± SE	6 ± 3	23 ± 4	28 ± 5
% pat +ve	27	18.5	45
Lines containing pat amplicon	X20, 2G11.2, 2G11.3, 2G12.2, 2M3.2, 3K1.1	2J9.5, 2Z9.1, 2Z9.2, 3F6.1, 3H2.1	2E3.4, 2E3.13, 2E7.2, 2E8, 2E9, 2E11, 2E12, 2I4, 2S2.3, 2S4, 2S12, 3B7, 3E8, 3E10

5.3.2.3 Phenotypic analysis

Plants in the glasshouse (three to four months old) were sprayed with Buster (4 l/ha) in order to confirm phenotypic expression of the *pat* gene. The results are summarised in Table 5.5 and the visual difference in the responses of sensitive and resistant plants is shown in Figure 5.8. Only a relatively small proportion of the plants displayed complete resistance to the herbicide (19, 11 and 18 % of the plants regenerated via direct and indirect (leaf disc and callus) morphogenesis, respectively. The majority of plants regenerated via all three routes showed severe phytotoxic symptoms and died as a result of being sprayed, confirming the PCR results which indicated a high level of escapes. This is likely because of the low levels of geneticin employed during selection.

Table 5.5 Summary of phenotypic expression of the *pat* gene as measured by resistance to Buster (4 l/ha) application in the glasshouse. Plants were three to four months old when sprayed with herbicide and phytotoxic effects were assessed after three weeks.

Regeneration route	***No. plants sprayed***	***% lines showing the following response***		
		Resistant lines	Susceptible (plants killed)	Recovery lines*
Direct	31	19.3 (2E3.4, 2E3.13, 2E8, 2E11,3B7, 3E10)	54.9	25.8 (2E7.2, 2E9, 2E12, 2I4, 2S2.3, 2S4,2S12, 3E8)
Indirect (leaf disc)	27	11.1 (2Z9.1, 3F6.1, 3H2.1)	81.5	7.4 (2J9.5, 2Z9.2)
Indirect (callus)	22	18.2 (X20, 2G12.2, 2M3.2, 3K1.1)	72.7	9.1 (2G11.2, 2G11.3)

*plants which displayed the recovery phenotype showed phytotoxic symptoms on those leaves sprayed with herbicide, but after three weeks new leaves emerged and were asymptomatic. Untransformed plants died.

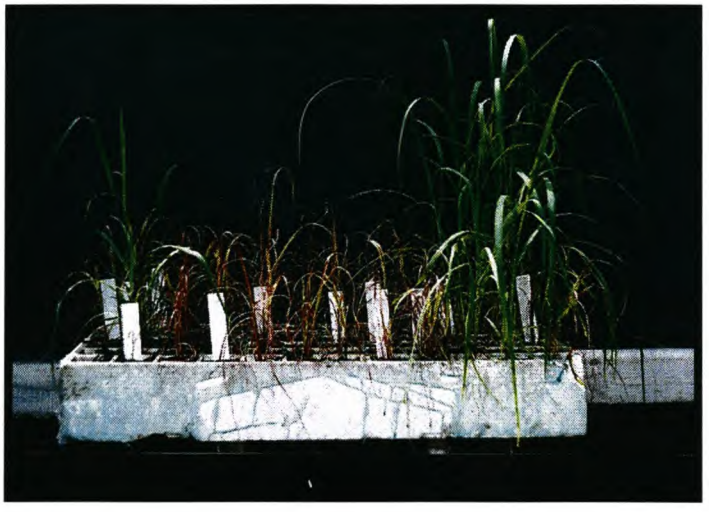


Figure 5.8 Speedling trays with plants that had been sprayed with herbicide one month previously. Buster (4 l/ha) was applied to three-month old plants. Plants on right and left are resistant to the herbicide, while plants in centre were susceptible and died.

A phenotypic response termed 'recovery' was observed in 26% of direct route-derived plants and in smaller proportions of indirect route plants (7 and 9% from bombarded leaf discs and callus, respectively) after herbicide application. These plants displayed phytotoxic symptoms after herbicide application, but unlike untransformed control plants, did not die, but recovered and the new emerging leaves were asymptomatic.

5.3.2.4 Molecular characterisation of resistant and 'recovery' plants by Southern blot analysis

Plants which were resistant to the herbicide and those displaying the recovery phenotype were characterised on a molecular level by Southern blot analysis to determine gene copy number and integration profiles. It was postulated that those plants displaying the recovery phenotype were expressing the *pat* gene at low levels, possibly because of a low copy number. The Southern blots for plants regenerated via the three different routes were probed with the *pat* and *nptII* genes and are presented in Figure 5.9. *Pat* gene copy numbers ranged from three to 11. Three clones (2G12.2 and 3K1.1 (bombarded callus) and 2Z9.1 (bombarded leaf disc regenerated via the indirect route)), showed identical *pat* gene integration profiles, although they were from three independent bombardment events. There is some evidence in the literature to suggest that foreign DNA integrates in the same place in genomic DNA (Kohli *et al.*, 1998; Linacero *et al.*, 2000; Kumar and Fladung, 2001), and it appears as though this may be occurring here.

None of the phenotypically resistant plants derived from the direct route, or any of the plants displaying the recovery phenotype, regardless of the regeneration route, hybridised to the *pat* gene probe. Because these plants all contained the *pat* amplicon when PCR was carried out, Southern analysis was repeated using double the DNA concentration Twenty micrograms of DNA was digested and blotted, and a separate Southern blot with unrestricted DNA was prepared as well. However, no signal was observed in either autoradiograph (results not shown), suggesting that the *pat* gene was below detectable levels or had been lost. In addition, no evidence of *pat* gene transcripts was observed when RT-PCR was carried out (results not shown).

Membranes were stripped and reprobed using labeled *nptII* (Figure 5.9B). All of the transformants that contained the *pat* gene, also contained the cobombarded *nptII* gene. Transformation efficiencies for the indirect approach were 1.2 and 2.5%, from bombarded callus and leaf discs, respectively.

5.3.3 Investigations into the incidence of chimaerism

Although a proportion (45%) of the plants regenerated via direct embryogenesis showed the resistant or recovery phenotype when sprayed in the glasshouse, none contained the *pat* gene when analysed by Southern blots. Similarly, two lines regenerated via indirect embryogenesis from both bombarded leaf discs and embryogenic callus that displayed the recovery phenotype,

showed no evidence of stable *pat* gene integration. It is hypothesised, therefore, that these plants were chimaeric for the *pat* gene. This would account for the ability to detect the *pat* gene by PCR in young plants and the resistant/recovery phenotype when three to four-month old plants were sprayed with the herbicide.

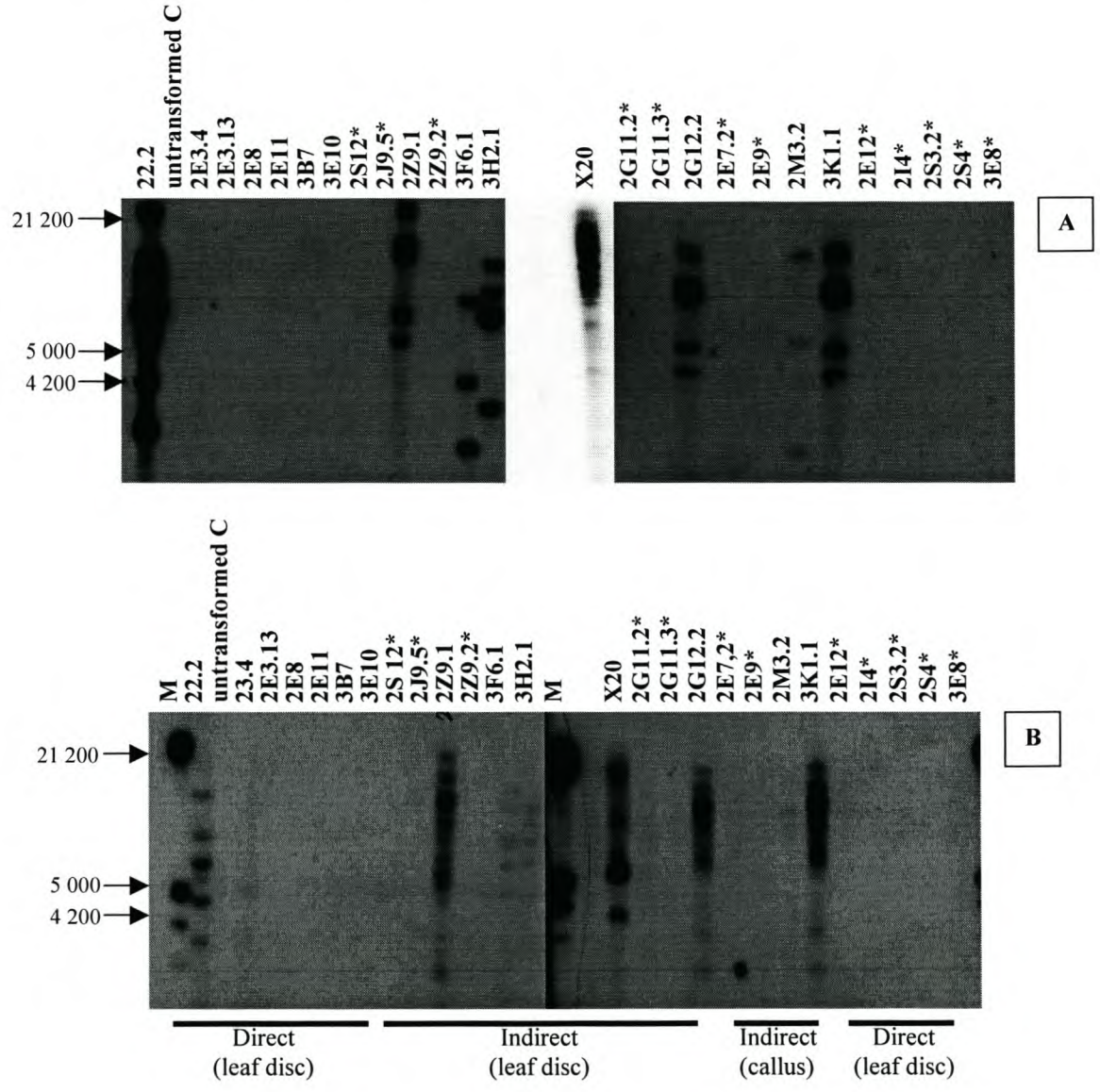


Figure 5.9 Southern blot analysis of genomic DNA from sugarcane plants regenerated on selection medium via direct and indirect morphogenesis. Lanes represent phenotypically resistant plants (unmarked) and plants which displayed the recovery phenotype after herbicide application (marked with *). None of the recovery plants showed hybridisation to the *pat* gene probe.

A. Blots were probed with a 430 bp PCR-generated *pat* gene fragment. DNA samples from plants produced via three regeneration routes are as follows: **direct (leaf disc)**: 2E3.4, 2E3.13, 2E8, 2E11, 3B7, 3E10, 2S12*, 2E7.2*, 2I4*, 2S3.2*, 3E8*; **indirect (leaf disc):** 2J9.5*, 2Z9.1, 2Z9.2*, 3F6.1, 3H2.1; **indirect (callus):** X20, 2G11.2*, 2G11.3*, 2G12.2, 2M3.2, 3K1.1.

B. Blots were probed with an 800 bp PCR-generated *nptII* fragment. The blot presented in Figure 5.8A was stripped and reprobed with *nptII*. M is a molecular weight marker (Lambda DNA restricted with Eco*RI* and Hind*III*).

It is postulated that as the plants matured, the small transformed sector of cells was diluted out in the primary tiller, and subsequent tillers did not contain any transformed cells, explaining why the mature plants did not appear to contain the herbicide resistance gene. Pending the outcome of the investigation into the chimaeric status of plants derived from the direct route, no further investigation was carried out on plants derived via indirect embryogenesis.

5.3.3.1 Verification of the chimaeric status of plants

Transgenic plants can be routinely recovered from chimaeric plants by placing the plants in culture again (Fromm *et al.*, 1990; Lowe *et al.*, 1995). In an attempt to recover fully transformed plants from putative transgenic sectors of mature plants regenerated via direct embryogenesis, the following two experiments were carried out:

- Firstly, stem sections (setts) were cut from stalks of mature plants. These were planted out and allowed to germinate. Young, three-month old plants were sprayed with Buster (4 l/ha). Line X20, a resistant line regenerated via indirect embryogenesis from bombarded callus, was used as a positive control in each case. When plants were re-sprayed with herbicide, none except those from line X20 survived.
- Secondly, leaf roll discs from plants regenerated via direct embryogenesis were removed from mature plants and placed on culture medium containing geneticin (45 mg/l). None of the material survived in culture, except the positive control (X20), which formed callus and regenerated plants.

This indicates that mature plants derived from the direct route do not contain the *pat* gene. It is likely that the transformed cells present in young chimaeric plants have been diluted out and are below detectable levels.

5.3.3.2 The effects of a more stringent selection regime and bombarding leaf discs at an earlier stage

It is likely that factors such as the low stringency selection regime, and time of DNA delivery could have allowed the growth of chimaeric plants. In an attempt to address both these issues, leaf discs intended for regeneration via direct somatic embryogenesis after bombardment were cultured on MS0.3 with increased levels of geneticin (45 mg/l) and were bombarded at an earlier stage. A bombardment time-course assay using cultivar N12 was carried out to see whether bombardment of discs at an earlier stage, day three or six after initiation, affected the success of transformation when regeneration occurred via direct embryogenesis. The numbers of plants regenerated from leaf discs bombarded at four different times were compared and are shown in Table 5.6. Selection on higher levels of geneticin reduces the regeneration

capacity, as the number of plantlets dropped to a third of the amount regenerated on 15 mg/l geneticin. No response was observed from leaf discs bombarded three days after culture initiation, suggesting that cells targeted at this early stage are not receptive to DNA uptake. When leaf discs were bombarded on days six and 10 post-initiation, 8.3 and 6.6 plants regenerated per 100 bombardments, respectively. The highest number of regenerated plants was observed when 14-day old leaf discs were bombarded, and a regeneration efficiency of 18% was observed. Although bombardment of leaf discs at this age yielded the most plants, these plants needed to be characterised for the incidence of chimaerism. Plants were evaluated by PCR, Southern blots and phenotypically, by spraying with Buster.

Table 5.6 A comparison of regeneration efficiency from leaf discs bombarded at day three, six, 10 and 14 after placement in culture. Mean numbers of plants regenerated per 100 bombardments are shown and are followed by SE (n=60 bombardments). Following bombardment, leaf discs were cultured on MS medium containing 0.3 mg/l 2,4-D with geneticin (45 mg/l) for 6-8 weeks before transfer to regeneration medium for a further six to eight weeks.

Number of days in culture prior to bombardment	*Total number of plants regenerated*	*Regeneration efficiency (%)*
3	0	0
6	5	8.3 ± 3.6
10	4	6.6 ± 3.2
14	11	18 ± 5

Only one plant, regenerated from a leaf disc bombarded on day 14 after placement on MS0.3, line 4E14.12, was resistant when sprayed with herbicide. The *pat* gene amplicon was detected after PCR (with AmpliTaq Gold DNA polymerase) (Figure 5.10A) and transcription of the gene was shown by the production of the *pat* transcript in a RT-PCR (Figure 5.10B). Southern blotting showed that four copies of the gene were present in 4E14.12. However, the hybridisation was only visible when 20 µg of genomic DNA was used (Figure 5.10C). A transformation efficiency of 1.7% was calculated for the direct route.

5.4 DISCUSSION

Because of the low transformation efficiency reported in the previous chapter when embryogenic callus was bombarded, this chapter focused on the following:

- Development of a tissue culture protocol for direct somatic embryogenesis from leaf discs,
- Use of leaf discs as target material for bombardment,
- Reduction of selection pressure from 45 to 15 mg/l geneticin, and

- Comparison of regeneration and transformation efficiencies via direct and indirect embryogenesis.

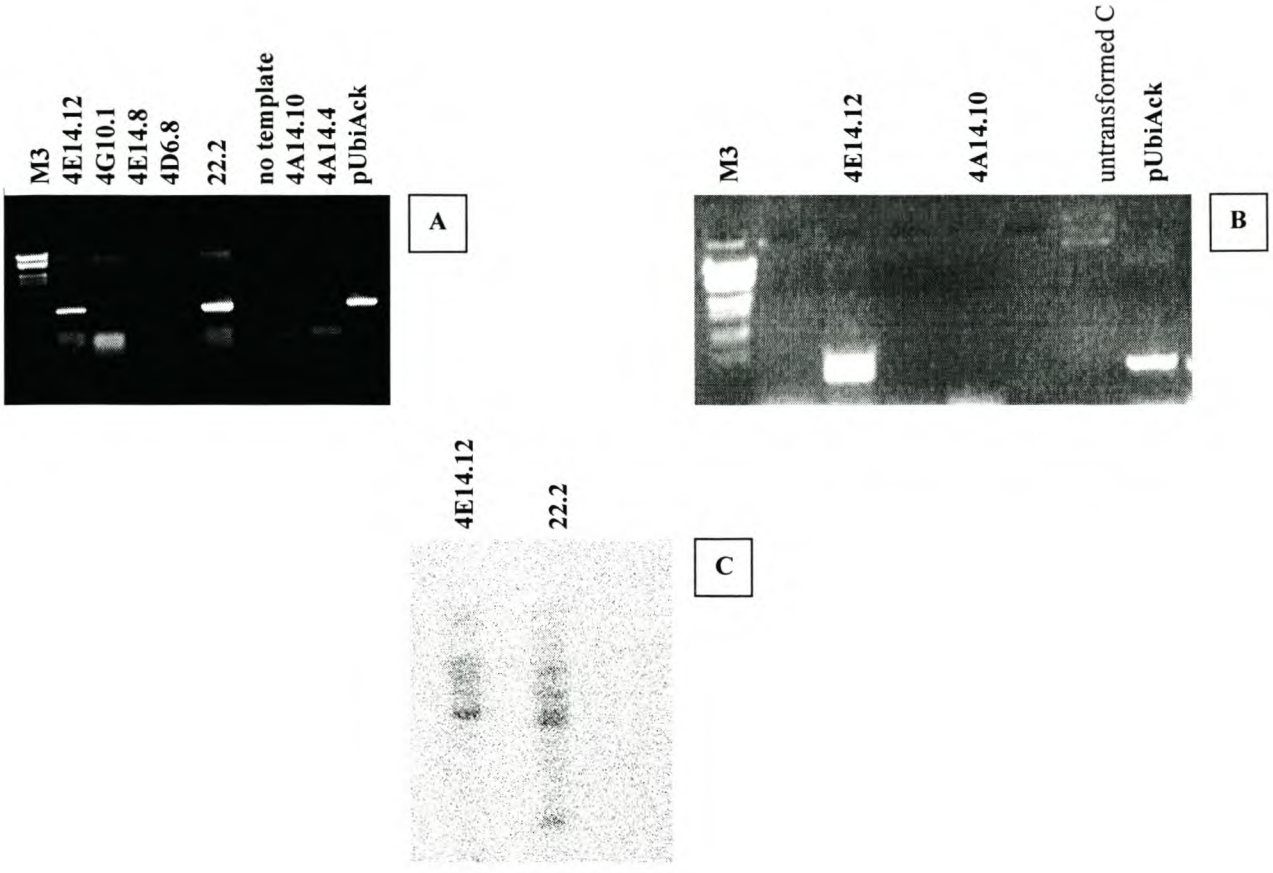


Figure 5.10 Molecular analysis of a single line regenerated via direct embryogenesis on selection medium containing geneticin (45 mg/l). Line 4E14.12 arose from leaf discs bombarded at day 14 after placement on MS0.3.

A. PCR amplification of the *pat* gene from genomic DNA. No amplification of line 4E14.12 was possible using the regular source of Taq polymerase enzyme (described in 5.3.2.2), so AmpliTaq Gold polymerase (Applied Biosystems) was used, and amplification was observed in lines 4E14.12, 22.2 and plasmid pUbiAck. The 50 µl reaction mixture consisted of the following: DNA template (100-150 ng), $MgCl_2$ (1.5 mM), dNTP (0.2 mM), primer (0.2 µM), AmpliTaq Gold DNA polymerase (2.5 U per reaction) and buffer (1X). Amplification conditions were as described in Figure 5.7, but a 'hot start' of 94°C for 8 mins was included.

B. Gel electrophoresis of RT-PCR products (Titan one tube system) obtained with *pat* specific primers. The lane preceding line 4E14.12 is the RNA that was used as a template for PCR to confirm that there was no contaminating DNA. No transcript was observed in line 4A14.10.

C. Southern blot analysis of line 4E14.12. The radioactively labeled *pat* gene fragment was used to probe 20 µg of genomic DNA restricted with *Hind*III. Line 22.2 (5 µg DNA) was included for comparative purposes.

5.4.1 The route of direct somatic embryogenesis in sugarcane tissue culture

The regeneration of plants directly from organised tissue (apical meristems, axillary buds and leaf tissue) has been widely used in micropropagation systems as it is rapid, and reduces the

potential for somaclonal variation (George, 1993). Although the production of somatic embryos directly from leaf material has been reported in sugarcane previously (Aftab and Iqbal, 1999), the morphogenic process had not been well described or documented. The work carried out in this study therefore offers a more detailed characterisation of the process of direct somatic embryo production from leaf roll discs in terms of auxin dependency, culture times and the potential to reduce somaclonal variation.

5.4.1.1 Auxin dependency

The only other report on direct somatic embryo production from leaf material in sugarcane utilised the auxin, 2,4-D, at lower levels than that required for callus initiation (Aftab and Iqbal, 1999). Because it appeared as though direct somatic embryo production was triggered by low auxin levels, a comparison of a range of 2,4-D concentrations was carried out in this study, and it was shown that embryos could be produced directly on sugarcane leaf discs on MS medium containing 0.3 mg/l 2,4-D, 10 times less than the concentration required for embryogenic callus initiation (Table 5.1).

Similarly, low concentrations of the synthetic auxins, 2,4-D and dicamba, have been used to initiate direct formation of embryos on organised plant tissue in millet (Vikrant and Rashid, 2001), orchardgrass (Denchev *et al.*, 1997) and *Arabidopsis* (Gaj, 2001). In orchid, however, leaves were not responsive when cultured on 2,4-D, and only produced adventitious embryos when cultured on medium containing a cytokinin, thidiazuron (Chen *et al.*, 1999). As thidiazuron has recently been found to stimulate shoot formation from callus in sugarcane (Gallo-Meagher *et al.*, 2000; Chengalrayan and Gallo-Meagher, 2001), the response of immature sugarcane leaf roll discs to this growth hormone in a direct regeneration system may be investigated in the future.

5.4.1.2 Reduction of time in culture between initiation and plant regeneration

Regeneration of sugarcane plants via direct somatic embryogenesis can be observed in culture from as early as six weeks after culture initiation (Table 5.2). From microscopy, it is apparent that the process of embryogenesis is initiated early, with semi-organised or globular stage sugarcane embryoids visible by two weeks (Figure 5.3), and embryo development to the heart-shaped stage visible by four weeks (Figure 5.4). If leaf discs are placed on regeneration medium at that stage, plantlets can be observed as early as six weeks after placement of discs on culture medium (Figure 5.5). Similar time frames for the production of plants via direct

embryogenesis have been reported in orchardgrass (Conger *et al.*, 1983) and millet (Vikrant and Rashid, 2001). By comparison, the formation of somatic embryos via indirect embryogenesis in sugarcane requires higher levels of 2,4-D and takes longer (Chen *et al.*, 1988; Ho and Vasil, 1983).

5.4.1.3 Potential to reduce somaclonal variation

The detection of subtle genetic variation at the phenotypic and DNA sequence levels (Zhang *et al.*, 1999; Linacero *et al.*, 2000; Bouman and De Klerk, 2001) has dispelled the view that micropropagated plants, even from somatic embryogenesis, retain complete genetic fidelity. Somaclonal variation has been reported in sugarcane at both a molecular level (Harrison *et al.*, 2001; Arencibia *et al.*, 1999; Sala *et al.*, 1999; Harrison *et al.*, 2001) and morphologically (Grof, 2001; Grof and Campbell, 2001). Somaclonal variation has been reported in several cereal crops, and has been attributed to the tissue culture process in rice (Arencibia *et al.*, 1998) and rye (Linaccero *et al.*, 2000), and more specifically, the length of time in culture in barley (Zhang *et al.*, 1999).

As the nature and extent of genomic change has not been assessed in plants derived from direct embryogenesis, this could form part of future work to determine whether somaclonal variation can be avoided/minimised by employing this route of morphogenesis.

5.4.2 The direct route in a transformation programme

5.4.2.1 Potential cost benefit

Regeneration via direct embryogenesis can offer advantages such as high throughput, reduced labour requirement and potential reduction in phenotypic 'off-types'. Various components of this route were compared to the more conventionally used indirect embryogenic route with a view to using the direct route in a transformation programme. Labour and medium costs were calculated to produce a transgenic plant via each route, and the results are presented in Table 5.7. The route with the most efficient regeneration capacity and shortest time frame for the production of transgenic plants, is that of direct embryogenesis from bombarded leaf discs. Consequently it is the most cost-effective route for the production of transgenic plants. The bombardment of leaf discs followed by regeneration via indirect embryogenesis also appears to be an effective approach to reduce overall time spent in culture. However, even if the overall transformation efficiency via direct embryogenesis is lower and therefore less cost effective than the indirect routes, the approach resulting in the least amount of genetic variation in the

transformants would be the more advantageous route to use.

Table 5.7 A comparison of factors affecting the production of transgenic sugarcane via direct and indirect morphogenesis.

	Indirect morphogenesis		*Direct morphogenesis*
Target material	Embryogenic callus	leaf roll discs	leaf roll discs
2,4-D (mg/l)	3	3	0.3
No. of subcultures	15	12	10
Time taken to generate plant (weeks)	24-36	18-26	13-22
Labour input required to generate transgenic plant (min/plant)	22.6	21.1	19.6
Amount of medium required to generate transgenic plant (ml/plant)	290	268	241
Comparative cost ratio of producing 1 transgenic plant	2.4	1.5	1

5.4.2.2 Incidence and implications of chimaerism

Although it was probable that the high level of untransformed plants observed in this study, regardless of the regeneration route, may have been due to a low stringency selection procedure, a more complex picture was emerging for plants regenerated via direct embryogenesis. 55% of these plants were susceptible to Buster, and a further 26% of plants that were sprayed showed mild phytotoxic symptoms and then recovered, suggesting initially that this may have been due to a low gene copy number or poor gene expression. However, no signal could be detected on a Southern blot from genomic DNA extracted from mature plants (Figure 5.9), despite the observation of a *pat* gene amplicon in half of the plants when they were younger (3-4 months old) (Figure 5.7 and Table 5.4). These observations led to the proposal that the 14 phenotypically resistant/recovery plants regenerated via direct embryogenesis from bombarded leaf discs were chimaeric, as were four plants regenerated via indirect embryogenesis from bombarded leaf discs and embryogenic callus. This would explain the ability to detect a PCR signal and phenotypic expression in immature chimaeric plants, and the absence of the *pat* gene in Southern blots conducted when the plants were mature, as the *pat* gene had by that stage been diluted out and was below detectable levels.

Initially it was thought that chimaerism in direct route-derived plants may be due to the reduced selection pressure and rapid regeneration potential. However, even once the selection

pressure had been increased to 45 mg/l geneticin and discs were bombarded at an earlier stage (Table 5.6), only one plant was transformed. For this plant, a Southern blot result could only be obtained when 20 µg of genomic DNA was loaded on the gel (Figure 5.10C). To determine whether this is a result of chimaerism or low copy gene number would require further analysis. It appears as though solving the chimaeric problem is a more complex issue.

The production of transgenic plants by direct morphogenesis is novel in the Poaceae and was first reported in orchardgrass (Denchev *et al.*, 1997). Here, leaf segments were bombarded and a high transformation efficiency of 16% was observed. A relatively high level of escapes (54%) and chimaeras (30%) were observed, as determined by GUS histochemical staining. The authors postulated that chimaeras arose if cells were transformed after the first cell division. An additional problem with this approach may be the lack of synchrony with regard to cell division and embryo initiation (Fromm *et al.*, 1990).

The production of transgenic chimaeric plants in sugarcane has been reported before, also in an attempt to reduce the tissue culture component of the transformation process (Gambley *et al.*, 1993, 1994). These plants were produced when sugarcane meristems were bombarded with the GUS reporter gene (Gambley *et al.*, 1993, 1994). The pattern of gene expression was that of blue streaks running up the leaf and was consistent with observations made in soybean plants, where embryonic axes had been bombarded (Christou, 1995). It was suggested that the GUS activity was as a result of a single transformed cell in the meristem which underwent periclinal cell division.

Although chimaeras may prove to be a problem for the expression of agronomically important genes, it is posssible to recover fully transformed plants by placing chimaeric plants in to culture again (Raemakers *et al.*, 1995; Fromm *et al.*, 1990) or by using axillary bud culture (Lowe *et al.*, 1995; Zhong *et al.*, 1996). Although both techniques were used in this study, neither allowed the recovery of transgenic plants. Had these manipulations been carried out at an earlier stage, perhaps they could have been more effective in recovering transgenic plants. However, the time spent having to retrieve transgenic plants from chimaeras in this way would negate the advantages of the short time frame offered by direct embryogenic plant production.

The importance of a stringent selection pressure has been emphasised previously (Fromm *et al.*, 1990), as too low a pressure results in escapes and chimaeras and cross protection or

'rescuing' of untransformed cells by transformed ones (Park *et al.*, 1998). It is now apparent that the most effective concentration of the selection agent must be determined empirically for each type of explant, and as this was not carried out for leaf discs in this study, this will form part of future optimisation of the bombardment of leaf discs, followed by direct morphogenesis.

5.4.2.3 Future work required to optimise the transformation protocol

Because leaf discs have potential to be used as targets for gene delivery, future work will concentrate on two aspects:

- Investigating the timing of gene delivery and the time at which bombarded leaf discs should be placed on selection medium for regeneration by direct embryogenesis, perhaps by using a visible reporter gene, such as GFP, to track transformed cells and somatic embryos, and,
- Determining a more efficient selection regime for leaf discs, whether regenerating by direct or indirect embryogenesis. Discs may have to be thinner or even cut up into smaller sections in order to expose pre-embryogenic cells or developing embryos to selection medium.

5.5 CONCLUSIONS

A regeneration protocol via direct somatic embryogenesis from leaf discs was developed. This was found to occur optimally on MS medium with 0.3 mg/l 2,4-D, and plants could be regenerated in six to 10 weeks. A novel approach for the production of transgenic plants involved the use of leaf discs as target material for microprojectile bombardment. The regeneration of transgenic plants can be obtained in half the amount of time compared with the conventional embryogenic callus approach if regeneration proceeds via direct embryogenesis, and a saving of eight to 12 weeks is obtained when bombarded leaf discs regenerate via indirect embryogenesis. Refinement of the approach is necessary in order to eliminate chimaerism, possibly with the establishment of a new selection kill curve for leaf discs.

CHAPTER 6
STABILITY OF GENE EXPRESSION AND AGRONOMIC PERFORMANCE OF A TRANSGENIC HERBICIDE-RESISTANT SUGARCANE LINE

6.1 INTRODUCTION

The development of herbicide resistance in crop plants using genetic engineering was one of the first applications of this technology to a commercially important objective (Mullineaux, 1992; Tsaftaris, 1996; Freyssinet and Cole, 1999). In genetically modified sugarcane, resistance to PPT via the *bar* (Gallo-Meagher and Irvine, 1996; Enriquez-Obregon *et al.*, 1998; Falco *et al.*, 2000) and *pat* (Snyman *et al.*, 1998) genes has been reported. Although commercial application of herbicide resistant crop plants in the field have been reported in tobacco and potato (de Greef *et al.*, 1989), sugarbeet (Buckmann *et al.*, 2000) and rice (Jiang *et al.*, 2000), little published information is available on the agronomic performance of herbicide resistant sugarcane.

Transgene stability in vegetatively propagated monocotyledonous plants has not been well documented, but studies in transgenic sugarcane to date have indicated that expression of a herbicide resistance gene was stable over two to three vegetative propagation cycles or ratoons (Gallo-Meagher and Irvine, 1996; Snyman *et al.*, 1998). However, in tall fescue, four to five rounds of vegetative propagation were necessary to select stable transformants (Bettany *et al.*, 1998).

In addition to stable gene expression, another aspect of importance is the stability of the plant genome as a whole which should be unaffected by the transformation process. The frequency of genomic change has been measured in transgenic rice (Jiang *et al.*, 2000; Labra *et al.*, 2001), barley (Bregitzer *et al.*, 1998b; Choi *et al.*, 2000a), oat (Choi *et al*, 2000b) and sugarcane (Arencibia *et al.*, 1999, 2000; Sala *et al.*, 1999). Differences were observed in both morphological traits and field performance, and these can be attributed to either somaclonal variation or the effect of the transformation process, e.g. gene inactivation by positional effects.

Acceptance of transgenic crops for commercialisation is only possible if it can be demonstrated that the introduced foreign gene is expressed in a plant that retains its agronomic characteristics. In this chapter the effect of the expression of the *pat* herbicide resistance gene on the agronomic potential of sugarcane was investigated. The aims, using line 22.2 as the

model transformed plant, were 1) to establish the stability of the gene over several ratoons and herbicide sensitivity at different application rates in a preliminary trial and 2) to compare morphological and agronomic characteristics of transformed and untransformed NCo310 grown in the field in a large-scale field trial. In 2), parameters such as stalk morphology, susceptibility to pests and diseases and yields were compared. In addition, the efficacy of the herbicide Buster in a weed control programme was compared to hand-weeding and the conventionally used cocktail of pre- and post-emergence herbicides (currently used by small-scale and large commercial sectors of the sugar-growing industry, respectively) in an attempt to asses the economic viability of herbicide resistant sugarcane.

6.2 TRIAL DESIGN

The field experiments were carried out at the South African Sugar Association in Mount Edgecombe, KwaZulu Natal (29° 42'S and 31°02'E) under natural rainfall conditions (1 045 mm per annum).

6.2.1 Preliminary field trial to assess herbicide sensitivity and resistance stability

A small-scale field trial was carried out to establish herbicide sensitivity of line 22.2 and to determine the stability of the resistance gene over several rounds of vegetative propagation. Transformed (line 22.2) and untransformed NCo310 lines were germinated in polystyrene seedling trays and transplanted into 7 x 18 m rows, separated by concrete strips (0.5 m wide). Plants were approximately three months old when planted in the field. Supplementary irrigation was provided. Once the plants had tillered, Buster (active ingredient: glufosinate ammonium 200 g/l; Aventis) was applied to the foliage in approximately 200 l water/ha using a lever-operated knapsack fitted with a 110° Teejet nozzle. Rates from 1 to 7 l/ha commercial preparation were used, and these were applied at increments of 1 liter per row. Damage was assessed three weeks after application. The plant crop was cut back after six months and allowed to ratoon. The two subsequent ratoons each received a single application of Buster at 7 l/ha after four months, resulting in one of the rows of transgenic cane having received a total of 21 l/ha from plant to second ratoon.

Material for a large-scale field trial to assess characteristics such as stalk morphology and pest and disease ratings, as well as yield data of transgenic plants, was obtained from bulking-up plots.

6.2.2 Large-scale field trial to compare both morphological and yield characteristics of herbicide resistant sugarcane in four weed control programmes

A large-scale field trial was conducted to compare line 22.2 with untransformed NCo310, using four different weed control treatments, in terms of:

- stalk morphology (height, diameter, population density) and fibre content,
- response to diseases smut and SCMV and the insect pest, eldana, and
- yield (both cane biomass and sucrose production).

The trial was planted with stalk sections (setts) of line 22.2, obtained from bulking plots when the sugarcane was 12 months old. During the field preparation, grass seeds (*Panicum maximum, Sorghum bicolor* and *Rottboellia conchinchinensis*) were introduced into the soil to increase weed pressure to the level normally found under industry conditions. Stalk sections were dipped in Panoctine fungicide (1% (v/v); active ingredient: guazatine 400 g/l; Rhone-Poulenc Agrichem, SA) before planting. Fertiliser (N (160 kg/ha), P (52 kg/ha) and K (160 kg/ha)) was applied in the planting furrows and as a top-dressing along the rows. The trial had a randomised block design with split plots, each with six rows, 7.5 m in length and spaced at 1.2 m (Appendix 2). Transformed and untransformed sugarcane in the following four weed control treatments were compared and each was replicated eight times:

1. Repeated Buster application (T1)

Buster (5 l/ha) was applied to sugarcane once weed emergence was complete. Three applications in the plant crop and two in the first ratoon were necessary to keep weeds under control.

2. Pre-emergence herbicides, followed by repeated Buster application (T2)

The plant crop was treated with a pre-emergence herbicide 'cocktail' comprising Visor (2 l/ha; active ingredient: thiazopyr 240 g/l; Rohm and Haas), Harness (1.5 l/ha; active ingredient: acetochlor 900 g/l; Monsanto) and Diuron 800 SC (2.5 l/ha; active ingredient: diuron 800 g/l; Sanachem) followed by two Buster (5 l/ha) applications. The first ratoon was hand-weeded once using eight labour units (1 labour unit= 1 labourer/day/ha) before receiving Har-i-cane (2 l/ha; active ingredient: acetochlor 960 g/l; Monsanto) and Diuron 800 SC (2.5 l/ha). One Buster application (5 l/ha) was necessary in the first ratoon.

3. Conventional weed control programme (T3)

The plant crop received a pre-emergence application of Visor (2 l/ha), Harness (1.5 l/ha) and

Diuron 800 SC (2.5 l/ha), followed by a post-emergence application of MCPA (3 l/ha; 400g active ingredient/l; Sanachem), Gesapax (4 l/ha; 500 g ametryn/l; Syngenta) and Reverseal 10 (adjuvant; 0,5% (v/v)). The plant crop was hand-weeded using four labour units. The ratoon crop was hand-weeded with eight labour units and then sprayed with Har-i-cane (2 l/ha) and Diuron 800 SC (2.5 l/ha). Finally, weeds were removed by hand-weeding with eight labour units two months later.

4. Hand-weeding (T4)

Five operations using hand-hoes and a total of 78 labour units were required in order to keep weeds under control in the plant crop. Three hand-weeding operations were necessary in the first ratoon crop (total of 34 labour units).

For the above four treatments, records were kept of labour use per hectare, operational times and amounts of herbicide used. Herbicides were applied as described in 6.2.1. The number of stalks was counted in two rows (7.5 m with 1.2 m spacing) in each plot to give an estimate of stalk population density per hectare. Stalk height (measured from base to visible dewlap) and diameter (measured at the centre of the stalk) were recorded in each of 20 stalks per plot. The incidence of smut (causal agent *Ustilago scitaminea*) and SCMV (sugarcane mosaic virus) was determined by field inspection carried out when the ratoon crop was 10 months old, directly prior to harvest. Similarly, an eldana survey was conducted to record damage by the borer *Eldana saccharina* (Walker; Lepidoptera: Pyralidae) by recording % internodes bored in each of 25 stalks per plot, sampled from harvested material. These surveys were carried out by SASEX inspection teams from Pathology and Entomology departments, respectively. The trial was harvested when the plant crop was 12.5 months old and the ratoon 10 months of age. The cane was cut by hand, weighed and sampled for direct analysis of sucrose and fibre (Anonymous, 1985) at the SASEX mill room. The estimated recoverable crystal (ERC) % cane, used to calculate the RV (recoverable value) payment system, was calculated according to the following formula:

ERC=a*S-b*N-c*F (where S=sucrose % cane, N=non-sucrose % cane, F=fibre % cane, a= losses other than molasses and bagasse (value of 0.978), b=losses in molasses (0.539), c=losses in bagasse, which is dependant on the fibre content (0.019) (Anonymous, 2000b)).

Data were subjected to analysis of variance (ANOVA) to determine the effect of the four weed control treatments on phenotypic characteristics, pest and disease resistance and yields of

transgenic and untransformed cane.

6.3 RESULTS

6.3.1 Stability of *pat* gene expression over multiple ratoons and rates of Buster application in the field

Having established that a rate of 4 l/ha Buster was lethal to untransformed plants in the glasshouse (4.2.10), a small-scale field trial was conducted using transgenic line 22.2 to determine whether this rate was sufficient to kill untransformed plants in the field. Buster was applied to transformed and untransformed cane planted in seven rows at 1 litre/ha increments. Transgenic plants showed no signs of damage after application of rates as high as 7 l/ha (Figure 6.1A). Untransformed cane plants displayed phytotoxic symptoms at all rates tested (Figure 6.1B), but damage was extreme above rates of 4 l/ha and plants died after application of 5 l/ha and above. Having established that line 22.2 was resistant to up to 7 l/ha Buster in the plant crop, and untransformed plants died at rates of 5 l/ha and above, it was decided to use the latter rate in the large-scale field trial.

Buster was applied to two subsequent crops at a rate of 7 l/ha, so that the transgenic cane to which 7 l/ha Buster was applied in the plant crop, received a total dose of 21 l/ha of Buster. This repeated application of the herbicide in successive ratoon crops, with no phytotoxic symptoms, indicates that the introduced gene is stable and expressed over successive crops. Such stability has been reported in sugarcane previously (Gallo-Meagher and Irvine, 1996).

6.3.2 Large-scale field trial to determine agronomic performance of transgenic cane under four weed control programmes

Preliminary assessment of line 22.2 indicated that the *pat* herbicide resistance gene was stably expressed over two ratoons (6.3.1), and no gross phenotypic abnormalities were apparent. However, the agronomic field performance of the line under field conditions needed to be established in terms of stalk characteristics, as these might impact on yield. In addition, the effects of selected pests and diseases were established and a yield comparison was done. Sucrose yields were measured under four weed control programmes to establish which treatment was the most viable economically.

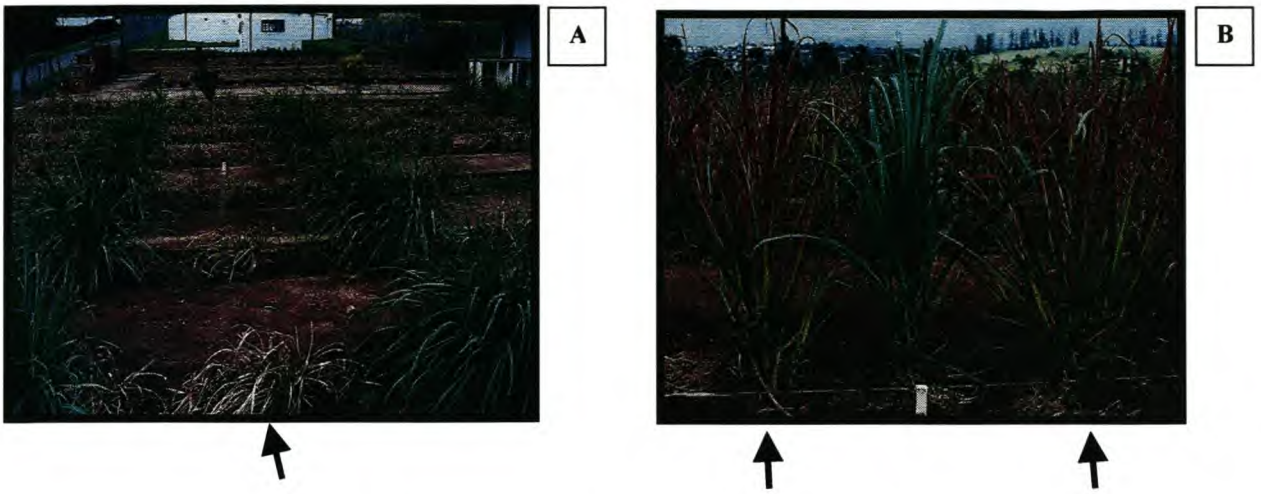


Figure 6.1 Phytotoxic damage to untransformed sugarcane when sprayed with Buster.

A. Transgenic and untransformed sugarcane plants two months after Buster application. Buster was applied in 1 liter increments to seven rows of plant cane. The untransformed cane (indicated with arrows) displayed phytotoxic symptoms at all the rates applied. Symptoms were severe above 4 l/ha and at rates above 5 l/ha, the herbicide was lethal.

B. Transgenic and untransformed plants three weeks after Buster application. Phytoxic symptoms were expressed by untransformed cane (arrows) at all the rates tested. Transgenic cane displayed no symptoms at any rate applied.

6.3.2.1 A comparison of stalk parameters for transformed and untransformed sugarcane in each weed treatment

Early studies on sugarcane somaclonal variation reported changes in sucrose content (Liu, 1990), gross morphology (Heinz and Mee, 1969, 1977) and isoenzyme profiles (Larkin and Scowcroft, 1981). More recently, in an attempt to determine field performance of sugarcane genetically modified with a *B. thuringiensis* transgene, stalk diameter and number of stalks per stool were found not to be significantly different when compared to conventionally propagated and untransformed *in vitro*-derived material (Arencibia *et al.*, 1999, 2000; Sala *et al.*, 1999). There was, however a significant increase in stalk height in the transformed plants. In this study, stalk height, diameter and population measurements were compared for line 22.2 and conventionally propagated NCo310 sugarcane in the first ratoon in four weed control treatments. Data from the first ratoon as opposed to that from plant cane was used as a basis for comparison. This was due to the findings reported by Bailey and Bechet (1989), where growth characteristics of conventionally propagated sugarcane setts were compared to tissue culture (callus)-derived plantlets. Their results suggested that the latter compared favourably to conventional sugarcane in the first ratoon. In this study, comparisons of mid-stalk diameter, height, population density per hectare and fibre content for first ratoon plants are summarised in Figures 6.2A, 6.2B, 6.2C and 6.2D, respectively. As untransformed cane treated with Buster

in treatment one died during the plant crop, no measurements could be taken from these plots in the ratoon crop.

Sugarcane stalk diameters can vary widely, depending on the cultivar and on growing conditions (van Dillewijn, 1952). In this study, untransformed cane had thicker stalks than transformed cane in all treatments, but these differences were not significant (Figure 4A) (ANOVA SED=0.57; LSD (0.05)=1.17). In addition, there were no significant differences in stalk diameter between any of the weed control treatments (ANOVA SED=0.59; LSD (0.05)=1.25).

Sugarcane stalk heights were measured when the first ratoon was harvested. Results are presented in Figure 6.2B. Stalk height ranged from 185.6-204.5 cm and there were no significant differences between untransformed and transformed cane in treatments T2, T3 and T4 (ANOVA SED=4.63; LSD (0.05)=9.6). The shortest stalks (186-190 cm) were observed in T4, where weeds were removed by hand-hoeing. It is possible that mechanical damage to the roots caused by hoeing stunted the growth of the cane.

The number of stalks per hectare were counted to compare the population of stalks, which may influence yield. The results are summarised in Figure 6.2C. The treatment that included Buster (T2) was the only treatment where significant differences in stalk population between transformed and untransformed sugarcane was observed. This is because the untransformed cane is susceptible to Buster and some stalks did not survive the herbicide application.

The fibre content of sugarcane was determined at harvest in order to compare transformed and untransformed sugarcane. These results are presented in Figure 6.2D. When results were analysed statistically, no significant differences in fibre content were observed (ANOVA SED=0.22; LSD (0.5)=0.46), although transformed cane in T3 and T4 contained more fibre than the untransformed plants from the same treatment.

6.3.2.2 The effect of pests and diseases on transgenic sugarcane

Varying levels of smut resistance have been observed in previous field studies carried out on tissue culture-derived (Bailey and Bechet, 1989) and transgenic (Arencibia *et al.*, 2000) sugarcane. In addition, cultivar NCo310 is susceptible to both smut and SCMV (Bailey, 1979). Therefore, an assessment of resistance to major pests and diseases was undertaken to compare

responses of transgenic and untransformed sugarcane in the four weed control treatments in this study. The ratings were carried out by an inspection team from the SASEX Plant Pathology Department directly prior to harvesting of the first ratoon crop, and the proportion of plants showing symptoms of smut and SCMV are presented in Figure 6.3. Although 1.4 - 5.1% plants were infected with smut (Figure 6.3A) and 1.3 - 5.9% with mosaic (Figure 6.3C), there were no significant differences between the ratings of transformed and untransformed cane in any of the treatments for both smut (ANOVA SED=1.67; LSD (0.05)=3.44) (Figure 6.3B) and mosaic (ANOVA SED=1.74; LSD (0.05)=3.59) (Figure 6.3D).

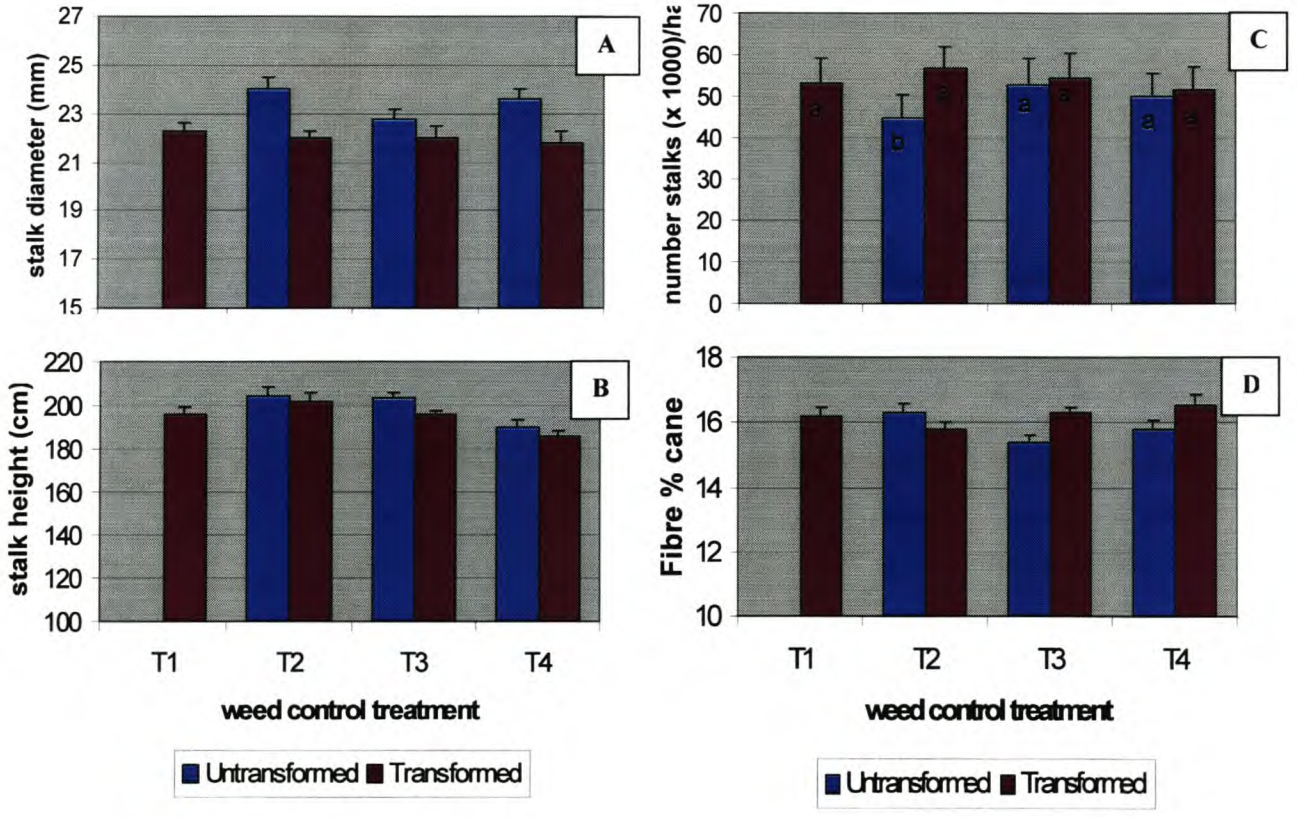


Figure 6.2 A comparison of morphological stalk characteristics and fibre content. Measurements were taken when the crop was harvested at the age of 10 months. Treatments T1-T4 are as follows: T1-Buster only, T2-pre-emergence and Buster application, T3-conventional weed control programme, T4-hand-hoeing. Standard errors are shown by vertical lines above the bars (n=8).

A. Stalk diameter measurements for transformed and untransformed cane in four weed control treatments. Measurements (mm) were taken around the centre of stalks.
B. A comparison of stalk heights of ratoon transgenic and untransformed sugarcane at harvest.
C. Stalk population comparison between untransformed and transformed sugarcane prior to harvesting the ratoon crop. Bars with different alphabetical letter are significantly different from others (ANOVA SED=3995; LSD (0.05) =8246).
D. Fibre content (fibre is the insoluble portion of a sugarcane stalk and is expressed as a percentage of the fresh mass of the stalk; fibre % cane) in transformed and untransformed sugarcane in the four weed control treatments.

Similarly, the inspection team from the Entomology Department assessed ratoon cane for damage by the stalk-boring insect, eldana, in order to see whether there was a difference in response of transformed and untransformed sugarcane between the four weed control treatments. Percentage internodes damaged by eldana are summarised in Figure 6.4. When weed control treatments were compared to each other (ANOVA SED=0.86; LSD (0.05)=1.83), stalks in hand weeded plots (T4) had significantly more internodes bored, 7.8%, compared to 4.6%, 5.4% and 4.4% for T1, T2 and T3, respectively. Hand-hoeing may stress sugarcane by disturbing or damaging roots, which can cause the plants to be more susceptible to eldana (Leslie, personal communication). Stress has been found to increase the incidence of diseases in sugarcane (Bailey, 1979). In untransformed cane in treatments T2 and T4, a greater proportion of internodes were bored when compared to the untransformed cane. Although there are no statistical differences in % internodes bored between transformed and untransformed cane for each treatment (ANOVA SED=1.3; LSD (0.05)=2.7), the transformed samples had less internode damage than the untransformed samples.

It is thought that an increase in fibre content of sugarcane may deter eldana from feeding on the plant (Leslie, personal communication). However, the differences in eldana susceptibility cannot be accounted for by 'hardness' of the cane, as there were no significant differences in fibre content (Figure 6.2D).

6.3.2.3 Yield comparisons for transformed and untransformed sugarcane in each weed control treatment

A comparison was made of transformed and untransformed cane yields in order to determine whether the growth and therefore yield capacity of the plants was affected by the transformation process. In addition, four different weed control treatments were compared to see whether Buster compared favourably with other weed control methods. Yields of sugarcane can be measured in terms of the stalk fresh mass of the crop at harvest (cane Mg /ha), sucrose yield (estimated recoverable sucrose (ERC) Mg/ha) derived from the fresh mass and sucrose content, or by cane quality (expressed as the proportion of sucrose per plant (ERC % cane)). Results are shown in Table 6.1. It was expected that T3 untransformed would yield well, as this is a treatment that is widely used in the South African sugar growing industry. Treatments in which both high cane biomass and cane quality were observed are T3 untransformed and T2 transgenic cane. In T2, the untransformed cane yielded poorly due to the phytotoxicity of multiple Buster applications. In T3 and T4 where Buster was omitted, transformed cane

yielded less than the untransformed equivalent. This indicates that conventionally propagated sugarcane is at a competitive advantage in the field when compared to *in vitro*-derived, transgenic plants.

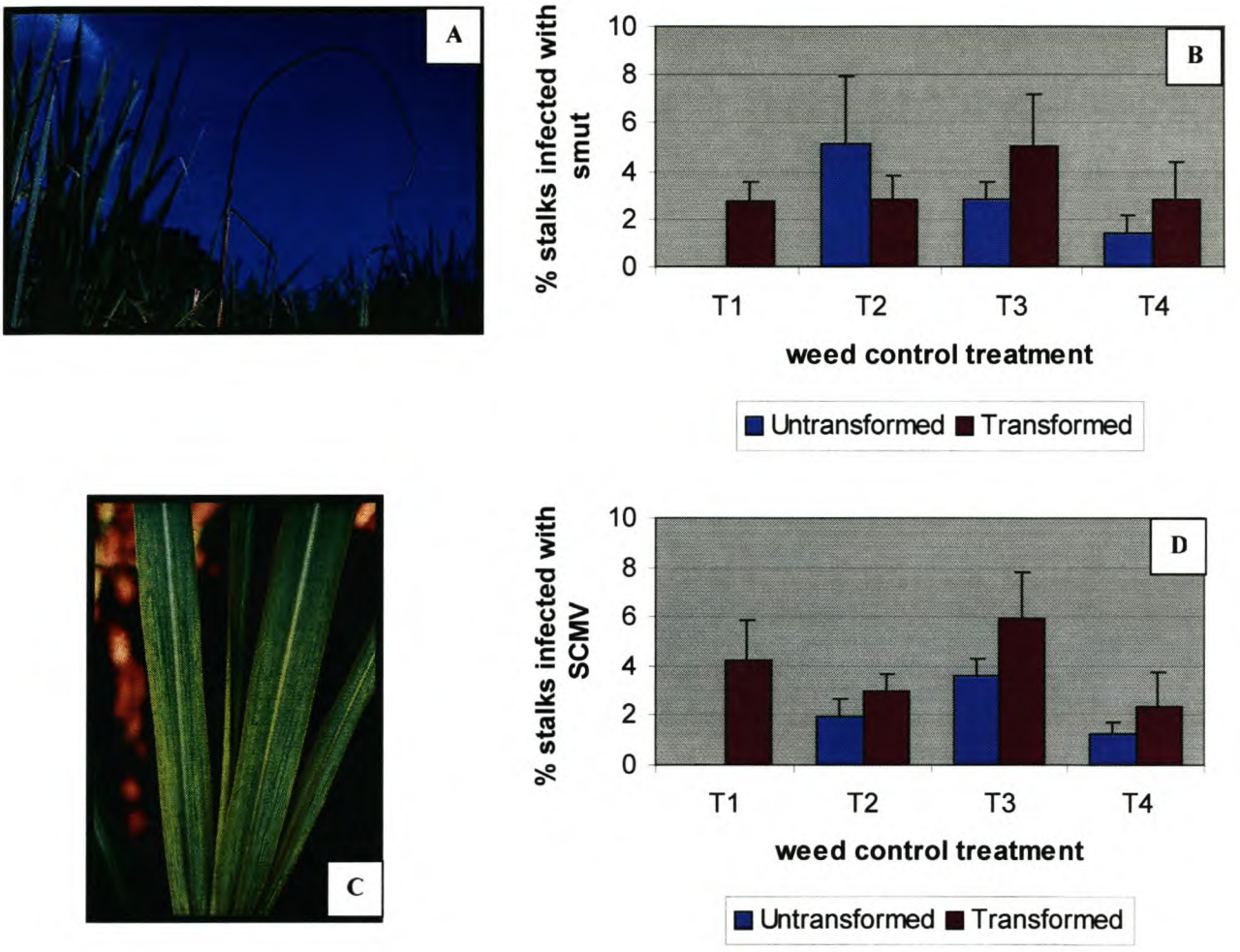


Figure 6.3 Incidence of smut and SCMV on transformed and untransformed sugarcane in four weed control treatments.

A. Typical smut whip on an infected plant (photo courtesy of Sharon MacFarlane).
B. Differences in % stalks infected with smut. Smut inspections were carried out by the SASEX Pathology inspection team at harvest, when the ratoon crop was 10 months old. Vertical lines above bars indicate SE (n=8).
C. Sugarcane plant infected with SCMV. Note the pale green streaks, indicative of infection with SCMV (photo courtesy of Sharon MacFarlane).
D. Incidence of stalks infected with SCMV. Observations were carried out by the Plant Pathology inspection team directly prior to harvesting, when the ratoon crop was 10 months old. Vertical lines above bars are SE (n=8).

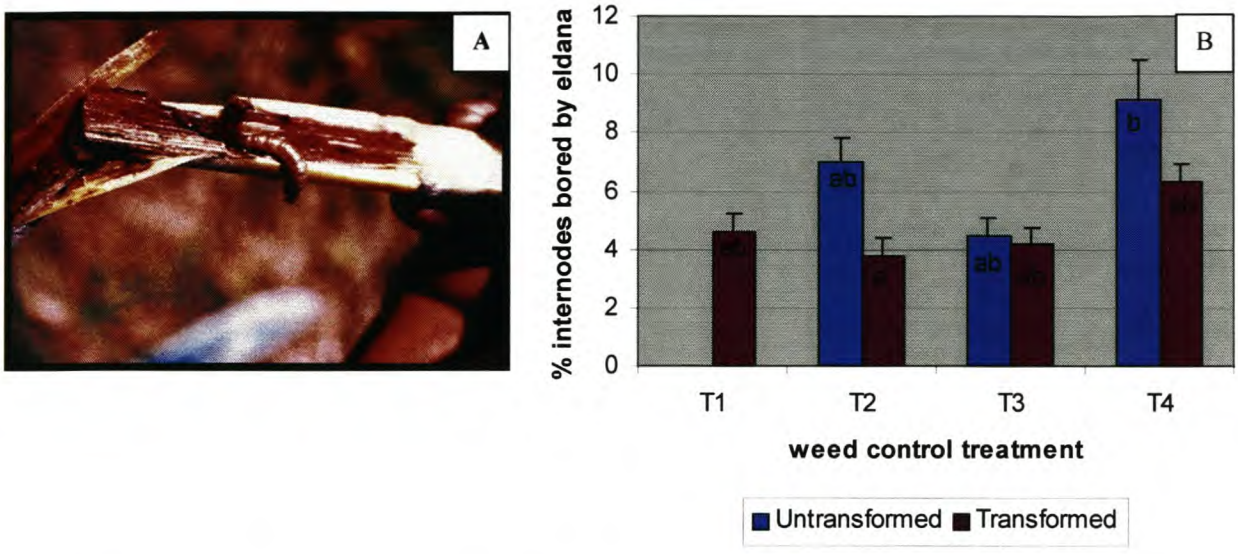


Figure 6.4 Damage caused to sugarcane stalks by the eldana stalk borer.
A. Eldana larva boring in a sugarcane stalk internode (photo courtesy of Graham Leslie).
B. A comparison of internodes bored by eldana in transformed and untransformed sugarcane. Eldana damage was assessed when the ratoon was 10 months old, directly prior to harvesting. Vertical lines above bars represent SE (n=8). Different alphabetical letters represent statistical differences (ANOVA SED=1.3; LSD (0.05)=2.71).

Untransformed cane in T1 died because it was susceptible to Buster. Weed control in T1 plots was not as effective as in T2, where pre-emergence herbicides were employed in combination with Buster. T2 untransformed cane yielded significantly less than T2 transformed cane, indicating the sensitivity of untransformed cane to Buster. Although the use of a single herbicide is unlikely to be used commercially in a large-scale operation, this treatment may have an application as a 'chemical' hoe to replace the conventionally used hand-hoeing in small-scale growing operations. Cane yields in T4 transformed were the lowest obtained in the trial, suggesting that transformed cane does not respond well to hand weeding.

When comparing treatments on the basis of sucrose yield, the only treatments with significantly higher yields are T2 transformed and T3 untransformed. This suggests that the weed control in these two cases is successful, and also that the *in vitro* culture- and transformation-derived plants are not compromised in terms of yield potential. There were no significant differences in cane quality between any of the treatments. Because the two highest yielding treatments, T2 transformed and T3 untransformed, showed no significant differences in yield, the economic advantage of using transformed sugarcane was investigated.

Table 6.1 A comparison of sugarcane biomass (cane Mg /ha), sucrose yields (ERS Mg/ha) and cane quality (ERS % cane) for the four weed control treatments in transformed and untransformed cane in the ratoon crop. Means are of eight replicates and are followed by different alphabetical letters if differences are significant (ANOVA).

Weed control treatment for 1st ratoon	*Category*	*Cane mass (Mg/ha)*	*Sucrose yield (ERC Mg/ha)*	*Cane quality (ERC % cane)*
T1 (Buster)	Untransformed	0	0	0
	Transformed	59.8 ab	8.2 a	13.8
T2 (cocktail plus Buster)	Untransformed	56.3 a	7.7 a	13.6
	Transformed	68 b	9.5 bc	13.9
T3 (conventional)	Untransformed	67.5 b	9.7 c	14.3
	Transformed	60.1 ab	8.3 ab	13.9
T4 (hand-hoeing)	Untransformed	60.5 ab	8.2 a	13.6
	Transformed	53.9 a	7.4 a	13.7
ANOVA comparison of category means				
SED		1.8	0.31	0.21
LSD (0.05)		3.8	0.65	0.44
ANOVA comparison of treatment means				
SED		3.72	0.62	0.37
LSD (0.05)		7.93	1.32	0.79

6.3.2.4 A comparison of weed control costs for four different treatments

In order for the cultivation of transgenic sugarcane to be commercially viable, there should be a financial advantage to the growers. Although yields do not differ significantly between T2 transgenic and T3 untransformed cane, the profit margin will be determined by the pricing of the herbicide to which resistance has been genetically engineered. A costing exercise was done using three different herbicide prices of R150/l, R60/l and R30/l. The return per Rand spent on weed control was calculated where the value of the crop (calculated on the price of R865/Mg sucrose) and the cost of weed control were taken into account. The return per Rand spent on weed control of the two Buster treatments (T1 and T2) were compared to T3 untransformed (a conventional weed control programme adopted by large-scale commercial growers) and T4 untransformed (weed control programme currently adopted by the majority of small-scale growers). Results are presented in Table 6.2. As it is unlikely that a single herbicide would be used to control weeds in large commercial fields due to the potential development of resistance to a single product, treatment T2 is likely to be used as a means of 'chemical hoeing' by small-scale growers. This was therefore compared to conventional hand-hoeing costs in T4 untransformed. In this scenario, even if the herbicide to which resistance has been engineered in sugarcane is relatively high (R150/l), there will be no loss in income to the growers. However, if the herbicide is priced at R60 or R30, the return to the growers can be increased by 2.3 and 4.2 times, respectively.

The return per Rand spent on weed control for transgenic cane in T2 ranges from 3.8-8.6, depending on the price of the herbicide to which resistance has been conferred. As the return

per Rand spent on conventional herbicides is 7.1, and this return is only comparable in T2 at a cost of R30/l, careful consideration will need to be given to the pricing of herbicides to which resistance is genetically engineered if transgenic plants are to be a commercially attractive option to growers.

Table 6.2 A comparison of the return per Rand spent on weed control. Transgenic sugarcane in T1 and T2 was considered, and untransformed sugarcane in T3 (treated with the conventional pre-and post-emergent herbicide cocktail) and T4 (hand weeded). Sucrose value calculations were done by multiplying sucrose yield by sucrose price (R865/Mg (2001 season)).

Treatment	*Sucrose value (R/ha)*	*Total weed control costs (R/ha)*	*Return per Rand spent on weed control if cost of herbicide to which resistance has been engineered is as follows:*		
			R150/l	R60/l	R30/l
T1 trans	7 093	1 618	4.4	10.3	18.3
T2 trans	8 218	2 182	3.8	6.6	8.6
T3 untrans	8391	1 004	7.1	7.1	7.1
T4 untrans	6 401	1 496	4.3	4.3	4.3

6.4 DISCUSSION

This research assesses a range of agronomic characteristics in transgenic sugarcane and the stability of the transgene over multiple ratoons. Use was made of a small-scale field trial to assess the rate of Buster that was toxic to transgenic cane in the field and to establish the stability of the *pat* gene over three rounds of vegetative propagation. The model study investigated whether tissue culture involving indirect embryogenesis influenced morphological parameters such as stalk height, diameter and stalk population, pest and disease resistance ratings and yield characteristics of untransformed and transformed cane in four weed control treatments. Cane and sucrose yields were compared for the different treatments to determine whether transgenic sugarcane would be as competitive as that grown commercially.

6.4.1 Transgene stability

A small-scale field trial, carried out over two ratoons, was sprayed with concentrations of Buster ranging from 1-7 l/ha and showed that the herbicide resistance gene was stably expressed over three rounds of vegetative propagation at all the concentrations tested (Figure 6.1). This was confirmed by the absence of phytotoxic symptoms to Buster in transformed plants. In contrast, the untransformed plants showed phytotoxic symptoms at all the concentrations tested, but they were severe at concentrations of 4 l/ha (Figure 6.1) and above 5 l/ha, plants died. The stability of a herbicide-resistance transgene in sugarcane over multiple ratoons has been previously reported (Gallo-Meagher and Irvine, 1996).

Stable inheritance of a transgene in field trials has been reported in transgenic crops such as barley (Horvath *et al.*, 2001) and rice (Jiang *et al.*, 2000). However, the importance of thorough screening of transgenic plants that are propagated vegetatively was stressed in a paper on transgenic tall fescue (Bettany *et al.*, 1998). It was suggested that stable transgene expression was only observed after three to four rounds of vegetative propagation and that environmental effects could account for a loss of gene expression in some tillers in the plant.

Occasionally transgenic plants fail to express a protein product as a result of silencing (Covey and Al-Kaff, 2000; Fagard and Vaucheret, 2000; Iyer *et al.*, 2000). In addition to complete silencing of transgenes, variation in expression levels among transformants is believed to be due to the transgene integrating at different chromosomal locations (Topping *et al.*, 1991). This variation in expression has been observed in rice (Chareonpornwattana *et al.*, 1999; Kohli *et al.*, 1999), sugarcane (Gallo-Meagher and Irvine, 1996) and tobacco (Mannerlof and Tenning, 1997). Only one line was tested in this study, and perhaps not all transformants would perform as well agronomically.

6.4.2 Phenotypic differences between herbicide-resistant and untransformed sugarcane

Parameters such as stalk height, thickness, population and susceptibility to a stalk borer, eldana, as well as incidence of smut and SCMV diseases were compared for transformed and untransformed cane in 4 weed control treatments. There was little variation in morphological parameters such as stalk diameter (Figure 6.2A), stalk height (Figure 6.2B) or in disease ratings to smut or SCMV (Figure 6.3) infection. A parameter which showed statistical differences was that of stalk population, which was significantly lower in T2 untransformed (Figure 6.2C). This response is due to tiller mortality because of the susceptibility of the untransformed cane to Buster.

Transformed and untransformed cane differed in their response to eldana damage (Figure 6.4), where significantly less internode damage was observed in T2 transgenic sugarcane. Although it was thought that the altered resistance to eldana may have been due to an increase in fibre, the insoluble portion of dry matter measured when sugarcane is harvested, there were no differences in fibre content when transformed and untransformed treatments were compared (Figure 6.2D). Changes in susceptibility to eldana between transformed and untransformed cane may be attributed to somaclonal variation.

Somaclonal variation has been observed in tissue culture-derived sugarcane plants and initial expectations were that it would give rise to promising agronomic genotypes (Heinz and Mee, 1969; Liu, 1990). However, not all changes are favourable and careful screening needs to be carried out to ensure that plants regenerated *in vitro* retain characteristics of parent plants. In a study carried out on tissue culture-derived and conventionally propagated South African cultivars NCo310, NCo376 and N12, disease susceptibility increased and double the number of smut whips was observed in all propagated plants (Bailey and Bechet, 1989).

In addition to the effects of *in vitro* culture, somaclonal changes may be induced by collateral gene damage as a result of the transformation process. This was demonstrated in *Bt* transgenic sugarcane in Cuba, where a detailed comparison between morphological traits was made between *Bt* transgenic cane, *in vitro*-derived untransformed cane and conventionally propagated cane (Arencibia *et al.*, 1999, 2000; Sala *et al.*, 1999). Transgenic populations presented the largest variance for two of five characteristics tested (stalk diameter, number per stool, affected internodes, stalk height and % dead stalks) and showed significant heterogeneity for the latter two variables, (Arencibia *et al.*, 1999). The unintended effects of genetic engineering in plants is the topic of a recently initiated assessment in Europe (http://www.entransfood.com.RTDprojects/ GMCARE).

Although somaclonal changes appear to be negligible in this study, reports on transgenic barley (Choi *et al.*, 2000) and rice (Jiang *et al.*, 2000) showed differences in transgenic plants which can be attributed to somaclonal variation. One of the long-term aims in this type of work will be to identify and reduce the stresses associated with tissue culture and transformation processes, so that transgenic plants are, with the exception of the introduced trait, phenotypically and genetically identical to parent plants.

6.4.3 Yield differences and economics

Yields are an important consideration in any commercial operation. Although the first paper reporting on field performance in tobacco and potato was published in 1989 (de Greef *et al.*, 1989), there is very little published information on yields in transgenic crops. In this study, sugarcane yield was compared using stalk fresh mass, sucrose mass and cane quality. The highest mass of cane at harvest was observed in T3 untransformed cane and T2 transformed cane (Table 6.1). In addition, the highest ERC % cane and ERC yields were observed in T3 untransformed, indicating that sugarcane treated with conventional weed control procedures

has the highest yields. Because there are very few statistical differences in stalk morphology between transformed and untransformed cane, differences in yield may be attributed to the success of the weed control programme. As the conventional programme (T3) has been optimised for use in weed control in sugarcane, superior weed control and yields can be expected from this treatment. Sucrose yields and therefore monetary value is greatest from this treatment.

The highest cane yield and second best sucrose yield were observed in T2 transformed cane, indicating that transgenic sugarcane is competitive when compared to untransformed cane. In addition, efficient weed control in the field is provided by the pre-emergent cocktail followed by Buster treatment. Because herbicides that are not approved for weed control in sugarcane are usually not as reasonably priced as those commonly used in the sugar industry, the herbicide to which resistance has been genetically engineered has to be competitively priced at R30/l or less, in present conditions (Table 6.2) in order for the weed control programme chosen for transgenic cane to be competitive with the conventional programme.

In recent reports on other transgenic crop yields, *Bt* rice showed a five times increase in yield when compared to a susceptible crop (Tu *et al.*, 2000b). However, in rice resistant to bacterial blight, there was no yield difference between transformed and untransformed controls (Tu *et al.*, 2000a). Transgenic barley showed lower yields and reduced height when field-tested (Bregitzer *et al.*, 1998b). However, when this line was cross-bred with another cultivar, the yield increased (Horvath *et al.*, 2001).

The highest return per Rand was calculated for T1 transformed cane when the herbicide to which resistance had been engineered was priced at R30/l (Table 6.2). However, it is unlikely that a single herbicide would be used on large-scale commercial practice due to the greater potential for the development of resistance when a single product is used. It is plausible that small-scale growers would use a single herbicide as a means of 'chemical' hoeing. If so, the return per Rand could be increased by three times when compared to that from hand-weeded, untransformed cane.

6.5 CONCLUSIONS

Field-testing of herbicide resistant transgenic sugarcane line 22.2 showed that the *pat* gene was

stably expressed over three rounds of vegetative propagation. Morphological and agronomic characters of transgenic cane such as stalk height, diameter, population, fibre, disease resistance and yield were not significantly different to untransformed sugarcane. The most economical weed control treatment is dependant on the cost of the herbicide to which resistance has been engineered, but at a cost of R30/l, the most cost effective weed control treatments are those employing Buster and a conventional pre-emergence cocktail, and employing chemical hoeing using Buster.

CHAPTER 7
CONCLUDING REMARKS

Genetic transformation has enormous potential for producing novel and useful plants in most crop species, but significant problems have arisen with the following:

- the development of reproducible, efficient transformation systems for commercial germplasm (Lowe *et al.*, 1995; Birch, 1997; Iser *et al.*, 1999; Pastori *et al.*, 2001),
- variation, where genotypically and phenotypically abnormal plants have been produced as a result of the tissue culture process (somaclonal variation), or collateral gene damage (Bregitzer *et al.*, 1998b; Zhang *et al.*, 1999; Choi *et al.*, 2000a, 2000b), and
- transgene expression and stability (Register *et al.*, 1994; Kumpatla *et al.*, 1997, 1998; Mannerlof and Tenning, 1997).

Initial reports of successful transformation in many of the cereals were highly dependent on the use of particular cultivars, which are amenable to *in vitro* growth and subsequent regeneration of fertile plants. In barley and wheat, the lack of culturing techniques to ensure long-term regenerability of transformed tissue hindered progress in achieving transformation of commercial germplasm until recently (Lemaux *et al.*, 1999; Iser *et al.*, 1999). In sugarcane, genotypic differences in response to *in vitro* culture have been observed (Taylor *et al.*, 1992b; Snyman *et al.*, 1996). A range of cultivars have been transformed, albeit at a range of efficiencies (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996). The work carried out in this study showed that several South African cultivars can be transformed, although the *in vitro* response of each new cultivar may have to be examined and established prior to genetic manipulation.

It has long been acknowledged that dedifferentiation of plant cells, which takes place when cells go through a callus stage, can result in the production of somaclonal variants (Larkin and Scowcroft, 1981). Prolonged periods in culture and the exposure to auxin in the growth medium have been implicated in genetic and phenotypic variability (Larkin and Scowcroft, 1981; Irvine *et al.*, 1991; Karp, 1995; Jain, 2001). One way in which the issue of somaclonal variation can be addressed is by reducing the tissue culture component of the transformation process Therefore, transformation protocols that reduce or preferably eliminate the tissue culture phase should be considered to reduce the risk of *in vitro*-culture induced variation. In this context, transformation protocols that are based on the utilisation of organised plant tissue

such as shoot apices, axillary buds and leaf tissue as target material could be advantageous.

Bombardment of leaf base segments in oat (Gless *et al.*, 1998), followed by embryogenic callus production, reduced the length of time spent in culture. Transgenic plants could be produced at a high efficiency, and did not show any evidence of somaclonal variation. The use of shoot apices (Gambley *et al.*, 1993, 1994; Christou, 1995; Lowe *et al.*, 1995) for the production of transgenic sugarcane, maize and soybean, respectively, resulted in the production of chimaeric plants. Although fully transformed plants could be recovered by placing chimaeric plants back in culture, this approach still required a fairly extensive culturing stage.

Transgenic orchardgrass plants were produced by a novel approach of bombardment of leaf tissue, followed by the formation of, and subsequent regeneration from, somatic embryos, directly on leaf material (Denchev *et al.*, 1997). Although chimaeric plants were observed with this route, a high transformation efficiency was reported. Furthermore, direct embryogenesis has the advantages of being simple and rapid, and therefore has the potential to reduce somaclonal variation. These sentiments have been echoed in several recent publications reporting on the establishment of direct embryogenic pathways in sugarcane (Aftab and Iqbal, 1999), millet (Vikrant and Rashid, 2001), orchid (Chen *et al.*, 1999), and even in a traditional 'model' plant system such as *Arabidopsis* (Gaj, 2001). It is expected that the advantages of direct somatic embryogenesis will be applied to other plant species with a view to utilisation in transformation programmes.

In this study, a novel alternate regeneration pathway for sugarcane was developed and characterised. Direct somatic embryogenesis was achieved by reducing 2,4-D levels to 1/10th of that used for the induction of embryogenic callus. Although this pathway requires further optimisation, it has the potential to reduce culture times, which would result in a rapid throughput and a more cost effective method for the production of transgenic plants. Due to reduced auxin levels and limited time *in vitro*, it also has the potential to reduce 'off-types' which have been reported in several field trials with transgenic sugarcane lines (Grof, 2001; Grof and Campbell, 2001).

One limitation of current methods of transformation is that insertions occur randomly and the location of the insertion in the plant genome might not be optimal for gene expression. Another problem is that the introduced DNA is often present in multiple, tandemly arrrayed copies

(Kumar and Fladung, 2001). Having multiple copies of the gene can lead to gene inactivation and genetic instability (Matzke and Matzke, 1995). However, neither tandem repeats nor position effects are likely to explain variability in expression among progeny derived from the same parent (Wan and Lemaux, 1994). Therefore the only definitive way in which to screen large numbers of transformants is to monitor gene expression in the field. Literature dealing with performance in the field of transgenic plants is limited. The field trial carried out in this study was one of the most extensive reported for transgenic sugarcane, as it focused not only on yield data, but also on resistance to pests and diseases. In addition the response to several weed control programmes was determined. There was no evidence to suggest that somaclonal variation was causing negative effects in the single line tested, but the outcomes may not be applied to all lines. In particular, the economic exercise was valuable in terms of realising the returns for the investment in genetic engineering.

REFERENCES

Aftab F and Iqbal J (1999) Plant regeneration from protoplasts derived from cell suspension of adventive somatic embryos in sugarcane (*Saccharum* spp. hybrid cv. CoL-54 and cv. CP-43/33). Plant Cell, Tissue and Organ Culture 56:155-162.

Ahloowalia BS and Maluszynski M (2001) Induced mutations- A new paradigm in plant breeding. Euphytica 118:167-173.

Ahloowalia BS (1984) Forage grasses. In: Handbook of plant cell culture. Volume 3. Eds: Ammirato PV, Evans DA, Sharp WR and Yamada Y. Macmillan Publishing Company, New York. pp 91-125.

Alam MF, Oliva NP, Zapata FJ and Datta SK (1995) Fertile transgenic indica rice plants by PEG-mediated protoplast transformation. Journal of Genetics and Breeding 49:303-308.

Albert H, Dale EC, Lee E and Ow DW (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. The Plant Journal 7:649-659.

Al-Kaff NS, Krieke MM, Covey SN, Pitcher R, Page AM and Dale PJ (2000) Plants rendered herbicide-susceptible by cauliflower mosaic virus-elicited suppression of a 35S promoter-regulated transgene. Nature Biotechnology 18:995-999.

Allen GC, Spiker S and Thompson WF (2000) Use of matrix attachment regions (MARs) to minimise transgene silencing. Plant Molecular Biology 43:361-376.

Altpeter F, Vasil V, Srivastava V, Stoger E and Vasil IK (1996) Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Reports 16:12-17.

Altpeter F and Xu J (2000) Rapid production of transgenic turfgrass (*Festuca rubra* L.) plants. Journal of Plant Physiology 157:441-448.

Altscul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Amoah BK, Wu H, Sparks C and Jones HD (2001) Factors influencing *Agrobacterium*-mediated transient expression of *uidA* in wheat inflourescence tissue. Journal of Experimental Botany 52:1135-1142.

Ammirato PV (1987) Organizational events during somatic embryogenesis. In: Plant tissue and cell culture. Eds: Green Ce, Somers DA, Hackett WP and Bisboer DD. Alan R Liss Inc, New York. pp57-81.

Anonymous (2000a) South Afican Sugar Association industry directory. Published by SASA Public Affairs Division.

Anonymous (2000b) Information sheet 11.8. The RV payment system. South African Sugar Association Experiment Station HH Dodds Library.

Anonymous (1985) Laboratory manual for South African sugar factories including the Official methods. Published by the South African Sugar Technologists Association.

Arceneaux G (1965) Cultivated sugarcanes of the world and their botanical derivatives. Proceedings of the 12th International Society for Sugar Cane Technologists: 844-855.

Arencibia A, Vazquez RI, Prieto D, Tellez P, Carmona ER, Coego A, Hernandez L, de la Riva GA and Selman-Housein G (1997) Transgenic sugarcane plants resistant to stem borer attack. Molecular Breeding 3:247-255.

Arencibia AD, Gentinetta E, Cuzzoni E, Castiglione S, Kohli A, Vain P, Leech M, Christou P and Sala F (1998a) Molecular analysis of the genome of transgenic rice (*Oryza sativa* L.) plants produced via particle bombardment or intact cell electroporation. Molecular Breeding 4:99-109.

Arencibia AD, Carmona ER, Tellez P, Cahn M-T, Yu S-M, Trujilo LE and Oramas P (1998b) An efficient protocol for sugarcane (*Saccharum spp.* L.) transformation mediated by *Agrobacterium tumefaciens*. Transgenic Research 7:213-222.

Arencibia AD, Carmona ER, Cornide MT, Castiglione S, O'Relly J, Chinea A, Oramas P and Sala F (1999) Somaclonal variation in insect-resistant transgenic sugarcane (*Saccharum* hybrid) plants produced by cell electroporation. Transgenic Research 8:349-360.

Arencibia AD, Carmona E, Cornide MT, Menendez E and Molina P (2000) Transgenic sugarcane (*Saccharum* spp.). In: Biotechnology in agriculture and forestry 46. Transgenic crops 1. Ed: Bajaj SS. Springer, Heidelberg. pp 188-206.

Armitage P, Walden R and Draper J (1988) Vectors for the transformation of plant cell using *Agrobacterium*. In: Plant genetic transformation and gene expression. A laboratory manual. Eds: Draper J, Scott R, Armitage P and Walden R. Blackwell Scientific Publications, Oxford UK. pp 1-68.

Armstrong CL and Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207-214.

Asano Y, Otsuki Y and Ugaki M (1991) Electroporation-mediated and silicon carbide whisker-mediated DNA delivery in *Agrostis alba* L. (Redtop). Plant Science 79:247-252.

Asano Y, Ito Y, Fukami M, Sugiura K and Fujiie A (1998) Herbicide-resistant transgenic creeping bentgrass plants obtained by electroporation using an altered buffer. Plant Cell Reports 17:963-967.

Azhakanandam K, McCabe MS, Power B, Lowe KC, Cocking EC and Davey MR (2000) T-DNA transfer, integration, expression and inheritance in rice: effects of plant genotype and *Agrobacterium* super-virulence. Journal of Plant Physiology 157:429-439.

Bailey RA (1979) An assessment of the status of sugarcane diseases in South Africa. Proceedings of the South African Sugar Technologists Association 53: 120-129.

Bailey RA and Bechet GR (1989) A comparison of seedcane derived from tissue culture with conventional seedcane. Proceedings of the South African Sugar Technologists Association 63:125-129.

Banerji J, Rusconi S and Schaffner W (1981) Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell 27:299-308.

Barandiaran X, Di Pietro A and Martin J (1998) Biolistic transfer and expression of a *uidA* reporter gene in different tissues of *Allium sativum* L. Plant Cell Reports 17:737-741.

Barcelo P, Hagel C, Becker D, Martin A and Lorz H (1994) Transgenic cereal (*tritordeum*) plants obtained at high efficiency of microprojectile bombardment of influorescence tissue. The Plant Journal 5:583-592.

Barnes AC (1974) The sugarcane. 2nd edition. Leonard Hill Books, London UK. pp 572.

Barro F, Cannell ME, Lazzeri PA and Barcelo P (1998) The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants. Theoretical and Applied Genetics 97:684-695.

Baszcyznski CL (1990) A rapid immunoprecipitation assay for neomycin phosphotransferase II expression in transformed bacteria and plant tissues. Biochemical and Cell Biology 68:983-987.

Battraw M and Hall TC (1991) Stable transformation of *Sorghum bicolor* protoplasts with chimaeric neomycin phosphotransferase II and β-glucuronidase genes. Theoretical and Applied Genetics 82:161-168.

Battraw M and Hall TC (1992) Expression of a chimeric neomycin phosphotransferase II gene in first and second generation transgenic rice plants. Plant Scienc 86:191-202.

Baulcombe DC (1996) RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Molecular Biology 32:79-88.

Baulcombe DC, Saunders GR, Bevan MV, Mayo MA and Harrison BD (1986) Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature 321:446-449.

Beachy RN, Loesch S and Turner NE (1990) Coat protein mediated resistance against virus infection. Annual Review of Phytopathology 28:451-455.

Beck E, Ludwig EA, Reiss B and Schaller H (1982) Nucleotide sequence and exact localisation of the neomycin phosphotransferase gene from transposon Tn5. Gene 19:327-336.

Becker D, Brettschneider R and Lorz H (1994) Fertile transgenic wheat from microprojectile bombardment of scuttellar tissue. The Plant Journal 5:299-307.

Beeckman T and Engler G (1994) An easy technique for the clearing of histochemically stained plant tissue. Plant Molecular Biology Reporter 12:37-42.

Bellucci M, Alpini A, Paolocci F, Damiani F and Arcioni S (1999) Transcription of a maize cDNA in *Lotus corniculatus* is regulated by T-DNA methylation and transgene copy number. Theoretical and Applied Genetics 98:257-264.

Benkirane H, Sabounji K, Chlyah A and Chayah H (2000) Somatic embryogenesis and plant regeneration from fragments of immature floescenses and coleoptiles of durum wheat. Plant Cell, Tissue and Organ Culture 61:107-113.

Bennet MD and Smith JD (1976) Nuclear DNA amounts in Angiosperms. Phil Trans Roy Soc London Ser B 274:227-274.

Berding N and Roach RT (1987) Germplasm collection, maintenance and use. In: Developments in Crop Scienceence III. Sugarcane improvement through breeding. Ed: Heinz D. Elsevier, The Netherlands. pp143-210.

Beruto M, Beruto D and Debergh P (1999a) Influence of agar on *in vitro cultures*: I. Physiochemical properties of agar and agar gelled media. In Vitro Cellular and Developmental Biology-Plant 35:86-93.

Beruto M, Curir P and Debergh P (1999b) Influence of agar on *in vitro* cultures: II. Biological performance of *Ranunculus* on media solidified with three different agar brands. In Vitro Cellular and Developmental Biology-Plant 35:94-101.

Bettany AJE, Dalton SJ, Timms E and Morris P (1998) Stability of transgene expression during vegetative propagation of protoplast-derived tall fescue (*Festuca arundinaceae* Schreb.) plants. Journal of Experimental Botany 49:1797-1804.

Bevan MW, Flavell RB and Chilton MD (1983) A chimeric antibiotic resistance marker gene as a selectable marker for plant cell transformation. Nature 304:184-187.

Bhaskaran S and Smith RH (1989) Control of morphogenesis in sorghum by 2,4-dichlorophenoxyacetic acid and cytokinins. Annals of Botany 64:217-224.

Bhaskaran S and Smith RH (1990) Regeneration in cereal tissue culture: a review. Crop Science 30:1328-1337.

Bhau BS and Koul V (1998) Switching on *Bacillus thuringiensis* to reduce selection for resistance. Current Science 75:771-777.

Birch RG (1997) Plant transformation: problems and stategies for practical application. Annual Review of Plant Physiology and Plant Molecular Biology 48:297-326.

Birch RG, Bower R, Elliott A, Basnake S and Zhang L (2000) Regulation of transgene expression: progress towards practical development in sugarcane, and implications for other plant species. In: Plant genetic engineering: towards the third millennium: Proceedings of the international symposium on plant genetic engineering. Havana, Cuba 6-10 December, 2000.

Birch RG and Franks T (1991) Development and optimisation of microprojectile systems for plant genetic transformation. Australian Journal of Plant Physiology 18:453-469.

Biswas GCG, Chen D-F and Elliott MC (1998) A routine system for generation of transgenic rice (*Oryza sativa* L.) plants by microprojectile bombardment of embryogenic cell clusters. Plant Science 133:203-210.

Bommineni VR and Jauhar PP (1997) An evaluation of target cells and tissues used in genetic transformation of cereals. Maydica 42:107-120.

Botterman J and Leemans J (1988) Engineering herbicide resistance in plants. Trends in Genetics 4:219-222.

Boumann H and De Klerk G-J (2001) Measurement of the extent of somaclonal variation in begonia plants regenerated under various conditions. Comparison of three assays. Theoretical and Applied Genetics 102:111-117.

Bowen B (1992) Anthocyanin genes as visual markers in transformed maize tissue. In: GUS protocols: using the GUS gene as a reporter of gene expression. Ed: Galagher S. Academic Press, New York. pp 163-177.

Bower R and Birch RG (1992) Transgenic sugarcane plants via microprojectile bombardment. The Plant Journal 2:409-416.

Bower R, Elliott AR, Potier BAM and Birch RG (1996) High-efficiency, microprojectile-mediated cotransformation of sugarcane, using visible or selectable markers. Molecular Breeding 2:239-249.

Bregitzer P, Campbell RD and Wu Y (1995) Plant regeneration from barley callus: effcts of 2,4-dichlorophenoxyacetic acid and phenylacetic acid. Plant Cell, Tissue and Organ Culture 43:229-235.

Bregitzer P, Dahleen LS and Campbell RD (1998a) Enhancement of regeneration from embryogenic callus of commercial barley cultivars. Plant Cell Reports 17:941-945.

Bregitzer P, Halbert SE and Lemaux PG (1998b) Somaclonal variation in the progeny of transgenic barley. Theoretical and Applied Genetics 96:421-425.

Breiman A, Felsenburg T and Galun E (1987) *Nor* loci analysis in progenies of plants regenerated from the scutellar callus of bread-wheat. A molecular approach to evaluate somaclonal variation. Theoretical and Applied Genetics 73:827-831.

Bremer G (1961) Problems in breeding and cytology of sugarcane. IV The origin of the increase of chromosome number in species hybrids *Saccharum.* Euphytica 10:325-342.

Brett PGC (1974) Early experiments on the artificial induction of flowering at Mount Edgecombe. Proceedings of the South African Sugar Technologists Associationoc 48:78-79.

Brettschneider R, Backer D and Lorz H (1997) Efficient transformation of scutellar tissue of immature maize embryos. Theoretical and Applied Genetics 94:737-748.

Brisibe EA, Nishioka D, Miyake H, Taniguchi T and Maeda E (1993) Developmental electron microscopy and hitochemistry of somatic embryo differentiation in sugarcane. Plant Science 89:85-92.

Brisibe EA, Miyake H, Taniguchi T and Maeda E (1994) Regulation of somatic embryogenesis in long-term callus cultures of sugarcane (*Saccharum officinarum* L.). New Phytologist126:301-307.

Brisibe EA, Gajdosova, Olesen A and Anderson SB (2000) Cytodifferentiation and transformation of embryogenic callus lines derived from anther culture of wheat. Journal of Experimental Botany 51:187-196.

Brown DCW, Tian L, Buckley DJ, Lefebvre M, McGrath A and Webb J (1994) Development of a simple particle bombardment device for gene transfer into plant cells. Plant Cell, Tissue and Organ Culture 37:47-53.

Buckmann H, Petersen J, Schlinker G and Marlander B (2000) Weed control in genetically modified sugarbeet-two year experiences in a field trial series in Germany. Z PflKrankh PflSchutz, Sonderh XVII:353-362.

Burner DM and Legendre BL (1994) Cytogenetic and fertility charcateristics of elite sugarcane clones. Sugar Cane 1:6-10.

Bull TA and Tovey DA (1974) Aspects of modelling sugarcane growth by computer simulation. Proceedings of the International Society of Sugar Cane Technologists 15:1021-1032.

Burnside OC (1992) Rationale for developing herbicide-resistant crops. Weed Technology 6:621-625.

Butterfield MK. Plant Breeding Department, SA Sugar Association Experiment Station, P/B X02, Mount Edgecombe, 4300

Butterfield MK and Thomas DW (1996) Sucrose, yield and disease resistance characteristics of sugarcane varieties under test in the SASEX selection programme. Proceedings of the South African Sugar Technologists Association 70:103-105.

Cachita-Cosma D (1991) The effect of the nature and origin of explants on micropropagation. In: Biotechnology in agriculture and forestry, Vol 17. Ed: Y.P.S. Bajaj. Springer-Verlag, Heidelberg, Germany, pp143-166.

Cannell ME, Doherty A, Lazzeri PA and Barcelo P (1999) A population of wheat and tritordeum transformants showing a high degree of marker gene stability and heritability. Theoretical and Applied Genetics 99:772-784.

Cao J, Duan X, McElroy D and Wu R (1992) Regeneration of herbicide resistant rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Reports 11:586-591.

Carlson AR, Letarte J, Chen J and Kasha KJ (2001) Visual screening of microspore-derived transgenic barley (*Hordeum vulgare* L.) with green-flourescent protein. Plant Cell Reports 20:331-337.

Cary JW, Rajasekaran K, Jaynes JM and Cleveland TE (2000) Transgenic expression of a gene encoding a synthetic antimicrobial peptide results in inhibition of fungal growth *in vitro* and in planta. Plant Science 154:171-181.

Castillo AM, Vasil V and Vasil IK (1994) Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). Bio/Technology 12:1366-1371.

Chamberlain DA, Brettell RIS, Last DI, Witrzens B, McElroy D, Dolferus R and Dennis ES (1994) The use of the Emu promoter with antibiotic and herbicide resistance genes for the selection of transgenic wheat callus and rice plants. Australian Journal of Plant Physiology 21:95-112.

Chan MT, Chang HH, Ho SL, Tong WF and Yu SM (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. Plant Molecular Biology 22:491-506.

Chapman A, Blervacq A-S, Tissier J-P, Delbreil B, Vasseur J and Hilbert J-L (2000) Cell wall differentiation during early somatic embryogenesis in plants. 1. Scanning and transmission electron microscopy study on embryos originating from direct, indirect and adventitious pathways. Canadian Journal of Botany 78:816-823.

Chareonpornwattana S, Thara KV, Wang L, Datta SK, Panbangred W and Muthukrishnan (1999) Inheritance, expression, and silencing of a chitinase transgene in rice. Theoretical and Applied Genetics 98:371-378.

Chawla HS, Cass LA and Simmonds JA (1999) Development and environmental regulation of anthocyanin pigmentation in wheat tissues transformed with anthocyanin regulatory genes. In Vitro Cellular and Developmental Biology-Plant 35:403-408.

Chen JT, Chang C and Chang WC (1999) Direct somatic embryogenesis on leaf explants of *Oncidium* Gower Ramsdey and subsequent planst regeneration. Plant Cell Reports 19:143-149.

Chen L, Marmey P, Taylor NJ, Brizard J-P, Espinoza C, D'Cruz P, Huet H, Zhang S, de Kochko A, Beachy RN and Fauquet CM (1998a) Expression and inheritance of multiple transgenes in rice plants. Nature Biotechnology 16:1060-1064.

Chen L, Zhang S, Beachy RN and Faquet CM (1998b) A protocol for consistent, large-scale production of fertile transgenic rice plants. Plant Cell Reports 18:25-31.

Chen WH, Gartland KMA, Davey MR, Sotak R, Gartland JS, Mulligan BJ, Power JB and Cocking EC (1987) Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. Plant Cell Reports 6:297-301.

Chen WH, Davey MR, Power JB and Cocking EC (1988a) Control and maintenance of plant regeneration in sugarcane callus cultures. Journal of Experimental Botany 39:251-261.

Chen WH, Davey MR, Power JB and Cocking EC (1988b) Sugarcane protoplasts: factors affecting division and plant regeneration. Plant Cell Reports 7:344-347.

Cheng M.et al. (1997) Genetic transformation of wheat mediated by *Agrobacterium* tumefaciens. Plant Physiology 115:971-980.

Chengalrayan K and Gallo-Meagher M (2001) Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cellular and Developmental Biology-Plant 37:434-439.

Chibbar RN, Kartha KK, Leung N, Qureshi J and Caswell K (1991) Transient expression of marker genes in immature zygotic embryos of spring wheat (*Triticum aestivum*) through microprojectile bombardment. Genome 34:453-460.

Christensen AH and Quail P (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Research 5:13-18.

Christou P (1990) Morphological description of transgenic soybean chimeras created by delivery, integration and expression of foreign DNA using electric discharge particle acceleration. Annals of Botany 66:379-386.

Christou P (1993a) Philosophy and practice of variety-independent gene transfer into recalcitrant crops. In Vitro Cellular Developmental Biology-Plant 29:119-124.

Christou P (1993b) Particle gun mediated transformation. Current Opinions in Biotechnology 4:135-141.

Christou P (1995) Strategies for variety independent genetic transformation of important cereals, legumes and woody species utilising particle bombardment. Euphytica 85:13-27.

Christou P (1996) Transformation technology. Trends in Plant Science 1:423-431.

Christou P, Ford TL and Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9:957-962.

Christou P, Ford T and Kofron M (1992) The development of a variety-independent gene transfer method for rice. Trends in Biotechnology 10:239-246.

Cho M-J, Choi HW and Lemaux (2001) Transformed To orchardgrass (*Dactylis glomerata* L.) plants produced from highly regenerative tissues derived from mature seeds. Plant Cell Reports 20:318-324.

Cho M-J, Ha CD and Lemaux PG (2000) Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly regenerative tissues. Plant Cell Reports 19:1084-1089.

Cho M-J, Jiang W and Lemaux PG (1998) Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. Plant Science 138:229-244.

Choi C-H, Lamaux PG and Cho M-J (2000a) Increased chromosomal variation in transgenic versus nontransgenic barley (*Hordeum vulgare* L.) plants. Crop Science 40:524-533.

Choi C-H, Lamaux PG and Cho M-J (2000b) High frequency of cytogenetic aberration in transgenic oat (*Avena sativa* L.) plants. Plant Science 156:85-94.

Chowdhury MKU and Vasil IK (1992) Stably transformed herbicide resistant callus of sugarcane microprojectile bombardment of cell suspension cultures and electroporation of protoplasts. Plant Cell Reports 11:494-498.

Conger BV, Hanning GE, Gray DJ and McDaniel JK (1983) Direct embryogenesis from mesophyll cells of orchardgrass. Science 221:850-851.

Conkling MA, Cheng C-L, Yamamoto YT and Goodman HM (1990) Isolation of transcriptionally regulated root-specific genes from tobacco. Plant Physiology 93:1203-1211.

Cooley J, Ford T and Christou P (1995) Molecular and genetic characterisation of elite transgenic rice plants produced by electric-discharge particle acceleration. Theoretical and Applied Genetics 90:97-104.

Cornelissen BJC and Melchers LS (1993) Strategies for control of fungal diseases with transgenic plants. Plant Physiology 101:709-712.

Covey SN and Al-Kaff NS (2000) Plant DNA viruses and gene silencing. Plant Molecular Biology 43:307-322.

Dahleen LS, Okubara PA and Blechl AE (2001) Transgenic approaches to combat Fusarium head blight in wheat and barley. Crop Science 41:628-637.

Dalton SJ, Bettany AJE, Trimms E and Morris P (1995) The effect of selection pressure on transformation frequency and copy number in transgenic plants of tall fescue (*Festuca arundinacea* Schreb.). Plant Science 108:63-70.

Dalton SJ, Bettany AJE, Trimms E and Morris P (1998) Transgenic plants *of Lolium Multiflorum, Lolium perenne, Festuca arundinaceae* and *Agrostis stolonifera* by silicon carbide fibre-mediated transformation of cell suspension cultures. Plant Science 132:31-43.

Daniell H, Streatfield SJ and Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends in Plant Science 6:219-226.

Daniell H, Datta R, Varma S, Gray S and Lee S-B (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nature Biotechnology 16:345-348.

Daniels J and Roach BT (1987) Taxonomy and Evolution. In: Developments in Crop Science II. Sugarcane improvement through breeding. Ed:Heinz DJ. Elsevier, Amsterdam. pp 7-84

Datta K, Potrykus I and Datta SK (1992a) Efficient fertile plant regeneration from protoplasts of the Indica rice breeding line IR72 (*Oryza sativa* L.). Plant Cell Reports 11:229-233.

Datta SK, Datta K, Soltanifar G, Donn G and Potrykus I (1992b) Herbicide resistant indica rice plants from IRRI breeding line ER72 after PEG-mediated transformation of protoplasts. Plant Molecular Biology 20:619-629.

Dawson WO (1996) Gene silencing and virus resistance: a common mechanism. Trends in Plant Science 1:107-108.

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gossele V, Rao Movva N, Thompson C, Van Montagu M and Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO 6:2513-2518.

De Block M, Debrouwer D and Tenning P (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium* and the expression of the *bar* and *neo* genes in transgenic plants. Plant Physiology 91:694-701.

De Block M, De Sonville A and Debrouwer D (1995) The selection mechanism of phosphinothricin is influenced by metabolic status of the tissue. Planta 197:619-626.

De Greef W, Delon R, de Block M, Leemans J and Botterman J (1989) Evaluation of herbicide resistance in transgenic crops under field conditions. Bio/Technology 7:61-63.

D'Hont A, Grivet L, Feldman P, Rao S, Berding N and Glaszmann JC (1996) Characterisation of the double genome structure of modern sugarcane cultivars (*Saccharum* spp.) by molecular genetics. Moleular and General Genetics 250:405-413.

D'Hont A, Ison D, Alix K, Roux C and Glaszmann JC (1998) Determination of basic chromosome numbers in the genus Saccharum by physical mapping of ribosomal RNA genes. Genome 41:221-225.

De los Blanco M, Nieves N, Sanchez M, Borroto CG, Castillo R, Gonzales JL, Escalona M, Baez E and Hernandez Z (1997) Protein changes associated with plant regeneration in embryogenic calli of sugarcane (*Saccharum* spp.). Plant Cell, Tissue and Organ Culture 51:153-158.

De la Pena A, Lorz H and Schell J (1987) Transgenic rye plants obtained by injecting DNA into young floral tillers. Nature 325:274-276.

Dellaporta SJ, Wood J and Hicks JB (1985) Maize DNA miniprep. In: Molecular Biology of plants: A laboratory manual. Eds: Malmsberg R, Messing J and Sussex I. Cold Spring Harbour Press, New York. pp 36-37.

De Jong AJ, Schmidt EDL and de Vries SC (1993) Early events in higher plant embryogenesis. Plant Molecular Biology 22:367-377.

Dekeyser RB, Claes M, Marichal M, van Montagu M and Caplan A (1989) Evaluation of selectable markers for rice transformation. Plant Physiology 90:217-223.

Dekeyser RA, Claes B, De Rycke RMU, Habets ME, van Montagu M and Caplan A (1990) Transient expression in intact and organised rice tissues. Plant Cell 2:591-602.

De Maagd RA, Bosch D and Stiekema W (1999) *Bacillus thuringiensis* toxin-mediated insect resistance in plants. Trends in Plant Science 4:9-13.

Denchev PD, Songstad DD, McDaniel JK and Conger BV (1997) Transgenic orchardgrass (*Dactylis glomerata*) plants by direct embryogenesis from microprojectile bombarded leaf cells. Plant Cell Reports 16:813-819.

Dennehey BK, Petersen WL, Ford-Santino C, Pajeau M and Armstrong CL (1994) Comparison of selective agents for use with the selectable marker gene *bar* in maize transformation. Plant Cell, Tissue and Organ Culture 36:1-7.

D'Halluin K, Bossut M, Bonne E, Mazur B, Leemans J and Botterman J (1992) Transformation of sugarbeet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Bio/Technology 10:309-314.

Dodeman VL, Ducreux G and Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. Journal of Experimental Botany 48:1493-1509.

Dong J, Teng W, Buchholz WG and Hall TC (1996) *Agrobacterium*-mediated transformation of *Japonica* rice. Molecular Breeding 2:267-276.

Dougherty WG, Lindbo JA, Smith HA, Parks TD, Swaney S and Proebsting WM (1994) RNA-mediated virus resistance in transgenic plants: exploitation of a cellular pathway possibly involved in RNA degradation. Molecular and Plant-Microbe Interactions 7:544-552.

Draper J and Scott R (1988) The isolation of plant nucleic acids. In: Plant genetic transformation and gene expression. A laboratory manual. Eds: Draper J, Scott R, Armitage P and Walden R. Blackwell Scientific Publications, Oxford UK. pp 199-236.

Duncan DR, Williams ME, Zehr BE and Widholm JM (1985) The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. Planta 165:322-332.

Dupuis I and Page GM (1993) Gene transfer to maize male reproductive structure by particle bombardment of tassel primordia. Plant Cell Reports 12:607-611.

Dunstan DI, Short KC and Thomas E (1978) The anatomy of secondary morphogenesis in cultured scutellum tissues of *Sorghum bicolor* (L.). Protoplasma 97:251-260.

Dunwell JM (1999) Transgenic crops: the next generation, or an example of 2020 vision. Annals of Botany 84:269-277.

Elliot AR, Campbell JA, Brettell RIS and Grof CPL (1998) *Agrobacterium*-mediated transformation of sugarcane using GFP as a screenable marker. Australian Journal of Plant Physiology 25:739-743.

Endo S, Kasahara T, Sugita K, Matsunaga E and Ebinuma H (2001) The isopentenyl transferase gene is effective as a selectable marker gene for plant transformation in tobacco (*Nicotiana tabacum* cv. Petite Havana SRI). Plant Cell Reports 20:60-66.

English JJ, Mueller E and Balcombe DC (1996) Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell 8:179-188.

Enriquez-Obregon GA, Vazquez-Padron RI, Prieto-Samsonov DL, de la Riva GA, Selman-Housein G (1998) Herbicide resistant sugarcane plants by *Agrobacterium*-mediated transformation. Planta 206:20-27.

Escudero J, Neuhaus G, Schlappi M and Hohn B (1996) T-DNA transfer in meristematic cells of maize provided with intracellular *Agrobacterium*. The Plant Journal 10:355-360.

Estruch JJ, Carozzi NB, Desai N, Duck NB, Warren GW and Koziel MG (1997) Transgenic plants: an emerging approach to pest control. Nature Biotechnology 15:137-141.

Evans DA, Sharp WR and Flick CE (1981) Growth and behaviour of cell cultures: embryogenesis and organogenesis. In: Plant tissue culture: methods and applications. Ed: Thorpe TA. Academic Press, New York. pp 43-113.

Fagard M and Vaucheret H (2000) (Trans)gene silencing in plants: how many mechanisms? Annual Review of Plant Physiology and Plant Molecular Biology 51:167-194.

Falco MC, Tulmann Neto A and Ulian EC (2000) Transformation and expression of a gene for herbicide resistance in Brazilian sugarcane. Plant Cell Reports 19:1188-1194.

Finer JJ, Vain P, Jones MW and McMullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Reports 11:323-328.

Finnegan J and McElroy D (1994) Transgene inactivation: plants fight back! Bio/Technology 12:883-888.

Fisk HJ and Dandekar AM (1993) The introduction and expression of transgenes in plants. Scienta Horticulturae 55:5-36.

Fitch MM and Moore PH (1990) Comparison of 2,4-D and picloram for selection of long-term totipotent green callus cultures of sugarcane. Plant Cell, Tissue and Organ Culture 20:157-163.

Fitch MM and Moore PH (1993) Long-term culture of embryogenic sugarcane callus. Plant Cell, Tissue and Organ Culture 32:335-343.

Fitchen JH and Beachy RN (1993) Genetically engineered protection against viruses in transgenic plants. Annual Review of Microbiology 47:739-763.

Folling L and Olesen A (2001) Transformation of wheat (*Triticum aestivum* L.) microspoe-derived callus and microspores by particle bombardment. Plant Cell Reports 20:629-636.

Frame BR, Drayton PR, Bagnal SV et al (1994). Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. The Plant Journal 6:941-948.

Frame BR, Zhang H, Cocciolone SM, Sidorenko LV, Dietrich CR, Pegg SE, Zhen S, Schnable PS and Wang K (2000) Production of transgenic maize from bombarded Type II callus: effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cellular and Developmental Biology-Plant 36:21-29.

Franks T and Birch RG (1991) Gene transfer to intact sugarcane cells using microprojectile bombardment. Australian Journal of Plant Physiology 18:471-480.

Freyssinet G and Cole DJ (1999) Herbicide tolerance in crops: a commercial reality. In: plant biotechnolgy and *in vitro* biology in the 21st century. Eds: Altman A, Ziv M and Izhar S.Kluwer Academic Publishers The Netherlands. pp 481-485.

Fromm ME, Taylor LP and Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. Nature 319:791-793.

Fromm ME, Morrish F, Armstrong C, Williams R and Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technology 8:833-839.

Funatsaki H, Kuroda M, Kihara PA, Muller E, Lorz H and Kishinami I (1995) Fertile transgenic barley generated by direct DNA transfer to protoplasts. Theoretical and Applied Genetics 91:707-712.

Futterer J and Portykus I (1995) Transformation of Poaceae and gene expression in transgenic plants. Agronomie 15:309-319.

Gaj MD (2001) Direct somatic embryogenesis as a rapid and efficient system for *in vitro* regeneration of *Arabidopsis thaliana*. Plant Cell, Tissue and Organ Culture 64:39-46.

Gallo-Meagher M and Irvine JE (1993) Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment, Plant Cell Reports 12:666-670.

Gallo-Meagher M and Irvine JE (1996) Herbicide resistant sugarcane containing the *bar* gene. Crop Science 36:1367-1374.

Gallo-Meagher M, English RG and Abouzid A (2000) Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. In Vitro Cellular and Developmental Biology-Plant 36:37-40.

Gambley RL, Ford R and Smith GR (1993) Microprojectile transformation of sugarcane meristems and regeneration of shoots expressing β-glucuronidase. Plant Cell Reports 12:343-346.

Gambley RL, Bryant JD, Masel NP and Smith GR (1994) Cytokinin-enhanced regeneration of plants from microprojectile bombarded sugarcane meristematic tissue. Australian Journal of Plant Physiology 21:603-612.

Gamborg L, Miller RA and Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151-158.

Gao A-G, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J and Rommens CMT (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nature Biotechnology 18:1307-1310.

Garside AL (1997) Yield decline research in the Australian sugar industry. Proceedings of the South African Sugar Technologists Association 71:3-8.

Garside AL, Bell MJ and Magarey RC (2001) Monoculture yield decline-fact not fiction. Proceedings of the International Society of Sugar Cane Technologists 24:16-21.

Gatz C and Lenk I (1998) Promoters that respond to chemical inducers. Trends in Plant Science 3:352-358.

Gelvin S (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annual Review of Plant Physiology and Plant Molecular Biology 51:223-256.

George EF (1993) Plant propagation by tissue culture. Part 1: The Technology. 2nd Edition. Butler and Tanner, London. p 574.

Giddings G, Allison G, Brookes D and Carter A (2000) Transgenic plants as factories for biopharmaceuticals. Nature Biotechnology 18:1151-1155.

Gless C, Lorz H and Jahne-Gartner J (1998) Transgenic oat plants obtained at high efficiency by micrprojectile bombardment of leaf base segments. Journal of Plant Physiology 152:151-157.

Gonzales RA and Franks E (1987) Induction of morphogenetically competent callus and suspension cell cultures from leaf explants and mature seeds of *Digitaria sanguinalis*. In Vitro Cellular and Developmental Biology-Plant 23:581-584.

Goodwin J, Chapman K, Swaney S, Parks TD, Wernsman EA and Dougherty WG (1996) Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. The Plant Cell 8:95-105.

Gopalakrishnan S, Garg GK, Singh DT and Singh NK (2000) Herbicide-tolerant plants in high yielding commercial wheat cultivars obtained by microprojectile bombardment and selection on Basta. Current Science 79:1094-1100.

Gordon-Kamm WT, Spencer ML, Mangano ML, Adams TR, Daines RJ, Tar, WG, O'Brien JV, Chambers SA, Adams WR, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP and Lemaux PG (1990) Transformation of maize cells and regeneration of fertile maize plants. Plant Cell 2:603-618.

Gould J, Devey M, Hasegawa O, Ulian EC, Peterson G and Smith RH (1991) Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot tip. Plant Physiology 95:426-434.

Graves AE, Goldman SL, Banks SW and Graves ACF (1988) Scanning electron microscope studies of *Agrobacterium tumefaciens* attachment to *Zea mays*, *Gladiolus* sp. and *Triticum aestivum*. Journal of Bacteriology 170:2395-2400.

Green CE and Phillips RL (1975) Plant regeneration from tissue cultures of maize. Crop Science 15:417-421.

Grimsley NH (1990) Agroinfection. Physiologia Plant 79:147-153.

Grimsley N, Hohn T, Davies JW and Hohn B (1987) *Agrobacterium* mediated delivery of infectious maize streak virus into maize plants. Nature 325:177-179.

Grisham MP and Bourg D (1989) Efficiency *of in vitro* propagation of sugarcane plants by direct regeneration from leaf tissue and by shoot- tip culture. Journal of the American Society of Sugarcane Technologists 9:97-102.

Grof CPL (2001) Molecular manipulation of sucrose metabolism. Proceedings of the International Society of Sugar Cane Technologists 24:586-587.

Grof CPL and Campbell JA (2001) Sugarcane sucrose metabolism: scope for molecular manipulation. Australian Journal of Plant Physiology 28:1-12.

Guiderdoni E and Demarly Y (1988) Histology of somatic embryogenesis in cultured leaf segments of sugarcane plants. Plant Cell, Tissue and Organ Culture 14:71-88.

Gupta HS and Pattanayak A (1993) Plant regeneration from mesophyll protoplasts of rice (*Oryza sativa* L.). Bio/Technology 11:90-94.

Ha S-B (2000) Transgenic tall fescue (*Festuca arundinacea*). In: Biotechnology in agriculture and forestry 46. Transgenic crops 1. Ed: Bajaj YPS. Springer, Heidelburg. pp165-171.

Hadi MZ, McMullen MD and Finer JJ (1996) Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Reports 15:500-505.

Haldrup A, Noerremark M and Okkels FT (2001) Plant selection principle based on xylose isomerase. In Vitro Cellular and Developmental Biology-Plant 37:114-119.

Hanna WW, Lu C and Vasil IK (1984) Uniformity of plants regenerated from somatic embryos of *Pannicum maximum* Jacq. (Giunea grass). Theoretical and Applied Genetics 67:155-159.

Hanning GE and Conger BV (1986) Factors influencing somatic mebryogenesis from cultured leaf segments of *Dactylis glomerata*. Journal of Plant Physiology 123:23-29.

Hansen G and Wright M (1999) Recent advances in the transformation of plants. Trends in Plant Science. 4: 226-231.

Hansom S, Bower R, Zhang L, Potier B, Elliott A, Basnayke S, Cordeiro G, Hogarth DM, Cox M, Berding N and Birch RG (1999) Regulation of transgene expression in sugarcane. International Society of Sugar Cane Technologists, India pp22-33.

Hansch R, Mendel RR and Schulze J (1998) A rapid and sensitive method to evaluate genotype specific tolerance to phosphinothricin-based selective agents in cereal transformation. Journal of Plant Physiology 152:145-150.

Harrison D, Waldron J, Ramage C, Peace C, Cox M, Birch RG and Carroll BJ (2001) New DNA technologies provide insights into the molecular basis of somaclonal variation in sugarcane. Proceedings of the International Sugar Cane Technologists, Australia 24:580-582.

Hartke S and Lorz HJ (1989) Somatic embryogenesis and plant regeneration from various indica rice (*Oryza sativum* L.) genotypes. Journal of Genetics and Breeding 43:205-214.

Hartman CL, Lee L, Day PR and Tumer NE (1994) Herbicide resistant turfgrass (*Agrostis palustris* Huds.) by biolistic tramsformation. Bio/Technology 12:919-923.

Harwood WA, Ross SM, Cilento P and Snape JW (2000) The effect of DNA/gold particle preparation technique, and particle bombardment device, on the transformation of barley (*Hordeum vulgare*). Euphytica 111:67-76.

Haseloff J, Siemering KR, Prasher DC and Hodge S (1997) Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proceedings of the National Acadamy of Science USA 94:2122-2127.

Hathway DE (1989) Improved resistance through genetic engineering. In: Molecular mechanisms of herbicide selectivity. Oxford University Press, England. pp146-158.

Hauptmann RM, Vasil V, Ozias-Akinns P, Tabaeizadeh Z, Rogers SG, Fraley RT, Horsch RB and Vasil IK (1988) Evaluation of selectable markers for obtaining stable transformants in the Gramineae. Plant Physiology 86:602-606.

He DG, Yang YM, Bertram J and Scott KJ (1990) The histological development of the regenerative tissue derived from cultured immature embryos of wheat (*Triticum sativum* L.). Plant Science 68:103-111.

He DG, Mouradov A, Yang YM, Mouradova E and Scott KJ (1994) Transformation of wheat (*Triticum aestivum* L.) through electroporation of protoplasts. Plant Cell Reports 14:193-196.

He GY, Lazzeri and Cannell ME (2001) Fertile transgenic plants obtained from tritordeum inflorescences by tissue electroporation. Plant Cell Reports 20:67-72.

Heinz DJ (1987) Sugarcane improvement through breeding. Elsevier, The Netherlands. pp1-6.

Heinz DJ and Mee GWP (1969) Plant differentiation from callus tissue of *Saccharum* species. Crop Science 9:346-348.

Heinz DJ, Krishnamurthi M, Nickell LG and Maretzki A (1977) Cell, tissue and organ culture in sugarcane improvement. In: Applied and fundamental aspects of plant cell, tissue and organ culture. Eds: Reinert J and Bajaj YPS. Springer, Berlin. pp3-17.

Henderson L, Rao AG and Howard J (1990) An immunoaffinity immobilised enzyme assay for neomycin phosphotransferase II in crude cell extracts. Annals of Biochemistry 194:64-68.

Hernalsteens JP, Van Vliet F, Beuckeleer MD, Depicker A, Engler G, Lemmers M, Holsters M, Van Montagu M and Schell J (1980) The *Agrobacterium tumefaciens* Ti plasmid as a host vector system for introducing foreign DNA in plant cells. Nature 287:654-656.

Herrera-Estrella L, De Block M, Messens E, Hernalsteens J-P, Van Montagu M and Schell J (1983a) Chimeric genes as dominant selectable markers in plants. EMBO J 2:987-995.

Herrera-Estrella LA, Depicker A, Van Montague M and Schell J (1983b) Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303:209-213.

Hiei Y, Ohta S, Komari T and Kumashiro,T (1994) Efficient transformation of rice *(Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant Journal 6:271-282.

Hiei Y, Komari T and Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens.* Plant Molecular Biology 35:205-218.

Higgs DC and Colbert JT (1993) β-glucuronidase gene expression and mRNA stability in oat protoplasts. Plant Cell Reports 12:445-452.

Ho W-J and Vasil IK (1983) Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) 1. The morphology and physiology of callus formation and the ontogeny of somatic embryos. Protoplasma 118:169-180.

Hobbs SAL, Warkentin TM and DeLong CMO (1993) Transgenic copy number can be positively or negatively associated with transgene expression. Plant Molecular Biology 21:17-26.

Hohn B, Koukolilova-Nicola Z, Bakkeren G and Grimsley N (1989) *Agrobacterium*-mediated gene transfer to monocots and dicots. Genome 31:987-991.

Hodal L, Bochardt A, Nielsen JE, Mattsson O and Okkels FT (1992) Detection, expression and specific elimination of endogenous β-glucuronidase activity in transgenic and non-transgenic plants. Plant Science 87:115-122.

Holmes-Davis R and Comai L (1998) Nuclear matrix attachment regions and plant gene expression. Trends in Plant Science 3:91-96.

Horn ME, Shillito RD, Conger BV and Harms CT (1988) Transgenic plants of orchardgrass (*Dactylis glomerata* L.) from protoplasts. Plant Cell Reports 7:469-472.

Horn R, Brahm L and Friedt W (1996) Recombination: novel gene and genome combinations for resistance breeding by interspecific hybridisation and genetic transformation. Progress in Botany 57:177-196.

Horsch RB, Fry JE, Hoffmann N, Eicholz D, Rogers SG and Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229-1231.

Horvath H, Jensen LG, Wong OT, Kohl E, Ullrich SE, Cochran J, Kannangara CG and von Wettstein D (2001) Stability of transgene expression, field performance and recombination breeding of transformed barley lines. Theoretical and Applied Genetics 102:1-11.

Ibaraki Y, Matsushima R and Kurata K (2000) Analysis of morphological changes in carrot somatic embryogenesis by serial observation. Plant Cell, Tissue and Organ Culture 61:9-14.

Iglesias VA, Gisel A, Bilang R, Ledue N, Potrykus I and Sautter C (1994) Transient expression of different marker genes in meristem cells of wheat immature embryos after ballistic microtragetting. Planta 192:84-91.

Iida A, Nagasawa A and Oeda K (1995) Positive and negative cis-regulatory elements in the soybean glycinin promoter identified by quantitative transient gene expression. Plant Cell Reports 14:539-544.

Ingelbrecht I, van Houdt H, Montagu MV and Depicker A (1994) Post-transcriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proceedings of the National Acadamy of Science USA 91:10502-10506.

Ingelbrecht IL, Irvine JE and Mirkov TE (1999) Pottranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploid genome. Plant Physiology 119:1187-1197.

Irvine JE (1984) The frequency of marker changes in sugarcane plants regenerated from callus culture. Plant Cell, Tissue and Organ Culture 3:201-209.

Irvine JE (1999) Saccharum species as horticultural classes. Theoretical and Applied Genetics 98:186-194.

Irvine JE and Benda GTA (1985) Sugarcane mosaic virus in plantlets regenerated from diseased leaf tissue. Plant Cell, Tissue and Organ Culture 5:101-106.

Irvine JE and Benda GTA (1987) Transmission of sugarcane diseases in plants derived by rapid regeneration of diseased leaf tissue. Sugar Cane 6:14-16.

Irvine JE, Benda GTA, Legendre BL and Machado GR (1991) The frequency of maker changes in sugarcane plants regenerated from callus culture. II. Evidence for vegetative and genetic transmission, epigenetic effects and chimeral disruption. Plant Cell, Tissue and Organ Culture 26:115-125.

Irvine JE, Fitch M and Moore PH (1983) The induction of callus in sugarcane tissue cultures by selected chemicals. Plant Cell, Tissue and Organ Culture 2:141-149.

Iser M, Fettig S, Scheyhing F, Viertel K and Hess D (1999) Genotype-dependent stable genetic transformation in German spring wheat varieties selected for high regeneration potential. Journal of Plant Physiology 154:509-516.

Ishida, Y et al. (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnology 14:745-750.

Iyer LM, Kumpatla SP, Chandrasekharan MB and Hall TC (2000) Transgene silencing in monocots. Plant Molecular Biology 43:323-346.

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J and Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. The Plant Journal 8:97-109.

Jahne A, Becker D, Brettschneider R and Lorz H (1994) Regeneration of transgenic, microspore derived, fertile barley. Theoretical and Applied Genetics 89:525-533.

Jahne A, Becker D and Lorz H (1995) Genetic engineering of cereal crop plants: a review. Euphytica 85:35-44.

Jain SM (2001) Tissue culture-derived variation in crop improvement. Euphytica 118:153-166.

Jaligot E, Rival A, Beule T, Dussert S and Verdeil J-L (2000) Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. Plant Cell Reports 19:684-690.

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS fusion system. Plant Molecular Biology Reporter 5:387-405.

Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO 6:3901-3907.

Jelenska J, Tietze E, Temp J and Brevet J (2000) Streptothricin resistance as a novel selectable marker for transgenic plant cells. Plant Cell Reports 19:298-303.

Jiang J, Linsconbe SD, Wang J and Oard JH (2000) High efficiency transformation of US rice lines from mature seed-derived calli and segragation of glufosinate resistance under field conditions. Crop Science 40:1729-1741.

Jiang W, Cho M-J and Lemaux PG (1998) Improved callus quality and prolonged regenerability in model and recalcitrant barley (*Hordeum vulgare* L.) cultivars. Plant Biotechnology 15:63-69.

Joersbo M and Okkels FT (1996) A novel principle for selection of transgenic plant cells: positive selection. Plant Cell Reports 16:219-221.

Joersbo M, Donaldson I, Kreiberg J, Petersen SG, Brunstedt J and Okkels FT (1998) Analysis of mannose selection used for transformation of sugarbeet. Molecular Breeding 4:111-117.

Johansen LK and Carrington JC (2001) Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. Plant Physiology 126:930-938.

Johnson E (1996) Edible plant vaccines. Nature Biotechnology 14:1532-1533.

Jones TJ and Rost T (1989) The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Botanical Gazette 150:41-49.

Jorgensen RA, Cluster P, Que Q, English J and Napoli C (1996) Chalcone synthase cosuppression phenotypes in Petunia flowers: comparison of sense vs. antisense constructs and single copy vs. complex T-DNA sequences. Plant Molecular Biology 31:957-973.

Joyce PA, McQualter RB, Handley JA, Dale JL, Harding RM and Smith GR (1998) Transgenic sugarcane resistant to sugarcane mosaic virus. Proceedings of the Australian Society of Sugar Cane Technologists 20:204-210.

Kaeppler HF, Carlson AR and Menon GK (2001) Routine utilization of green fluorescent protein as a visual selectable marker for cereal transformation. In Vitro Cellular and Developmental Biology-Plant 37:120-126.

Kaeppler HF, Somers DA, Rines HW and Cockburn AF (1992) Silicon carbide fiber-mediated stable transformation of plant cells. Theoretical and Applied Genetics 84:560-566.

Karp A (1995) Somaclonal variation as a tool for crop improvement. Euphytica 85:295-302.

Karp A, Steele SH, Paramr S, Jones HGK, Shwery PR and Breiman A (1987) Relative stability among barley plants regenerated from cultured immature embryos. Genome 29:405-412.

Kasschau KD and Carrington JC (1998) A counterdefensive strategy of plant viruses: suppression of post-transcriptional gene silencing. Cell 95:561-470.

Kavanagh TA and Spillane C (1995) Strategies for engineering virus resistance in transgenic plants. Euphytica 85:149-158.

Kempken F (2001) Plant biotechnology: transgenic crops for the third millennium. In: Progress in Botany Vol 62. Eds: Esser K, Luttge U, Kadereit J and Beyschlag W. Springer-Verlag, Heidelberg. pp114-139.

Kemper EL, da Sinva MJ and Arruda P (1996) Effect of microprojectile bombarment parameters and osmotic treatment on particle penetration and tissue damage in transiently transformed cultured immature maize (*Zea mays* L.) embryos. Plant Science 121:85-93.

Kim JK, Cao J and Wu R (1992) Regulation and interaction of multiple protein factors with the proximal promoter regions of a rice high pI α-amylase gene. Moleular and General Genetics 232:383-393.

Kishore GM, Padgette SR and Fraley RT (1992) History of herbicide-tolerant crops, methods of development and current state of the art- emphasis on glyphosate tolerance. Weed Technology 6:626-634.

Kirchner G, Kinslow CJ, Bloom GC and Taylor DW (1993) Nonlethal assay system of β-glucuronidase activity in transgenic tobacco roots. Plant Molecular Biology Reporter 11:320-325.

Klein TM, Arentzen R, Lewis PA and Fitzpatrick-McElligott S (1992) Transformation of microbes, plants and animals by particle bombardment. Bio/Technology 10:286-291.

Klein TM, Gradziel T, Fromm ME and Sanford JC (1988) Factors influencing gene delivery into *Zea mays* cells by high velocity microprojectiles. Bio/Technology 6:559-563.

Klein TM, Wolf ED, Wu R and Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids to living cells. Nature 327:70-73.

Kohli A, Leech M, Vain P, Laurie DA and Christou P (1998) Transgene organisation in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot-spots. Proceedings National Academy of Science USA 95:7203-7208.

Kohli A, Gahakwa D, Vain P, Laurie DA and Christou P (1999) Transgene expression in rice engineered through particle bombardment: molecular factors controlling stable expression and transgene silencing. Planta 208:88-97.

Koncz C, Olsson O, Langridge WHR, Schell J and Szlaly AA (1987) Expression and assembly of functional bacterial luciferase in plants. Proceedings of the National Acadamy of Science USA 84:313-315.

Koprek T, Hansch R, Nerlich A, Mendel RR and Schultze J (1996) Fertile barley of different cultivars obtained by adjustment of bombardment conditions to tissue response. Plant Science 119:79-91.

Kosugi, S, Ohash, Y, Nakajim, K and Ara Y (1990) An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses putative endogenous β-glucuronidase activity. Plant Science 70:133-140.

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M and Kadwell S (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology 11:194-200.

Kramer C, DiMaio J, Carswell GK and Shillito R (1993) Selection of transformed protoplast derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator chlorophenol red. Planta 190:454-458.

Kumar S and Fladung M (2000) Determination of transgene repeat formation and promoter methylation in transgenic plants. BioTechniques 28:1128-1137.

Kumar S and Fladung M (2001) Controlling transgene integration in plants. Trends in Plant Science 6:155-159.

Kumpatla SP, Chandrasekharan MB, Iyer LM, Li G and Hall TC (1998) Genome intruder scanning and modulation systems and transgene silencing. Trends in Plant Science 3:97-104.

Kumpatla SP, Teng W, Buchholz WG and Hall TC (1997) Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of a complex transgene in rice. Plant Physiology 115:361-373.

Kyozuka J, Olive M, Peacock WJ, Dennis ES and Shimamoto K (1994) Promoter elements required for developmental expression of the maize *Adh1* gene in transgenic rice. The Plant Cell 6:799-810.

Labra M, Savini C, Bracale M, Pelucchi N, Colombo L, Bardini M and Sala F (2001) Genomic changes in rice (*Oryza sativa* L.) plants produced by infecting calli with *Agrobacterium tumefaciens*. Plant Cell Reports 20:325-330.

Lacock L and Botha A-M (2000) Genotype variation in regeneration and transient expression efficiencies of 14 South African wheat cultivars. South African Journal of Plant Soil 17:170-174.

Larkin PJ and Scowcroft WR (1981) Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics 60:197-214.

Last DI, Brettell RIS, Chamberlain DA, Chaudhury AM, Larkin PJ, Marsh EL, Peacock WJ and Dennis ES (1991) pEmu: an improved promoter for gene expression in cereal cells. Theoretical and Applied Genetics 81:581-588.

Ledoux L and Huart R (1968) Integration and replication of DNA of *M. lysodeikticus* in DNA of germinating barley. Nature 218:1256-1259.

Ledoux L and Huart R (1969) Fate of exogenous bacterial deoxyribonucleic acids in barley seedlings. Journal of Molecular Biology 43:243-262.

Lee TSG (1987) Micropropagation of sugarcane (*Saccharum* spp.). Plant Cell, Tissue and Organ Culture 10:47-55.

Lehmann V (1999) A transgenic-centered approach to the biosafety assessment of transgenic herbicide-tolerant crops. Biotechnology and Development Monitor 38:6-10.

Leibbrandt NB and Snyman SJ (2001) Initial field testing of transgenic gluphosinate ammonuim resistant sugarcane. Proceedings of the South African Sugar Technologists Association 75: 108-111.

Leslie G. Head Entomology Department, SASA Experiment Station, P/B X01, Mount Edgecombe, South Africa, 4300.

Li L, Qu R, de Kochko A, Fauquet C and Beachy RN (1993) An improved rice transformation system using the biolistic method. Plant Cell Reports 12:250-255.

Liu MC (1990) Selection of agronomically useful mutants through plant cell culture systems. Taiwan Sugar 37:8-13.

Lichtenstein M, Keini G, Cedar H and Bergman Y (1994) B-cell-specific demethylation: a novel role for the intronic kappa-chain enhancer sequence. Cell 76:913-923.

Linacero R, Freitas Alves E and Vazquez AM (2000) Hot spots of DNA instability revealed through the study of somaclonal variation in rye. Theoretical and Applied Genetics 100:506-511.

Lindsey K and Jones MGK (1990) Selection of transformed cells. In: Plant cell line selection. Procedures and applications. Ed: Dix, PJ. VCH Publishers, Germany. pp317-339.

Linn F, Heidmann I, Saedler H, Meyer P (1990) Epigenetic changes in the expression of the maize A1 gene in petunia hybrids: Role of numbers of integrated gene copies and state of methylation. Molecular and General Genetics 222:239-336.

Litz RE and Grey DJ (1995) Somatic embryogenesis for agricultural improvement. World Journal of Microbiology and Biotechnology. 11:416-425.

Lonsdale D, Onde S and Cuming A (1990) Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. Journal of Experimental Botany 41:1161-1165.

Lorz H, Baker B and Schell J (1985) Gene transfer to cereal cells mediated by protoplast transformation. Molecular and General Genetics 199:178-182.

Lowe K, Taylor DB, Ryan PK and Paterson E (1985) Plant regeneration via organogenesis and embryogenesis in the maize inbred line B73. Plant Science 41:125-132.

Lowe K, Bowen B, Hoerster G, Ross M, Bond D, Pierce D and Gordon-Kamm B (1995) Germilne transformation of maize following manipulation of chimeric shoot meristems. Bio/Technology 13:677-682.

Ludwig SR, Bowen B, Beach L and Wessler SR (1990) A regulatory gene as a novel visible marker for maize transformation. Science 247:449-450.

Luo Z and Wu R (1988) A simple method for the transformation of rice via the pollen-tube pathway. Plant Molecular Biology Reporter 6:165-174.

Lusardi MC, Neuhaus-Url G, Potrykus I and Neuhaus G (1994) An approach towards genetically engineered cell fate mapping in maize using the *Lc* gene as a visible marker; transactivation capacity of *Lc* vectors in differentiated maize cells and microinjection of *Lc* vectors into somatic embryos and shoot apical meristems. The Plant Journal 5:571-582.

Lyznik LA, Ryan RD, Ritchie SW and Hodges TK (1989) Stable co-transformation of maize protoplasts with *gusA* and *neo* genes. Plant Molecular Biology 13:151-161.

MacKinnon C, Gunderson G and Nabors MW (1986) Plant regeneration by somatic embryogenesis from callus cultures of sweet sorghum. Plant Cell Reports 5:349-351.

Maheswaran G and Williams EG (1984) Direct somatic embryoid formation on immature embryos of *Trifolium repens, T. pratense* and *Medicago sativa*, and rapid clonal propagation of *T. repens*. Annals of Botany 54:201-211.

Maluszynski M, Ahloowalia BS and Sigurbjornsson B (1995) Application of *in vivo* and *in vitro* mutation techniques for crop improvement. Euphytica 85:303-315.

Maniatis T, Fritsch EF and Sambrook J (1982) Molecular cloning; A laboratory manual. Cold Spring Harbour Laboratory, USA. p468.

Mannerlof M and Tenning P (1997) Variability of gene expression in transgenic tobacco. Euphytica 98:133-139.

Mariani TS, Miyake H and Takeoka Y (2000) Critical factors for direct somatic embryogenesis of rice. Plant Production Science 3:114-123.

Martin T, Schmidt R, Altman T and Frommer WB (1992) Non-destructive assay system for detection of β-glucuronidase activity in higher plants. Plant Molecular Biology Reporter 10:37-46.

Matzke MA and Matzke AJM (1990) Gene interactions and epigenetic variation in transgenic plants. Genetic Development 11:214-223.

Matzke, MA and Matzke, AJM (1995) How and why do plants inactivate homologous (trans)genes?. Plant Physiology 107:679-685.

Matzke AJM, Neuhuber F, Park YD, Ambros PF and Matzke MA (1994). Homology-dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. Moleular and General Genetics 244:219-229.

McCabe DE and Christou P (1993) Direct DNA transfer using electric discharge particle acceleration (ACCELL technology). Plant Cell, Tissue and Organ Culture 33:227-236

McDonnell RE, Clark RD, Smith WA and Hinchee MA (1987) A simplified method for the detection of neomycin phosphotransferase II activity in transformed plant tissues. Plant Molecular Biology Reporter 5:380-386.

McElroy D and Brettell RIS (1994) Foreign gene expression in transgenic cereals. Trends in Biotechnology 12:62-68.

McElroy D, Zhang W, Cao J and Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163-171.

McKenzie MJ, Mett V and Jameson PE (2000) Modified ELISA for the detection of neomycin phosphotransferase II in transformed plant species. Plant Cell Reports 19:286-289.

McKinnon GE, Abedinia M and Henry RJ (1996) Expression of non-selectable markers in wheat and rice tissues. Plant Tissue Culture and Biotechnology 2:24-33.

Meijer EGM, Schilperoot RA, Rueb S, van Os-Ruygrok PE and Hensgens LAM (1991) Transgenic rice cell lines and plants: expression of transferred chimeric genes. Plant Molecular Biology 16:807-820.

Mendel RR, Muller B, Schulze J, Kolesnikov V and Zelenin A (1989) Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Theoretical and Applied Genetics 78:31-34.

Menossi M, Puigdomenech P and Martinez-Izquierdo JA (2000) Improved analysis of promoter activity in biolistically transformd plant cells. BioTechniques 28:54-57.

Merkle SA, Parrott WA and Williams EG (1990) Applications of somatic embryogenesis and embryo cloning. In: Plant tissue culture: applications and limitations. Developments in Crop Science 19. Ed: Bhojwani SS. Elsevier, Amsterdam. pp 67-101.

Metzlaff M, O'Dell M, Cluster PD and Flavell RB (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. Cell 88:1-20.

Meyer P (1995) Variation of transgene expression in plants. Euphytica 85:359-366.

Meyer P (1999) The role of chromatin remodelling in transgenic silencing and plant development. In Vitro Cellular and Developmental Biology-Plant 35:29-36.

Mooney PA, Goodwin PB, Dennis ES and Llewellyn DJ (1991) *Agrobacterium tumefaciens* gene transfer into wheat tissues. Plant Cell, Tissue and Organ Culture 25:209-218.

Moore PH and Irvine JE (1991) Genomic mapping of sugarcane and its potential contribution to improvement and to selection of new varieties. Proceedings of the South African Sugar Technologists Association 65:96-102.

Mordhorst AP and Lorz H (1992) Electrostimulated regeneration of plantlets from protoplasts derived from cell suspensions of barley (*Hordeum vulgare*). Physiologia Plant 85:289-294.

Mordhorst AP, Toonen AJ and de Vries SC (1997) Plant embryogenesis. Critical Reviews in Plant Science. 16:535-576.

Morino K, Olsen O-A and Shimamoto K (1999) Silencing of an aleurone-specific gene in transgenic rice is caused by a rearranged transgene. The Plant Journal 17:275-285.

Morrish FM, Hanna WW and Vasil IK (1990) The expression and perpetuation of inherent somaclonal variation in regenerents from embryogenc cultuers of *Pennisetum glaucum* (l) R Br (pearl millet). Theoretical and Applied Genetics 80:409-416.

Morrish F, Songstad DD, Armstrong CL and Fromm M (1993) Microprojectile bombardment: A method for the production of transgenic cereal crop plants and the functional analysis of genes. In: Transgenic plants. Fundamentals and applications. Ed: Hiatt, A. Marcel Dekker Inc, New York. pp133-172.

Moutia M and Dookun A (1999) Evaluation of surface sterilisation and hot water treatments on bacterial contaminants in bud culture of sugarcane. Experimental Agriculture 35:265-274.

Muller E, Brown PTH, Hartke S and Lorz H (1990) DNA variation in tissue-culture-derived rice plants. Theoretical and Applied Genetics 80:673-679.

Mullineaux PM (1992) Genetically engineered plants for herbicide resistance. In: Plant genetic manipulation for crop protection. Eds: Gatehouse AMR, Hilder VA and Boulter D. CAB International England. pp 75-108.

Murakami T, Anzai H, Imai S, Satoh A, Nagaoka D and Thompson CJ (1986) The bialaphos biosynthetic genes of *Streptomyces hygroscopicus:* molecular cloning and charcterisation of the gene cluster. Moleular and General Genetics 205:42-50.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plant 15:473-497.

Nadar HM, Soepraptopo S, Heinz DJ and Ladd SL (1978) Fine structure of sugarcane (*Saccharum* sp.) callus and the role of auxin in embryogenesis. Crop Science 18:210-216.

Nagel RJ, Manners JM and Birch RG (1992) Evaluation of an ELISA assay for rapid detection and quantification of neomycin phosphotransferase II in transgenic plants. Plant Molecular Biology Reporter 10:263-272.

Nandadeva YL, Lupi CG, Meyer CS, Devi PS, Potrykus I and Bilang R (1999) Micropojectile-mediated transient and integrative transformation of rice embryogenic suspension cells: effects of osmotic cell conditioning and of the physical configuration of plasmid DNA. Plant Cell Reports 18:500-504.

Nap JP (1999) A transgene-centred approach to the biosafety assessment of transgenic herbicide-tolerant plants. Monitor 38:6-10.

Nap JP, Bijvoet J and Stiekema W (1992) Biosafety of kanamycin-resistant transgenic plants. Transgenic Research 1:239-249.

Negrotto D, Jolley M, Beer S, Wenck AR and Hansen G (2000) The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. Plant Cell Reports 19:798-803.

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M and Kartha KK (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues for microprojectile bombardment with two distinct genes. The Plant Journal 5:285-297.

Neuhuber F, Park YD, Matzke AJM and Matzke MA (1994) Susceptibilty of transgene loci to homology-dependent gene silencing. Moleular and General Genetics 244:230-241.

Norris R, Smith RH and Vaughn KC (1983) Plant chimeras used to establish *de novo* origin of shoots. Science 220:75-76.

Nuss K. Plant Breeding Department, SA Sugar Association Experiment Station, P/B X02, Mount Edgecombe, South Africa, 4300

O'Kennedy MM, Burger JT and Watson TG (1998) Stable transformation of Hi-II maize using the particle inflow gun. South African Journal of Science 94:188-192.

Omirulleh S, Abraham M, Golovkin M, Stefanov I, Karabaev MK, Mustardy L, Morocz S and Dudits D (1993) Activity of a chimeric promoter with the doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize. Plant Molecular Biology 21:415-428.

Oard JH, Paige DF, Simmonds JA and Gradziel TM (1990) Transient gene expression in maize, rice and wheat cells using an airgun apparatus. Plant Physiology 92:334-339.

O'Reilly G (1998) The South African sugar industry. International Sugar Journal 100:266-268.

Ow DW, Wood KV, Deluca M, De Wet JR, Helsinki DR and Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234:856-859.

Owen MDK (2000) Current use of transgenic herbicide-resistant soybean and corn in the USA. Crop Protection 19:765-771.

Park YD, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJ and Matzke MA (1996) Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. The Plant Journal 9:183-94.

Park SH, Rose SC, Zapata C, Srivatanakul M and Smith RH (1998) Cross-protection and selectable marker genes in plant transformation. In Vitro Cellular and Developmental Biology-Plant 34:117-121.

Parveez GKA, Chowdhury MKU and Saleh NM (1997) Physical parameters affecting transient GUS gene expression in oil palm (*Elaeis guineensis* Jacq.) using the biolistic device. Industrial Crops and Products 6:41-50.

Pasternak TP, Rudas VA and Kumlehn J (1999) Embryogenic callus formation and plant regeneration from leaf base segments of barley (*Hordeum vulgare* L.) Journal of Plant Physiology 155:371-375.

Pastori GM, Wilkinson MD, Steele SH, Sparks CA, Jones HD and Parry MAJ (2001) Age-dependent transformation frequency in elite whaet cultivars. Journal of Experimental Botany 52:857-863.

Petolino JF, Hopkins NL, Kosegi BD and Skokut M (2000) Whisker-mediated transformation of embryogenic callus of maize. Plant Cell Reports 19:781-786.

Pfeilstetter E, Matzk A, Feldmann SD and Schiemann J (2000) Rapid and efficient screening of phosphinothricin tolerant oilseed rape (*Brassica napus*) with a novel germination test. Euphytica 113:119-124.

Phillips RL, Kaeppler SM and Olhoft P (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. Proceedings of the National Acadamy of Science USA 91:5222-5226.

Platt S and Yang N-S (1987) Dot assay for neomycin phosphotransferase activity in crude cell extracts. Annals of Biochemistry 162:529-535.

Popelka JC and Altpeter F (2001) Interactions between genotypes and culture media components for improved *in vitro* response of rye (*Secale cereale* L.) inbred lines. Plant Cell Reports 20:575-582.

Potrykus I (1991) Gene transfer to plants: assessment of published approaches and results. Annual Review of Plant Physiology and Plant Molecular Biology 42:205-225.

Price S (1967) Interspecific hybridisation in sugarcane breeding. Proceedings of the International Society of Sugar Cane Technologists 12:1021-1026.

Purvis MJ, Collier DC Wallis D (1964) Laboratory techniques in botany. Butterworths, England.

Raemakers CJJM, Jacobsen E and Visser RGF (1995) Secondary somatic embryogenesis and applications in plant breeding. Euphytica 81:93-107.

Raineri DM, Bottino P, Gordon MP and Nester EW (1990) *Agrobacterium*-mediated transformation of rice (*Oryza sativum*). Bio/Technology 8:33-38.

Rajasekaran K, Cary JW, Jacks TJ and Cleveland TE (2000) Inhibition of fungal growth *in planta* and *in vitro* by transgenic tobacco expressing a bacterial nonheme chloroperoxidase gene. Plant Cell Reports 19:333-338.

Rani V and Raina SN (2000) Genetic fidelity of organised meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cellular and Developmental Biology-Plant 36:319-330.

Rasco-Gaunt S, Riley A, Lazzeri P and Barcelo P (1999a) A facile method for screening for phosphinothricin (PPT)-resistant transgenic wheats. Molecular Breeding 5:255-262.

Rasco-Gaunt S, Riley A, Barcelo P and Lazzeri PA (1999b) Analysis of particle bombardment parameters to optimise DNA delivery into wheat tissues. Plant Cell Reports 19:118-127.

Rashid H, Yokoi S, Yoriyama K and Hinata K (1994) Transgenic plant production mediated by *Agrobacterium* in *Indica* rice. Plant Cell Reports 15:727-730.

Rathus C and Birch RG (1992) Stable transformation of callus from electroporated sugarcane protoplasts. Plant Science 82:81-89.

Rathus C, Bower R and Birch R (1993) Effects of promoter, intron and enhancer elements on transient gene expression in sugarcane and carrot protoplasts. Plant Molecular Biology 23:613-618.

Redway FA, Vasil V and Vasil IK (1990) Characterisation of wheat (*Triticum aestivum* L.) embryogenic cell suspension cultures. Plant Cell Reports 8:714-717.

Reed J et al (2001) Phosphomannose isomerase: an efficient selectable marker for plant transformation. In Vitro Cellular and Developmental Biology-Plant 37:127-132.

Register JC III, Peterson DJ and Bell PJ *et al* (1994) Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Molecular Biology 25:951-961.

Reiss B, Deprengel R, Will H and Schaller H (1984) A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene 30:211-218.

Repellin A, Baga M, Jauhar PP and Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: New challenges. Plant Cell, Tissue and Organ Culture 64:159-183.

Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D and Detmer JJ (1988) Genetically transformed maize plants from protoplasts. Science 240:204-207.

Richards HA, Rudas VA, Sun H, McDaniel JK, Tomaszewski Z and Conger BV (2001) Construction of a GFP-BAR plasmid and its use for switchgrass transformation. Plant Cell Reports 20:48-54.

Ritala A, Mannonen L, Aspegren K, Salmenkallio-Marttila M, Kurten U, Hannus R, Mendez Lozano J, Teeri TH and Kauppinen V (1993) Stable transformation of barley tissue by particle bombardment. Plant Cell Reports 12:435-440.

Ritchie SW, Lui C-N, Sellmer JC, Kononowicz H, Hodges TK and Gelvin SB (1993) *Agrobacterium tumefaciens*-mediated expression of gusA in maize tissues. Transgenic Research 2:252-265.

Rooke L, Byrne D and Salgueiro S (2000) Marker gene expression driven by the maize ubiquitin promoter in transgenic wheat. Annals of Applied Biology 136:167-172.

Russell JA, Roy MK and Sanford JC (1992) Major improvements in biolistic transformation of suspension-cultured tobacco cells. In Vitro Cellular and Developmental Biology-Plant 28:97-105.

Ryan CA (1990) Protease inhibitors in plant genes for improving defences against insects and pathogens. Annual Review of Phytopathology 28:425-429.

Sala F, Arencibia A, Castiglione S, Christou P, Zheng Y and Han Y (1999) Molecular and field analysis of somaclonal variation in transgenic plants. In: Plant biotechnology and *in vitro* biology in the 21st centuary. Eds: Altman A, Ziv M and Izhar S. Kluwer Academic Publishers, The Netherlands. p259-262.

Salgueiro S, Pignocchi C and Parry MAJ (2000) Intron-mediated *gus*A expression in tritordeum and wheat resulting from particle bombardment. Plant Molecular Biology 42:615-622.

Sanford JC (2000) The development of the biolistic process. In Vitro Cellular and Developmental Biology-Plant 36:303-308.

Sanford JC, Klein TM, Wolf ED and Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. Journal of Particle Science Technology 5:27-37.

Sanford JC, Smith FD and Russell JA (1993) Optimising the biolistic process for different biological applications. In: Methods in enzymology. Vol 217. Recombinant DNA. Part H. Ed.: Wu, R. Academic Press, California. pp 483-509.

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proceedings of the National Acadamy of Science USA 74:5463.

Sautter C (1993) Development of a microtargeting device for particle bombardment of plant meristems. Plant Cell, Tissue and Organ Culture 33:251-257.

Sautter C, Leduc N, Bilang R, Iglesias VA, Gisel A, Wen X and Potrykus I (1995) Shoot apical meristems as a target for gene transfer by microballistics. Euphytica 85:45-51.

Schenk RU and Hildebrandt AC (1972) Medium and techniques for induction of growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany 50: 166-204.

Semal J and Lepoivre P (1990) Application of tissue cultre variability to crop improvement. In: Plant tissue culture: applications and limitations. Ed: Bhojwani SS. Alsevier, Amsterdam. pp 310-315.

Sharp WR, Sondahl MR, Caldas LS and Maraffa SB (1980) The physiology of *in vitro* asexual embryogenesis. Horticultural Reviews 2:268-310.

Shen WH, Escudero J, Schlappi M, Ranos C, Hohn B and Koukolikova-Nicola Z (1993). T-DNA transfer to maize cells: histochemical investigation of β-glucuronidase activity in maize cells. Proceedings of the National Acadamy of Science USA 90:1488-1492.

Shenoy VB and Vasil IK (1992) Biochemical and molecular analysis of plants derived from embryogenic tissue cultures of napier grass (*Pennisetum purpureum* K. Shum). Theoretical and Applied Genetics 83:947-955.

Shillito RD, Saul MW, Paszowski J, Muller M and Potrykus I (1985) High efficiency direct gene transfer to plants. Bio/Technology 3:1099-1103.

Shimron-Abarbanell D and Breiman A (1991) Comprehensive molecular characterisation of tissue-culture-derived *Hordeum marinum* plants. Theoretical and Applied Genetics 83:71-80.

Simmonds J, Steward P and Simmonds D (1992) Regeneration of *Triticum aestivum* apical explants after microinjection of germ line progenitor cells. Physiologia Plantarum 85:197-206.

Skene KGM and Barlass M (1983) Studies on the fragmented shoot apex of grapevine. Journal of Experimental Botany 34:1271-1280.

Skoog F and Miller CO (1957) Chemical regulation of growth and organ formation in plant tissue cultured *in vitro*. Symposium of the Society of Experimental Biology 11:118-131.

Smith DL and Krikorian AD (1989) Release of somatic embryogenic potential from excised embryos of carrot and maintenance of polyembryonic cultures in hormone-free medium. American Journal of Botany 76:1832-1843.

Smith RH and Hood E (1995) *Agrobacterium tumefaciens* transformation of monocotyledons. Crop Science 35:301-309.

Snyman SJ, Leibbrandt NB and Botha FC (1998) Buster resistant sugarcane. Proceedings of the South African Sugar Technologists Association 72:138-139.

Snyman SJ, Meyer GM, Carson D and Botha FC (1996) Establishment of embryogenic callus and transient gene expression in selected sugarcane varieties. South African Journal of Botany 62:151-154.

Snyman SJ, Watt MP, Huckett BI and Botha FC (2000) Direct somatic embryogenesis for rapid, cost effective production of transgenic sugarcane (*Saccharum* spp. hybrids). Proceedings of the South African Sugar Technologists Association 74: 186-187.

Snyman SJ, Huckett BI, Botha FC and Watt MP (2001) A comparison between direct and indirect somatic morphogenesis for the production of transgenic sugarcane (*Saccharum* spp. hybrids). Acta Hortculturae no. 560:105-108.

Somers DA, Rines HW, Gu W, Kaeppler HF and Bushnell WR (1992) Fertile, transgenic oat plants. Bio/Technology 10:1589-1594.

Songstad DD, Halaka FG, DeBoer DL, Armstrong CL, Hinchee MAW, Santino F, Brown SM, Fromm ME and Horsch RB (1993) Transient expression of GUS and anthocyanin constructs in intact maize immature embryos following electroporation. Plant Cell, Tissue and Organ Culture 33:195-201.

Songstad DD, Somers DA and Griesbach RJ (1995) Advances in alternative DNA delivery techniques. Plant Cell, Tissue and Organ Culture 40:1-15.

Sorokin AP, Ke X-Y, Chen D-F and Elliott M (2000) Production of fertile transgenic wheat plants via tissue electroproation. Plant Science 156:227-233.

Spencer TM, Gordon-Kamm WJ, Daines RJ, Start WG and Lemaux PG (1990) Bialaphos selection of stable transformants from maize cell culture. Theoretical and Applied Genetics 79:625-631.

Spencer TM, O'Brian JV, Start WG, Adams TR, Gordon-Kamm WJ and Lemaux PG (1992) Segregation of transgenes in maize. Plant Molecular Biology 18;201-210.

Sreenivasan TV, Ahloowalia S and Heinz DJ (1987) Cytogenetics. In: Developments in Crop Scienceence III. Sugarcane improvement through breeding. Ed: Heinz D. Elsevier, Amsterdam. pp211-254.

Srinivasan C and Vasil IK (1986) Plant regeneration from protoplasts of sugarcane *(Saccharum officinarum* L.). Journal of Plant Physiology 126:41-48.

Staebell M, Tomes D, Weissinger A, Maddock S, Marsh W, Huffman G, Bauer R, Ross M and Howard J (1990) A quantitative assay for neomycin phosphotransferase activity in plants. Annals of Biochemistry 185:319-323.

Stam M, Mol JNM and Kooter JM (1998) The silence of genes in plants. Annals of Botany 79:3-12.

Stevenson GC (1965) Genetics and breeding of sugarcane. Longmans, London. pp284.

Steward FC (1958) Growth and development of cultivated cells. III. Interpretations of the growth from free cell to carrot plant. American Journal of Botany 45:709-713.

Stewart CN (2001) The utility of green flourescent protien in transgenic plants. Plant Cell Reports 20:276-382.

Stiff CM, Kilian A, Zhou H, Kudrna DA and Kleinhofs A (1995) Stable transformation of barley callus using biolistic particle bombardment and the phosphinothricin acetyltransferase (*bar*) gene. Plant Cell, Tissue and Organ Culture 40:243-248.

Sukhapinda K, Kozuch ME, Rubin-Wilson B, Ainley WM and Merlo DJ (1993) Transformation of maize (*Zea mays* L.) protoplasts and regeneration of haploid transgenic plants. Plant Cell Reports 13:63-68.

Sun SSM, Maretzki A, Nagai C, Houchens K and Bidney D (1993) Transformation of *Saccharum spontaneum* by particle bombardment. Sugar Cane 2:1-7.

Sweby DL, Huckett BI and Botha FC (1994) Minimising somaclonal variation in tissue cultures of sugarcane. Proceedings of the South African Sugar Technologists Association 66:29-37.

Tachibana K, Watanabe T, Sekizawa Y and Takematsu T (1986) Accumulation of ammonia in plants treated with bialaphos. Journal of Pesticide Science 11:33-37.

Takumi S and Shimada T (1996) Production of transgenic wheat through particle bombardment of scutellar tissues: frequency is influenced by culture duration. Journal of Plant Physiology 149:418-423.

Tanaka A, Satoru M, Ohta S, Kyozuka J and Shimamoto K (1990) Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron. Nucleic Acids Research 18:6767-6770.

Taylor PWJ, Ko H-L and Adkins SW (1992a) Factors affecting protoplast isolation and the regeneration of shoot-like stuctures from protoplast-derived callus of sugarcane (*Saccharum* spp. hybrids). Australian Journal of Botany 40:863-876.

Taylor PWJ, Ko H-L, Adkins SW, Rathus C and Birch RG (1992b) Establishment of embryogenic callus and high protoplast yielding suspension cultures of sugarcane (*Saccharum* spp. hybrids). Plant Cell, Tissue and Organ Culture 28:69-78.

Taylor MG, Vasil V and Vasil IK (1993) Enhanced GUS gene expression in cereal/grass cell suspensions and immature embryos using the maize ubiquitin-based plasmid pAHC25. Plant Cell Reports 12:491-495.

Thomas JC, Adams DG, Keppenne VD, Wasmann CC, Brown JK, Kanost MR and Bohnert HJ (1995a) *Manduca sexta* protease inhibitors expressed in *Nicotiana tabacum* provide protection against insects. Plant Physiology and Biochemistry 33:611-614.

Thomas JC, Adams DG, Keppenne VD, Wasmann CC, Brown JK, Kanost MR and Bohnert HJ (1995b) Protease inhibitors of *Manduca sexta* expressed in transgenic cotton. Plant Cell Reports 14:758-762.

Thompson CJ, Rao Movva N, Tizard R, CrameriR, Davies JE, Lauwereys M and Botterman J (1987) Characterisation of the herbicide-resistant *bar* gene from *Streptomyces hygroscopicus*. EMBO J 6:2519-2523.

Thompson JA, Drayton PR, Frame BR, Wang K and Dunwell JM (1995) Miaze transformation utilizing silicon carbide whiskers: a review. Euphytica 85:75-80.

Thornburg RW, An G, Cleveland TE Johson R and Ryan CA (1987) Wound-inducible expression of a potato inhibitor II-CAT gene fusion in transgenic tobacco plants. Proceedings of the National Acadamy of Science USA 84:744-748.

Thorpe TA (1990) The current status of plant tissue culture. In: Plant tisue culture: applications and limitations. Ed: Bhojwani SS. Elsevier, Amsterdam. pp 1-33.

Tian L, Brown DCW and Webb J (2000) Transient expression of a reporter genes changes significantly during somatic embryogenesis in alfalfa. Canadian Journal of Plant Science 80:765-771.

Tingay et al. (1997) *Agrobacterium tumefaciens*-mediated barley transformation. The Plant Journal 11:1369-1376.

Toki S (1997) Rapid and efficient *Agrobacterium*-mediated transformation of rice. Plant Molecular Biology Reporter 15:16-21.

Toki S, Takamatsu S, Nojiri C, Ooba S, Anzai H, Iwata M, Christensen AH, Quail P and Uchimiya H (1992) Expression of a maize ubiquitin gene promoter-*bar* chimeric gene in transgenic rice plants. Plant Physiology 100:1503-1507.

Toonen MAJ, Hendriks T, Schmidt EDL, Verhoeven HA, van Kammen A and de Vries SC (1994) Description of somatic embryo forming single cells in carrot suspension cultures employing video cell tracking. Planta 194:565-572.

Topfer R, Gronenborn B, Schafer S, Schell J and Steinbiss HH (1990) Expression of engineered wheat dwarf virus in seed derived embryos. Physiologia Plant 79:158-162.

Topping JF, Wei W and Lindsey K (1991) Functional tagging of regulatory elements in plant genomes. Development 112:1009-1019.

Tor M, Mantell SH and Ainsworth C (1992) Endophytic bacteria expressing β-glucuronidase cause false positives in transformation of *Dioscoria* species. Plant Cell Reports 11:452-456.

Torbet KA, Rines HW and Somers DA (1998) Transformation of oat using mature embryo-derived tissue cultures. Crop Science 38:226-231.

Toriyama K, Arimoto Y, Uchimiya H and Hinata K (1988) Transgenic rice plants after direct gene transfer to protoplasts. Bio/Technology 6:1072-1074.

Toyoda H, Yamaga T, Matsuda Y and Ouchi S (1990) Transient expression of the β-gluuronidase gene introduced into barley coleoptiles by microinjection. Plant Cell Reports 9:299-302.

Tsaftaris A (1996) The development of herbicide-tolerant transgenic crops. Field Crops Research 45:115-123.

Tu J, Datta K, Khush GS, Zhang Q and Datta SK (2000a) Field performance of *Xa21* transgenic indica rice (*Oryza sativa* L.), IR72. Theoretical and Applied Genetics 101:15-20.

Tu J, Zhang G, Datta K, Xu C, He Y, Zhang Q, Khush GS and Datta SK (2000b) Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ-endotoxin. Nature Biotechnology 18:1101-1104.

Tyagi A and Mohanty A (2000) Rice transformation for crop improvement and functional genomics. Plant Science 158:1-18.

Uze M, Potrykus I and Sautter C (1999) Single-stranded DNA in the genetic transformation of wheat (*Triticum aestivum* L.): transformation frequency and integration pattern. Theoretical and Applied Genetics 99:487-495.

Vain P, Finer KR, Engler DE, Pratt RC and Finer JJ (1996) Intron-mediated enhancement of gene expression in maize (*Zea mays* L.) and bluegrass (*Poa pratensis* L.). Plant Cell Reports 15:489-494.

Vain P, Keen N, Murillo J, Rathus C, Nemes C and Finer JJ (1993a) Development of the particle inflow gun. Plant Cell, Tissue and Organ Culture 33:237-246.

Vain P, McMullen MD and Finer JJ (1993b) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Reports 12:84-88.

Vain P, Worland B, Kohli A, Snape JW, Christou P, Allen GC and Thompson WF (1999) Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. The Plant Journal 18:233-242.

Van den Bulk RW (1991) Application of cell and tissue culture and *in vitro* selection for disease resistance breeding-a review. Euphytica 56:269-285.

Van Dillewijn C (1952) Botany of sugarcane. Waltham, Mass. USA. p 4.

Van Rensburg JBJ (1999) Evaluation of bt-transgenic maize for resistance to the stem borers *Busseola fusca* (Fuller) and *Chilo partellus* (Swinhoe) in South Africa. South African Journal of Plant Soils 61:38-43.

Vasic D, Alibert G and Skoric D (2001) Protocols for efficient repetitive and secondary somatic embryogenesis in *Hellianthus maximiliani* (Schrader). Plant Cell Reports 20:121-125.

Vasil IK (1987) Developing cell and tissue culture systems for the improvement of cereal and grass crops. Journal of Plant Physiology 128:193-218.

Vasil IK (1988) Progress in the regeneration and genetic manipulation of cereal crops. Bio/Technology 6:397-402.

Vasil IK and Hildebrandt AC (1965) Differentiation of tobacco plants from single, isolated cells in microcultures. Science 150:889-892.

Vasil V, Brown SM, Re D, Fromm ME and Vasil IK (1991) Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. Bio/Technology 9:743-747.

Vasil V, Castillo AM, Fromm ME and Vasil IK (1992) Herbicide resistant fertile transgenic wheat obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology 10:667-674.

Vasil V, Lu C-Y and Vasil IK (1985) Histology of somatic embryogenesis in cultured immature embryos of maize (*Zea mays* L.). Protplasma 127:1-8.

Vasil V and Vasil IK (1982) The ontogeny of somatic embryos of *Pennisetum americanum* (L.) K. Schum. in cultured immature embryos. Botanical Gazette 143:454-465.

Vasil V, Vasil IK and Lu C-Y (1984) Somatic embryogenesis in long-term callus cultures of *Zea mays* L. (Gramineae). American Journal of Botany 71:158-161.

Vikrant and Rashid A (2001) Direct as well as indirect somatic embryogenesis from immature (unemerged) inflrescence of a minor millet *Paspalum scrobiculatum* L. Euphytica 120:167-172.

Walters DA, Vetsch CS, Potts DE and Lindquist RC (1992) Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. Plant Molecular Biology 18:189-200.

Wan Y and Lemaux PG (1994) Generation of a large number of independantly transformed barley plants. Plant Physiology 104:37-48.

Wang AS, Evans RA, Altendorf PR, Hanten JA, Doyle MC and Rosichan JL (2000a) A mannose selection system for production of fertile transgenic maize plants from protoplasts. Plant Cell Reports 19:654-660.

Wang J, Jiang J and Oard JH (2000b) Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.). Plant Science 156:201-211.

Wang Y-C, Klein TM, Fromm M, Cao J, Sanford JC and Wu R (1988) Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. Plant Molecular Biology 11:433-439.

Ward KA and Jordan MC (2001) Callus formation and plant regeneration from immature and mature embryos of rye (*Secale cereale* L.). In Vitro Cellular and Developmental Biology-Plant 37:361-368.

Weber G, Monajembashi S, Wolfrum J and Greulich KO (1990) Genetic changes induced in higher plants by a laser microbeam. Physiologia Plant 79:190-193.

Weeks TJ, Anderson OD and Blechl AE (1993) Rapid production of multiple independent lines of fertile wheat (*Triticum aestivum*). Plant Physiology 102:1077-1084.

Wehrmann A, Van Vliet A, Opsomer C, Botterman J and Schulz A (1996) The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. Nature Biotechnology 14:1274-1278.

Wernicke W and Brettell RIS (1980) Somatic embryogenesis from *Sorghum bicolor* leaves. Nature 138:138-139.

Wernicke W and Milkovits L (1984) Developmental gradients in wheat leaves- responses of leaf segments in different genotypes cultured *in vitro*. Journal of Plant Physiology 115:49-58.

Wernicke W and Milkovits L (1987) Effect of auxin on the mitotic cell cycle in cultured leaf segments at different stages of development in wheat. Physiologia Plant 69:16-22.

Wernicke W, Potrykus I and Thomas E (1982) Morphogenesis from cultured leaf tissue of *Sorghum bicolor* (L.). The morphogenic pathways. Protoplasma 111:53-62.

Werr W and Lorz H (1986) Transient expression in a Gramineae cell line. Molecular and General Genetics 202:471-475.

Williams EG and Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Annals of Botany 57:443-462.

Wilmink A and Dons JJM (1993) Selective agents and marker genes for use in transformation of monoctyledonous plants. Plant Molecular Biology Reporter 11:165-185.

Wohlleben W, Arnold W, Broer I, Hillemann D, Strach E and Puhler A (1988) Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tu 494 and its expression in *Nicotiana tabacum*. Gene 70:25-37.

Xiao L and Ha S-B (1997) Efficient selection and regeneration of creeping bentgrass transformants following particle bombardment. Plant Cell Reports 16:874-878.

Yang N-S and Russell D (1990) Maize sucrose synthase-1 promoter directs phloem cell-specific expression of GUS genes in transgenic tobacco plants. Proceedings of the National Acadamy of Science USA 87:4144-4148.

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC and Davey MR (1988) Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Reports 7:379-384.

Zhang S, Cho M-J, Koprek T, Yun R, Bregitzer P and Lemaux PG (1999) Genetic transformation of commercial cultivars of oat (*Avena sativa* L.) and Barley (*Hordeum vulgare* L.) using *in vitro* shoot meristematic cultures derived from germinated seedlings. Plant Cell Reports 18:959-966.

Zheng MY and Konzak CF (2000) Effect of 2,4-dichlorophenoxyacetic acid on callus induction and plant regeneration in anther culture in wheat (*Triticum aestivum* L.). Plant Cell Reports 19:69-73.

Zhong G-Y (2001) Genetic issues and pitfalls in transgenic plant breeding. Euphytica 118:137-144.

Zhong H, Boylard MG, Srinivasan C and Sticklen MB (1993) Transgenic plants of turfgrass (*Agrostis palustris* Huds.) from microprojectile bombardment of embryogenic callus. Plant Cell Reports 13:1-6.

Zhong H, Srinivasan C and Sticklen MB (1992) *In-vitro* morphogenesis of corn (*Zea mays* L.). I. Differentiation of multiple shoot clumps and somatic embryos from shoot tips. Planta 187:483-489.

Zhong H and Sticklen MB (2000) Genetic engineering of corn: sustainability of shoot tip meristem in genetic transformation. In: Biotechnology in agriculture and forestry. Volume 46. Transgenic crops 1. Ed: Bajaj YPS. Springer-Verlag, Heidelberg. pp37-59.

Zhong H, Sun B, Warkentin D, Zhang S, Wu R, Wu T and Sticklen MB (1996) The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiology 110:1097-1107.

Zhou H, Stiff CM and Konzak CF (1993) Stably transformed callus of wheat by electroporation-induced direct gene transfer. Plant Cell Reports 12:612-616.

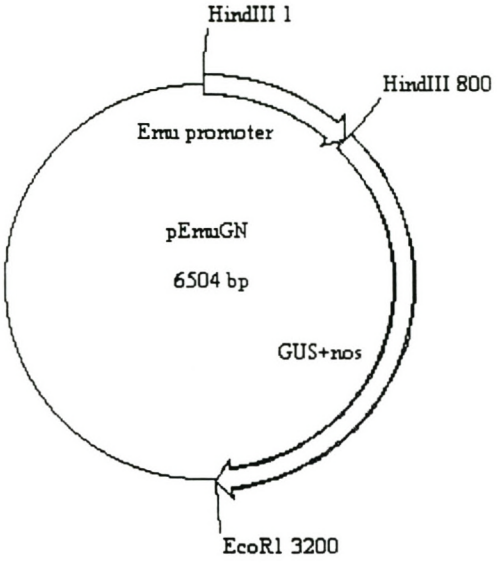
Zhou Q, Maher EA, Masoud S, Dixon RA and Lamb CJ (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Biotechnology 12:807-812.

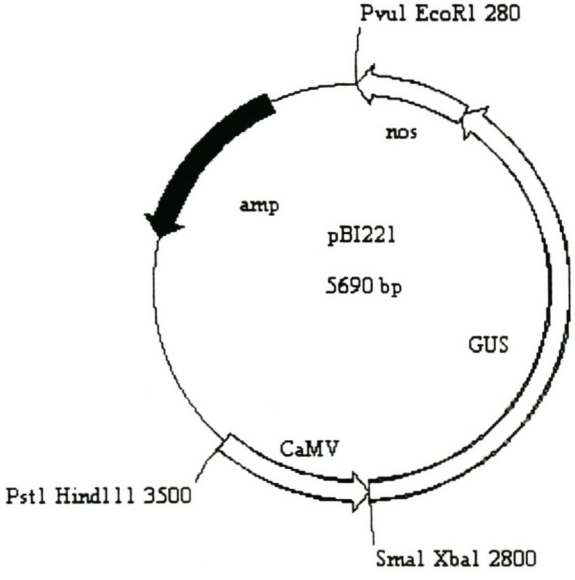
Zimmerman JL (1993) Somatic embryogenesis: a model study for early development in higher plants. Plant Cell 5:1411-1423.

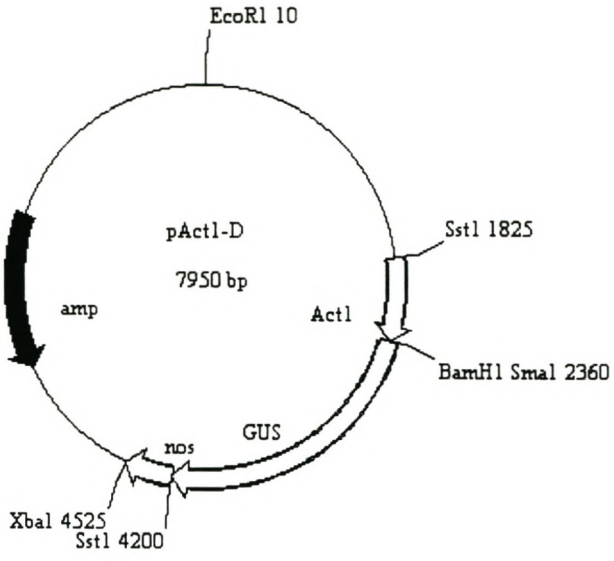
APPENDICES

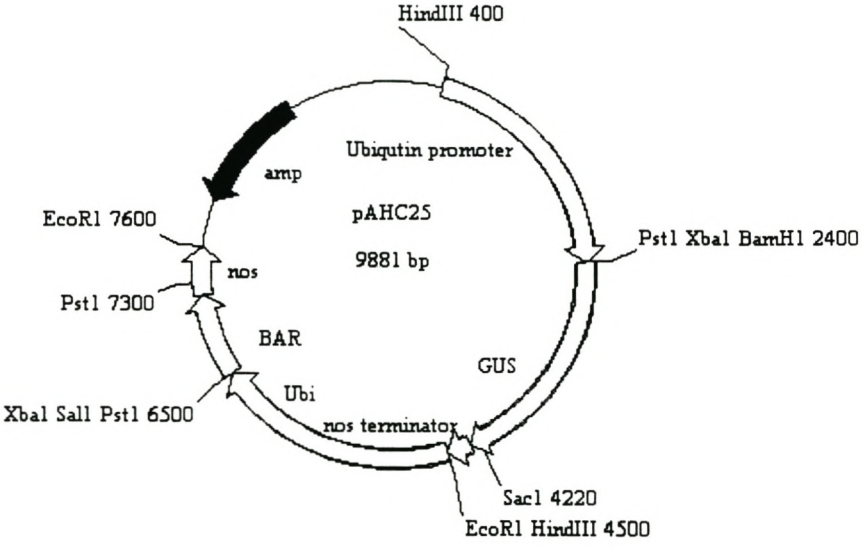
Appendix 1

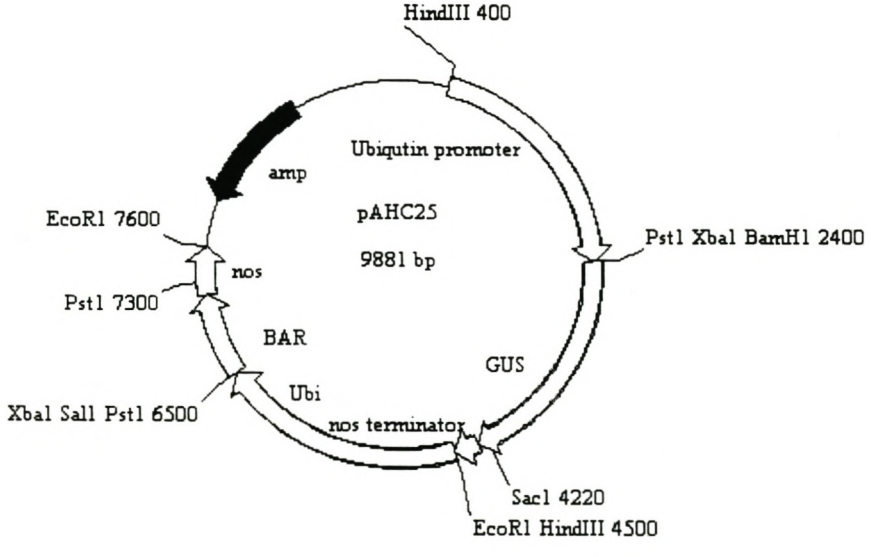
Plasmid maps for plasmids used for microprojectile bombardment in Chapter 3.











Appendix 2

Trial design for Chapter 6.

N ⟶

T3 C 8	T3 TG 7	T1 C 6	T1 TG 5	T4 TG 4	T4 C 3	T2 C 2	T2 TG 1
T2 TG 9	T2 C 10	T4 TG 11	T4 C 12	T3 C 12	T3 TG 14	T1 TG 15	T1 C 16
T1 C 24	T1 TG 23	T3 C 22	T3 TG 21	T2 C 20	T2 TG 19	T4 TG 18	T4 C 17
T4 TG 25	T4 C 26	T2 TG 27	T2 C 28	T1 TG 29	T1 C 30	T3 C 31	T3 TG 32
T1 TG 40	T1 C 39	T3 TG 38	T3 C 37	T2 TG 36	T2 C 35	T4 C 34	T4 TG 33
T2 C 41	T2 TG 42	T4 C 43	T4 TG 44	T1 C 45	T1 TG 46	T3 TG 47	T3 C 48
T4 C 56	T4 TG 55	T2 C 54	T2 TG 53	T3 TG 52	T3 C 51	T1 C 50	T1 TG 49
T3 TG 57	T3 C 58	T1 TG 59	T1 C 60	T4 C 61	T4 TG 62	T2 TG 63	T2 C 64

Treatments

T1 repeated Buster application

T2 pre-emergence plus Buster application

T3 conventional herbicide cocktail

T4 hand weeding

C (untransformed NCo310 plants)

TG (transgenic herbicide resistant NCo310 plants)

CURRICULUM VITAE

1985 Completed Bachelor of Science degree at the University of the Witwatersrand (Botany and Microbiology majors).

1986 Completed Bachelor of Science (Honours) degree, with distinction, at the University of the Witwatersrand (Project areas: tissue culture and plant pathology).

1992 Obtained a Master of Science degree at the University of Natal (Thesis title: Development of *in vitro* culture and gene transfer techniques in sugarcane (*Sachharum* spp. hybrids)).

1998 Enrolled for a PhD degree at the University of Stellenbosch. Experimental work was carried out in the Biotechnology Department, SASEX, Mount Edgecombe.
Current position held: Senior Research Officer, SASEX.

Publications arising from PhD thesis to date:

Snyman SJ, Watt MP, Huckett BI and Botha FC (2000) Direct somatic embryogenesis for rapid, cost effective production of transgenic sugarcane (*Saccharum* spp. hybrids). Proceedings of the South African Sugar Technologists Association 74: 186-187.

Leibbrandt NB and Snyman SJ (2001) Initial field-testing of a transgenic gluphosinate ammonium resistant sugarcane. Proceedings of the South African Sugar Technologists Association 75: 108-111.

Snyman SJ, Huckett BI, Botha FC and Watt MP (2001) A comparison of direct and indirect somatic morphogenesis for the production of transgenic sugarcane (*Saccharum* spp. hybrids). Acta Horticulturae no. 560:105-108.

Leibbrandt NB and Snyman SJ (2002) Stability of gene expression and agronomic performance of a transgenic herbicide-resistant sugarcane line in South Africa. Crop Science (submitted 7 February 2002).

International Conference presentations during the course of the study:

Snyman SJ, Leibbrandt NB and Botha FC (1998) Expression and stability of herbicide resistance in transgenic sugarcane. Poster presentation at IX International Congress on Plant Tissue and Cell Culture, Jerusalem, Israel (June 14 – 19, 1998).

Snyman SJ, Huckett BI, Botha FC and Watt MP (2000) A comparison of direct and indirect somatic morphogenesis for the production of transgenic sugarcane (*Saccharum* spp. hybrids). Oral presentation at Fourth International Symposium on *In Vitro* Culture and Horticultural Breeding, Tampere, Finland (July 2 – 7, 2000).