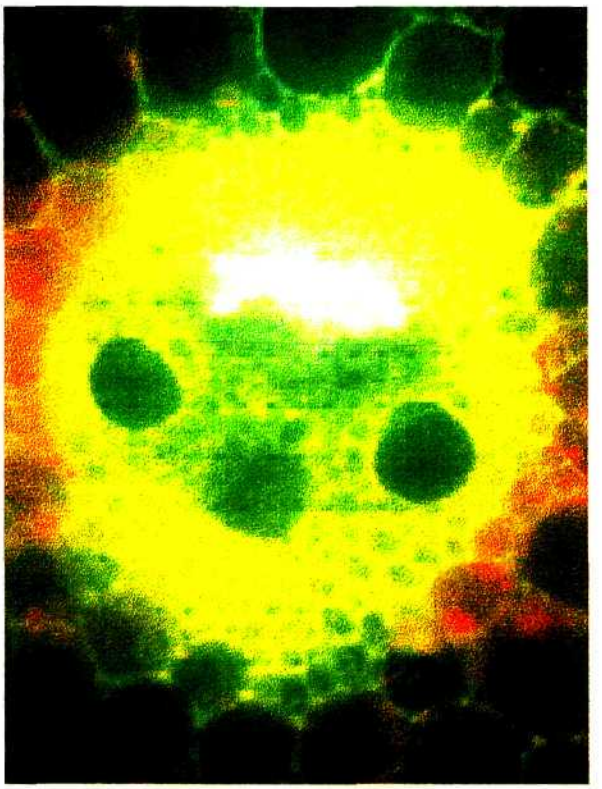

PROMOTERS FOR SUGARCANE TRANSFORMATION: ISOLATION OF SPECIFIC SEQUENCES AND EVALUATION OF *rolC*

by

Sarita Groenewald

Submitted in fulfilment of the academic
requirements for the degree of
Philosophiae Doctor
in the
Department of Botany,
University of Natal

Supervisor: Prof. F.C. Botha

Pietermaritzburg
1997

PREFACE

The experimental work described in this thesis was carried out in the Biotechnology Department of the South African Sugar Association Experiment Station (SASEX), Mount Edgecombe, from January 1994 to December 1997, under the supervision of Prof. F.C.Botha.

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

ACKNOWLEDGEMENTS

- My sincere thanks go to my supervisor, Prof. Frikkie Botha, for his advice, encouragement and support through the duration of this study. His enthusiasm and inquisitiveness have made this study an exciting experience.
- My thanks and appreciation are also extended to the following:
- Hennie Groenewald for his advice and assistance with the construction of expression vectors and photography;
- Dr. Barbara Huckett for her advice on plant molecular biology and Sandy Snyman for her help and advice on sugarcane tissue culture;
- Sharon McFarlane (Plant Pathology Department, SASEX), James Wesley-Smith (Electron microscopy unit, UND), Edith Elliot (Biochemistry Department, UNP) and Vihitha Beharee (Microbiology Department, UNP) for their assistance with the microscopy;
- Peter Hare (Botany Department, UNP) for the subcloning of the mGFP4 reporter gene into pBluescript(SK+);
- Chris Davies (CSIRO, Australia) for advice on northern blots;
- Julie Barnes for the careful and critical reading of this thesis, and for her friendship and enthusiasm;
- My colleagues in the Biotechnology Department (SASEX) for their friendship, support and encouragement;
- My family and friends for their love and support through the years;
- The South African Sugar Association and the Foundation for Research Development for financial support.
- A special thanks go to my husband, Hennie, for his love, patience and support during this study.
- I am most grateful for the privilege of studying God's creation at molecular level.

ABSTRACT

Increasing the sucrose yield and the disease resistance of plants are two major objectives of the transgenic sugarcane plant programme in South Africa. The sugarcane culm has thus been identified as one of the main target areas for transgene expression. A shortage of reliable promoter elements as well as patent limitations have necessitated the isolation of promoters that are preferentially expressed in the sugarcane culm. In the present study two different approaches were followed to isolate such promoters, and the bacterial promoter, *rolC*, was evaluated for tissue-specific expression in sugarcane.

Differential display is a non-directed technique that was used to identify genes that are differentially expressed in the mature sugarcane culm. The original method was modified, and four putative culm-preferential fragments were isolated. Sequence and hybridisation analyses revealed that these fragments were false positives, and could therefore not be used to obtain a culm-specific promoter.

Activity of the *Agrobacterium rolC* promoter was evaluated by analysing expression patterns of two reporter genes in the mature culm of transgenic sugarcane plants. Nucleic acid analyses indicated that the foreign DNA was incorporated into the sugarcane genome, and that mRNA transcripts were produced. Histochemical analysis was done to visualise *rolC*-driven GUS and GFP expression in the mature sugarcane culm. In both cases the reporter gene expression was restricted to the vascular bundles and specifically to the phloem.

A directed approach was followed to isolate the gene and subsequently the promoter of the β-subunit of pyrophosphate-dependent phosphofructokinase (PFP-β). An incomplete cDNA clone was obtained from a mature culm cDNA library, and was used for the screening of a sugarcane genomic library. Two clones containing different parts of the PFP-β gene were isolated. A Deletion Factory™ system was used to analyse the clone containing the 5' end of the gene. The first five exons and 1747 bp of the 5' flanking region of the gene were sequenced. Preliminary activity

analysis of the promoter region was done by constructing two expression vectors, and analysing transient GUS expression in sugarcane callus. Results indicated that the promoter is capable of driving foreign gene expression in callus. Transient expression levels were lower than that of the maize *Ubi-1* promoter. Further analysis of the 5' flanking region will be done to establish whether *cis*-acting elements outside the analysed area have an influence on the activity of the promoter.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2,4-D	2,4-dichloro-phenoxyacetic acid
35S	Cauliflower mosaic virus *35S* promoter
4-MUG	4-*methylumbelliferyl*-β-*glucuronide*
5' UTR	5' untranslated region
A	clockwise deletion subclones
aa	amino acid
ABA	abscisic acid
ABRE	abscisic acid response element
Act1	rice actin-1 promoter
Adh1	maize alcohol dehydrogenase-1 promoter
Arg	arginine
Asp	asparagine
ATP	adenosine 5'-triphosphate
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
bp	base pair
BP	band-pass
BSA	bovine serum albumin
cab	chlorophyll a/b binding protein
CaMV	cauliflower mosaic virus
cat	chloramphenicol acyltransferase
cDNA	complementary deoxyribonucleic acid
CLP	chlorallactophenol
dbEST	expressed sequence tag database
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DRE	dehydration response element
dT	deoxithymidine

EDD	enhanced differential display
EDTA	ethylenediaminetetraacetate
EMBL	European molecular biology laboratory
ERE	elicitor-responsive element
EST	expressed sequence tag
FITC	fluorescein isothiocyanate
Fru-1,6-P_2	fructose 1,6-bisphosphate
Fru-2,6-P_2	fructose 2,6-bisphosphate
Fru-6-P	fructose 6-phosphate
g	gravitational acceleration (9.806 m s^{-1})
G418	geneticin
GA	giberellic acid
GFP	green fluorescent protein
Gly	glycine
GUS	β-glucuronidase
HDC	high definition color
HSE	heat shock element
HSF	heat shock transcription factor
iPCR	inverse polymerase chain reaction
K	Counterclockwise deletion subclones
kb	kilobase pair
lac	β-galactosidase
LP	long-pass
LRP	light-responsive promoter
luc	firefly luciferase
mas	mannopine synthase
MAST	magnet-assisted subtraction technique
MJ	methyl jasmonate
MOPS	3-[*N*-morpholino]-propanesulphonic acid
MRC	Medical Research Council
mRNA	messenger ribonucleic acid

MS	Murashige and Skoog medium
M-MuLV	Moloney Murine Leukemia Virus
nos	nopaline synthase
NPT	neomycin phosphotransferase
NR	non-redundant
OCS	octapine synthase
OD	optical density
ORF	open reading frame
ori	origin of replication
PAT	phosphinothricin acyltransferase
PCR	polymerase chain reaction
PFK	phosphofructokinase
PFP	pyrophosphate-dependent phosphofructokinase
pfu	plaque forming units
PG	polygalacturonase
Pi	inorganic phosphate
PIG	particle inflow gun
PLRV	potato leafroll virus
PPi	pyrophosphate
PR	pathogenesis-related
PTT	phosphinotricin
Pu	purine
PVP	polyvinylpyrrolidone
R	pUC18/M13 reverse primer
RAPD	random amplification of polymorphic DNAs
rbcS	ribulose 1,5-bisphosphate carboxylase-oxygenase
Ri	*root-inducing*
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT	reverse transcription

RT-PCR	reverse transcription polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SASEX	South African Sugar Association Experiment Station
SBDS	Southern blot differential screening
SDS	sodium dodecyl sulphate
Ser	serine
Sh1	*Shrunken-1* (maize sucrose synthase1 gene)
spp.	species
TL-DNA	transfer DNA of *Agrobacterium*
Ti	tumor-inducing
Tm	temperature, melting
TMV	tobacco mosaic virus
tRNA	transfer RNA
Tris	Tris(hydroxymethyl)-aminomethane
Tyr	tyrosine
Ubi-1	maize polyubiquitin-1 promoter
UDPGlc	uridine 5'-diphosphate glucose
uidA	β-glucuronidase gene
UV	ultraviolet
vir	virulence gene
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-glucuronic acid

CHAPTER 1
INTRODUCTION

Spectacular progress has been made in the genetic manipulation of plants over the past two decades. Molecular techniques have now been applied to over 120 species in at least 35 families, including most major economical crops, vegetables, ornamental and medicinal plants (Birch, 1997). The refinement of techniques to introduce and express diverse foreign genes in plants and the development of sophisticated techniques to recover intact plants after transformation are the two major factors responsible for this progress. Transformation of sugarcane is also well established (Bower and Birch, 1992), and the genetic manipulation of this crop is now possible.

In South Africa the sugarcane breeders are faced with two major concerns, the first being the increase of sucrose yield and the second being resistance to the numerous pathogens and pests that cause major crop losses. Sucrose is the third most important agricultural export product in South Africa and a valuable source of revenue with a net income of approximately 3 600 million rand per season. An average annual harvest of 20 million tons of cane reaches the mills, yielding 2.3 million tons of sucrose. Improvement of sucrose yield is thus an important objective of the South African sugar industry for it to maintain international competitiveness. Over the past two decades, however, there has been little improvement in sucrose yield through conventional plant breeding, despite increased efforts in this regard. Although this is partly due to the difficulty of selecting for the desired phenotype in field trials, it may also be an indication that the genetic resources available in the parental germplasm have been substantially exploited already. If this is the case, genetic engineering could play a major role in the future.

The biotechnology programme at the South African Sugar Association Experiment Station (SASEX, Mount Edgecombe, Kwazulu Natal) is aimed at the improvement of the varietal potential of sugarcane, and is closely linked to the plant breeding

programme. One of the main focal points of the sugarcane biotechnology programme at SASEX is genetic engineering. The present study forms part of the transgenic plant programme.

To achieve effective transfer of foreign DNA into sugarcane cells, appropriate genetic constructs need to be made to facilitate integration and expression of the transgene. A typical genetic construct will contain a promoter, a transgene and a terminating signal. Several common promoters and genes that are active in different plants are currently available. There is, however, a commercial limitation to the use of many of these elements. Because the development of a promoter or resistance gene is effectively an invention, it is eligible for patent protection. Patents have already been issued on most established or promising genes and promoters, which has necessitated a renewed effort to isolate novel regulatory elements from plants. Another phenomenon that has been the cause of major concern in transgenic plant programmes is transgene silencing (Finnegan and McElroy, 1994). The mechanism of silencing is not clear, but it can occur at either transcriptional or post-transcriptional level, and from one generation to another, or somatically within a single plant (Jorgensen, 1995). The genetic material is not lost from the genome, but is simply no longer expressed. The use of stable promoters is thus a prerequisite for the successful production of transgenic plants.

The use of molecular technology to increase product yield through the manipulation of key enzymes requires strict control of transgene expression in certain tissue types or at specific developmental stages. This is especially true when attempts are being made to alter metabolism. In sugarcane, sucrose metabolism should be altered in the culm, preferably in the storage parenchyma, and not in the leaves or other tissues. Changes in sucrose metabolism in the whole plant will most probably have a negative affect on growth and yield.

The shortage of reliable regulatory elements to drive expression of transgenes in specific areas of the plant as well as patent considerations have made it necessary

to isolate specific promoters from sugarcane, and to investigate the performance of other possibly useful elements in sugarcane cells. Since sucrose is accumulated in the culm and some pathogens gain entry to the plant through the buds, the culm has become one of the major target areas for transgene expression in sugarcane and is thus the focus area from which possible promoters are isolated.

The aims of the present study was firstly to isolate promoter(s) that will drive stable transgene expression in the sugarcane culm, and secondly, to evaluate the use of the bacterial promoter, *rolC*, for tissue-specific expression in sugarcane.

Two distinct groups of techniques are available for the isolation of differentially expressed genes and these include non-directed and directed techniques. An overview of these techniques are discussed in Chapter 2. Both of these approaches have been followed in the present study.

The differential display technique is a non-directed technique that was developed to identify and characterise differentially expressed genes by detecting individual mRNA species, and to subsequently isolate and analyse the cDNA (Liang and Pardee, 1992). Polymerase chain reaction (PCR) is used to amplify cDNA fragments of different tissues after which amplification profiles are compared. The technique has been used to isolate differentially expressed genes in a number of experimental systems and a modified version of the technique was evaluated for the identification of culm-specific genes in sugarcane. Differential display analysis of four different sugarcane tissues, and refinements of the technique for use in sugarcane are discussed in Chapter 3.

Regulatory elements of certain genes in *Agrobacterium tumefaciens* and *A.rhizogenes* are not expressed in *Agrobacterium*, but become active on transfer to plant cells. One of these promoters, *rolC*, has been shown to drive phloem-specific gene expression in transgenic tobacco (Schmülling *et al.*,1989), rice (Matsuki *et al.*, 1989) and hybrid aspen (Nilsson *et al.*, 1996). The *rolC* promoter was also found to

be activated by sucrose in phloem cells of transgenic tobacco plants (Yokoyama *et al.*, 1994). Phloem-specific transgene expression in sugarcane will be advantageous in cases where resistance to phloem-feeders such as aphids and leafhoppers that carry viruses is conferred or where viral resistance genes are introduced into the plant. A sucrose-activated promoter is also of great benefit for studying sucrose metabolism in the sugarcane plant. In addition, phloem-specific gene expression may impose a decreased metabolic load on the plant in comparison to constitutive expression, and this may be crucial for the stable expression of transgenes.

The tissue-specificity of the *Agrobacterium rolC* promoter was investigated for the first time in sugarcane culm tissue. Transformation of sugarcane using two different reporter gene constructs, and the observed *rolC* expression patterns in mature sugarcane culm tissue are discussed in Chapter 4.

Another approach for the isolation of promoter elements is based on prior knowledge of the expression pattern of a particular gene or the sequence of the protein. The expression patterns of key enzymes involved in carbohydrate metabolism in sugarcane have been studied (Botha *et al.*, 1996). Although the expression (total activity per internode) of several of the enzymes increases with culm maturity one of these, the pyrophosphate-dependent phosphofructokinase (PFP), is of particular interest. PFP activity is negatively correlated with sucrose content in all analysed sugarcane varieties, and is apparently preferentially expressed in the peripheral areas of the culm (1996, unpublished[1]). These properties suggest that the PFP promoter might be an ideal candidate for the enrichment of transgene expression in the mature culm. The PFP enzyme is a heterotetramer with two α and two β-subunits, and the β-subunit has been shown to be the catalytic subunit (Yan and Tao, 1984; Carlisle *et al.*, 1990).

The gene sequence of PFP-β has been determined in castor bean and potato, and

---

[1]F.C.Botha, Institute for Plant Biotechnology, Private Bag X1, Matieland, 7602, South Africa

partially in rice. This information was used in a directed approach to isolate the gene and subsequently the promoter from sugarcane. Since this is the first reported investigation of the activity of the PFP-β promoter, two different constructs were used to evaluate its expression in sugarcane cells. Activity analyses of these two constructs containing the PFP-β promoter in sugarcane are discussed in Chapter 5.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

The present study was aimed at the identification and isolation of potential promoter elements for sugarcane transformation. Comprehensive reviews on plant promoters (Guilfoyle, 1997) and their use in transformation studies have been written in the past five years (An and Kim, 1993; Christou, 1996; Birch, 1997). This chapter is therefore not an attempt to cover all aspects of plant promoters, but is rather an overview of those issues that are relevant to the current work. For more detailed information the reader is referred to these review papers.

As is evident from the available literature, two distinct approaches can be used to identify and isolate new promoters. The first is independent of prior knowledge of a specific gene and is directed towards a group rather than an individual gene. The second is aimed at a specific gene and requires some information of the gene or gene product (protein). Both these approaches have been used in the current investigation.

However, due to the very limited information on sugarcane gene sequences and gene expression patterns, options in the second approach are limited. In previous work in the South African sugar industry, evidence was found that the expression of the pyrophosphate-dependent phosphofructokinase (PFP) is high in culm tissue and apparently increases in more mature tissues. This makes the PFP promoter an interesting candidate for culm directed gene expression. Some background information on PFP is therefore included in this chapter.

2.2 PLANT PROMOTERS

The regulation of gene expression in plants is controlled at both the transcriptional and post-transcriptional level. Multiple levels of control exist, including splicing, 3' end formation, stability, and translation of the mRNA as well as post-translational events

such as protein stability and modification (Sullivan and Green, 1993).

Transcription takes place in three stages, namely initiation, elongation and termination. Initiation begins with the binding of RNA polymerase to the promoter region of each gene, typically 40-200 nucleotides upstream of the translation initiation codon, ATG.

Plant genes can be divided into three classes which are defined by the type of promoter. Each class of gene promoter is recognised by a different polymerase enzyme: ribosomal RNA (rRNA) is transcribed by RNA polymerase I, messenger RNA (mRNA) by RNA polymerase II, and transfer RNA (tRNA) and other small RNAs by RNA polymerase III (An and Kim, 1993). In addition to RNA polymerases, *trans*-acting proteins or transcription factors are needed for transcription initiation. Elongation occurs by movement of the RNA polymerase along the DNA, extending the RNA chain until RNA synthesis is terminated at the transcription terminator region where the transcription complex dissociates (An and Kim, 1993).

Eukaryotic promoters are composed of many *cis*-acting control motifs that are recognised by specific *trans*-acting factors. The main characteristics of promoter motifs are distance-dependent activation and the requirement for location 5' to the cap site (Thangue and Rigby, 1988), an unusual 5'→5' triphosphate linkage involving a 7-methylguanine residue which is added after transcription (Grierson and Covey, 1984).

Most promoters contain a TATA box (TATA(T/A)A(T/A)), an AT-rich sequence located 21 to 35 nucleotides upstream from the transcription start site (Breathnach and Chambon, 1981). This region is surrounded by GC-rich sequences which may play a role in its function (Lefebvre and Gellatly, 1997) and interacts with a TATA-binding protein which comprises the TFIID transcription factor complex necessary for accurate initiation of promoters transcribed by RNA polymerase II (Rosenthal, 1987; Mukumoto *et al.*, 1993; Czarnecka-Verner *et al.*, 1994). Deletion of the TATA box

reduces promoter activity and results in multiple transcription start sites (An and Kim, 1993). In some promoters, such as those for the maize zein gene, two TATA boxes may be found as close as 10 nucleotides apart (Lefebvre and Gellatly, 1997).

Another control motif, the CCAAT box sequence, is located close to the -80 region of several, but not all plant promoters and is functional in both orientations (Thangue and Rigby, 1988). It has been established that a family of transcription factors recognise this motif and that factors binding to this sequence can co-operate with those binding to surrounding motifs (Thangue and Rigby, 1988).

A hexamer sequence, TGACGT, occurs in most constitutive promoters within a few hundred nucleotides from the transcription start site (An and Kim, 1993). These hexamer motifs are often found as repeats separated by six to eight nucleotides, and deletion analysis has indicated that these motifs are essential for the transcription activity of the cauliflower mosaic virus (CaMV) *35S*, octapine synthase (*ocs*) and nopaline synthase (*nos*) promoters (An and Kim, 1993). Genes for the transcription factor that specifically interacts with the hexamer motifs have been isolated from both dicotyledonous and monocotyledonous plant species (Katagiri *et al.*, 1989; Singh *et al.*, 1990).

Motifs that are found in promoters of genes that are environmentally inducible include the G-box sequence (CCACGTGG) in various photosynthesis gene promoters (An and Kim, 1993) and the H-box sequence (CCTACC) which, together with the G-Box sequence, is essential for the expression of the bean chalcone synthase gene (Arias *et al.*, 1993). AT-rich motifs appear to increase the amplitude of induction in heat-shock protein genes(Czarnecka-Verner *et al.*, 1994) and may play a regulatory role in several other higher plant genes (Forde, 1994). Examples of genes that contain AT-rich motifs are nodulin genes, seed-specific genes like lectin, trypsin inhibitor and β-phaseolin, photoregulated genes and some heat shock genes (Forde, 1994).

Another group of *cis*-acting regulatory sequences are called enhancers. These share

common characteristics with promoters and they are involved in increasing the level of transcription. Enhancers are typically 100-200 base pairs in length and can function from a distance, sometimes several thousand base pairs, independently of orientation and either from the 5' or 3' end of a gene (Lefebvre and Gellatly, 1997). They can also activate transcription from heterologous promoters with exceptional positional flexibility (Hatzopoulos *et al.*, 1988). These characteristics distinguish enhancers from other regulatory elements. In some cases, however, tandem copies of some promoter motifs can acquire enhancer properties (Thangue and Rigby, 1988). Enhancers from different eukaryotic organisms are very similar, for example many ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcS*) genes from dicots contain a sequence closely related to an animal enhancer, GTGTGG(T/C)(T/C)A(A/T)TA(T/A)G, at -140 from the transcriptional start site (Kuhlemeier *et al.*, 1987). Enhancer sequences can also function as gene silencers (Lefebvre and Gellatly, 1997).

Promoters are classified in three groups, namely constitutive, inducible and tissue-specifically or developmentally regulated. Plant organelles also contain their own class of promoters and some of these play an essential role in the control of gene expression during photosynthesis and CO_2 fixation. The promoters that are chosen for transformation studies will vary according to the expression pattern that is preferred (Gruber and Crosby, 1993).

2.2.1 Constitutive promoters

Constitutive promoters are active during most stages of development and in most plant tissues. A gene is classified as constitutive when its expression remains constant on a per cell basis under all conditions examined (Kuhlemeier *et al.*, 1987). This implies that there must be little variation of mRNA abundance or translational efficiency during development, in different organs or upon application of various endogenous or environmental stimuli (Kuhlemeier *et al.*, 1987). Examples of constitutive promoters are the nuclear gene promoter for the β-subunit of the mitochondrial ATPase complex (Boutry and Chua, 1985), and the rice actin promoter

Act1 (Zhang *et al.*, 1991).

2.2.2 Inducible promoters

There is a wide variety of inducible promoters, a few of which include temperature-inducible, hormone-inducible, pathogen-inducible and light-inducible promoters (Gruber and Crosby, 1993). Important *cis*-regulatory regions with consensus sequences identified in inducible promoters are summarised in Table 2.1.

2.2.2.1 Temperature-inducible promoters: Heat-shock promoters

The heat stress response in plants and other organisms comprises a highly complex but transient reprogramming of cellular activities necessary to protect the cells from extensive damage and to provide optimum conditions for recovery after the stress period (Scharf *et al.*, 1994). Heat shock proteins are sorted into four major classes on the basis of their sizes. They are encoded by nuclear genes but are localised in different cell compartments, including the cytoplasm, chloroplast, mitochondria and endoplasmic reticulum (Czarnecka-Verner *et al.*, 1994).

The thermo-inducible expression of heat shock proteins is attributed to the presence of controlling DNA sequences located in their promoters (for reviews see Czarnecka-Verner *et al.*, 1994; Scharf *et al.*, 1994). These conserved sequences were named heat shock elements, or HSEs (Scharf *et al.*,1994; Czarnecka-Verner *et al.*, 1994). Single, overlapping or multiple copies of the HSEs can occur in the promoter, usually within 15 to 18 bp upstream from the TATA box (Czarnecka-Verner *et al.*, 1994). The concise analysis of HSEs has resulted in a general structure which represents multimers of five nucleotide modules with alternating, palindromic orientation: 5'-(aGAAn)(nTTCt)(aGAAn)(nTTCt)-3' (Scharf *et al.*, 1994). Three consecutive modules are evidently the minimum combination required for an active heat shock promoter. In appropriate configurations, the HSE confers both high selectivity of heat shock induction and high overall efficiency of transcription initiation (Scharf *et al.*, 1994).

Most heat shock promoters contain TATA and CCAAT boxes. The requirement for

Table 2.1 *Cis*-regulatory regions identified in plant inducible promoters.

GENE	INDUCER	REGULATORY REGION	ELEMENT/ MOTIF	CONSENSUS SEQUENCE (5'-3')	REFERENCE
Temperature- and water stress-inducible promoters					
Heat shock proteins (several)	High temperatures	1)15-18 bp from TATA-box 2)21-35 bp from transcription start site 3)-80 region		1)[(aGAAn)(nTTCt)]2	Scharf *et al.*, 1994
			TATA-box	2)TATA(T/A)A(T/A)	Lefebvre and Gellatly, 1997
			CCAAT-box	3)CCAAT	Czarnecka *et al.*, 1989
Potato *ci7*	Low temperatures	-2000 to +1		1)[(G/A)CCGACAC]2	Kirch *et al.*, 1997
			G-box	2)[CACGTG]6	
Arabidopsis rd29a	Dehydration due to drought, low temperatures or salt stress	Not specific		TACCGACAT	Hajela *et al.*, 1990
Maize *rab28*	Water stress	Not specific		CCACGTTGG	Pla *et al.*, 1991
Hormone-inducible promoters					
Wheat embryo protein, *Em*	ABA or signals that cause changes in ABA levels		ABRE	TTGCCGGACACGTGGCGCGCGA	Quatrano *et al.*, 1993
Barley *HVA22*	ABA	1)-94 to -85 2)-64 to -56	ABRE3 CE1	1)GCCACGTACA 2)TGCCACCGG	Shen & Ho, 1995
Rab17	ABA	1)-191 2)-148		1)GGCCACCGA 2)CGTACGTGTA	Vilardell *et al.*, 1990
CdeT27-45	ABA	1)-213 2)-196		1)TTGCACCGT 2)GGCACGTATG	Michel *et al.*, 1993
HVA1	ABA	1)-79 2)-99		1)GAGCACCGC 2)CCTACGTGGC	Straub *et al.*, 1994
Lycopersicon esculentum LE25	ABA	1)-93 2)-130		1)ACTCACCAC 2)AAAACGTGTC	Cohen and Bray, 1992
Soybean *GH3* & *SAUR*	Auxin	-76 (*GH3*) -30 (*SAUR*)			Hagen,1994
Tobacco chitinase *chn48*	Ethylene	-503 to -358	GCC-box (x2)	[TAAGAGCCGCC]2	Shinshi *et al.*, 1995
Tobacco *prb-1b*	Ethylene	-187		CACGTG	Somssich, 1994
Bean chitinase *CH5B*	Ethylene	-236 to -305	ERE		Roby *et al.*,1991
Lycopersicon esculentum L. LeMTB	Ethylene			1)TGCRCBCG (R=purine, B= anything but A) 2)AATTCAAA	Whitelaw *et al.*, 1997
PRB1b	Ethylene	-213 to -141		TAAGAGCCGCC	Sessa *et al.*, 1995
Barley high pI α-amylase	GA	-203		1)TAACAAA 2)TATCCAC	Gubler & Jacobsen, 1992
Cereal α-amylase *Amy23b*	GA & ABA		GARE	1)CCTTTT 2)TATCCATGCAGTG	Lanahan *et al.*, 1992
Tomato lipoxygenases *tomlox A* & *tomlox B*	Methyl jasmonate (in seedlings)	-2400 to +1		GATAcAnnAAtnTGATG	Beaudain & Rothstein, 1997

Pathogen- inducible promoters and promoters induced in defense responses					
Tobacco class I β-1,3-glucanase B	Pathogen attack	-1452 to -1193		[AGCCGCC]2	Vögeli-Lange *et al.*, 1994
Tobacco chitinase *CHN50*	Elicitor	1)-698 to -637 2)-47 to -68 3)-539 to -518	AGC-box Silencer	1)AGCCGCC 3)GTCAGAAAAGTCAG	Fukuda & Shinshi 1994
PR-1a	SA and TMV infection	-902 to -625			Van de Rhee *et al.*, 1990
PR-5 thaumatin-like protein	TMV	-718 to -1364			Nelson *et al.*, 1992
Parsley *PR2*	Fungal elicitor	-52 to -168			Van de Löcht *et al.*, 1990
Parsley *CCoAOMT*	Stress/ elicitor	-139		(A/T)CCC(A/T)(T/A)C(A/T)(A/T)(C/G	Grimmig & Matern, 1997
Chitinase and glucanase	Bacteria, fungi, fungal elicitor, ethylene, SA, wounding and cytokinin	Not specific	AGC-box	AGCCGCC	Somssich, 1994
Proteinase inhibitor IIK	1)Wounding, elicitor, light 2)MJ	-136 to -557		1)AAGCGTAAGT 2)ACCTTGCC	Palm *et al.*, 1990 Kim *et al.*, 1992
Asparagus *AoPR1*	Wounding	-574		CACGTG	Warner *et al.*, 1993
Soybean CHS genes	Elicitor	-982 -175 to -134	G-box	Similar to ACCTGACC	Wingender *et al.*, 1990
Light-inducible promoters					
Soybean CHS genes	UV-light	-42		TACCTACCAA	Wingender *et al.*, 1990
Pea *rbcS-3A*	1)White light 2)UV-light 3)White light	1)-151 to -138 2)-211 to -201 3)-184 to -176	1)GT-box I 2)G-box 3)GA-1	1)GTGTGGTTAATATG 2)CACATGGCACT 3)ATGATAAGG	Gilmartin *et al.*, 1990
Lemna rbcS SSU5B	White/ red light	1)-156 to -141 2)-85 to -60	1)X-box 2)Z-box	1)GATAAGGATGTAATCC 2)GATCGAGCGATGGTAGCCGTAGAA	Buzby *et al.*, 1990
Arabidopsis FAD7	Light (tissue-specific)	-322		1)GGTATA 2)GGTGAA	Nishiuchi *et al.*, 1995
Lemna gibba cabAB19	Phytochrome	-134 to -106		1)AACCAATCCCAACC 2)AAGCTCCGATAGAGGG	Kehoe *et al.*, 1994
Wheat *cab1*	Phytochrome	-142 to -114		1)CACCAATGGCATCC 2)AAGCTGCAGATTTCT	"
Pea *cabAB80*	Phytochrome	-82 to -54		1)CTCCAACTAG 2)TAGCTTTAGATAACAC	"
Arabidopsis cab140	Phytochrome	-89 to -61		1)AGCCAATAGCAACCTC 2)AGAGATTGATATT	"
Maize *cab1*	Phytochrome	-92 to -64		1)AGCCAATGGCAACT 2)CGTCTTAAGATTCCA	"

these motifs together with the HSEs has been demonstrated by deletion analysis (Czarnecka *et al.*, 1989). Promoters of several soybean genes encoding heat shock proteins contain numerous blocks of contiguous AT-rich sequences located upstream from the TATA box. They vary in length and are not fixed to a specific position in the 5' flanking region. They do not show strict sequence conservation but frequently exist as repeats of TAAT or ATTA motifs (Czarnecka-Verner *et al.*, 1994).

Transcriptional activation of heat shock genes in response to elevated temperatures or chemical levels is mediated by the interaction of the *trans*-acting heat shock transcription factors (HSFs) with HSEs of the promoter. The genes for the HSFs have been cloned and characterised (Scharf *et al.*, 1994).

2.2.2.2 Temperature-inducible promoters: Cold-induced promoters

Plants from tropical and subtropical regions show injury symptoms when exposed to non-freezing low temperatures below 10 - 15°C. The extent of chilling injury depends on the duration and severity of treatment (Yoshida and Kato, 1994). Plant response to low temperature leads to alteration of the cellular metabolism and is correlated with significant changes in gene expression (for reviews see Guy, 1990; Palva, 1994). Cold-regulated genes have been isolated and characterised in several plant species including alfalfa (Wolfraim *et al.*, 1993), *Arabidopsis* (Hajela *et al.*, 1990), spinach (Neven *et al.*, 1993), tomato (Schaffner and Fischer, 1990), barley (Cattivelli and Bartels, 1990) and wheat (Houde *et al.*, 1992). In the *Arabidopsis rd29a* promoter a dehydration-responsive element (DRE) was identified (TACCGACAT). Within 1 kb of the transcription start site another two conserved sequence elements were identified and six G-box motifs (CACGTG) were found to be spread over the whole promoter (Kirch *et al.*, 1997).

2.2.2.3 Hormone-inducible promoters

Plant hormones are considered to be an important class of internal signal compounds which are easily transported to target cells and have been shown to be directly involved in transcriptional and translational control of gene activity in a number of

plant processes. Specific regulatory elements have been identified for hormone-responsiveness and several review papers have been written (Kuhlemeier *et al.*, 1987; Quatrano *et al.*,1993; Giraudat *et al.*, 1994; Barendse and Peeters, 1995; Gatz, 1997). A few of the important examples are discussed below.

The growth regulator abscisic acid (ABA) is known to induce the synthesis of specific proteins, among which are certain seed-storage proteins (Kuhlemeier *et al.*, 1987). Examples of genes that are expressed due to ABA influence include the beta conglycinin α-subunit, lectins and agglutinins, and these may be involved in maintaining seed dormancy, in protecting seeds against dessication, and in preventing deleterious effects of hydrolytic enzymes (Kuhlemeier *et al.*, 1987).

A protein that has been found to be ABA-inducible is the 10 kD soluble protein, *Em*, which accumulates in wheat embryos in the latter third of grain development (Williamson and Quatrano, 1988). When the embryos were removed from the grain in the first third of grain development, the *Em* protein did not accumulate and the embryos germinated precociously. In the presence of ABA, however, *Em* did accumulate in these embryos and normal germination occurred (Williamson and Quatrano, 1988). The *Em* gene is not expressed in response to auxin, cytokinin, salicylic acid (SA) or giberellic acid (GA). However, environmental signals that are believed to be transduced, at least in part, by changes in ABA levels, e.g. desiccation, temperature extremes, salinity and wounding, can result in *Em* gene expression (Quatrano *et al.*, 1993)

The *Em* promoter was functionally dissected and it was found that the 5' distal region consists, at least in part, of an AT-rich region that appears to be responsible for quantitative levels of expression through effects on transcription. The next region of regulatory sequences is the ABA response element (ABRE) which is responsible for the dramatic increase of expression in the presence of ABA. The consensus sequence of the ABRE is 20 bp in length with the sequence 5'-TTGCCGGACACGTGGCGCGA-3'. A third component of the *Em* gene 5' flanking

region, the 5' untranslated region (5'UTR), also appears to be involved in the ultimate level of expression from the *Em* promoter at a post-transcriptional level (Quatrano *et al.*, 1993).

The plant hormone gibberellic acid (GA) is produced upon the onset of germination and induces the synthesis of a number of enzymes involved in the breakdown of seed storage products, for example alpha-amylase (Kuhlemeier *et al.*, 1987). Deletion analysis of the promoter of a high-pI α-amylase gene of barley has shown that the major GA- and ABA-responsive elements are located between -174 and +53. The results showed that both the TAACAAA- and the TATCCAC-boxes play an important role in GA-regulated expression (Gubler and Jacobsen, 1992).

Auxins and cytokinins also influence expression of genes including those that are involved in maintenance of apical dominance, cell elongation and xylem differentiation (Kuhlemeier *et al.*, 1987). The promoter regions of two soybean auxin responsive genes, *GH3* and *SAUR,* have been analysed (Hagen *et al.*, 1994). The auxin-responsive elements were defined to a 76 bp region in the *GH3* promoter and to a 30 bp region in the *SAUR* promoter (Hagen *et al.*, 1994).

The plant responds to pathogen infection or other stress by producing the stress hormone ethylene and this is the signal for plants to activate the defense mechanism against pathogens. Inducible defense responses are mediated, at least in part, by ethylene-induced gene expression (Shinshi *et al.*, 1995). The promoter of an ethylene-induced tobacco class I basic chitinase gene, *Chn48*, has been examined in order to identify ethylene-responsive elements (Shinshi *et al.*, 1995). In transgenic tobacco plants a chimeric construct containing a 2 kb *Chn48* promoter fused to the β-glucuronidase (GUS) reporter gene was induced by ethylene in leaf tissues. Deletion analysis indicated that an ethylene-responsive element was located between -503 and -358 relative to the transcription initiation site. This 146 bp sequence conferred ethylene-responsive GUS expression in either orientation upstream of the heterologous promoter, indicating that the sequence functions as a regulatory

enhancer. The ethylene-responsive region contains two copies of the GCC-box (TAAGAGCCGCC), which is conserved in ethylene-responsive defence genes (Shinshi *et al.*, 1995).

2.2.2.4 Pathogen-inducible promoters

Plants are constantly exposed to environmental stress that could be detrimental to their survival. Apart from fluctuations in temperature, changes in nutrient composition and salinity of the soil, flooding, drought, etc., plants are continuously exposed to air- and soilborne bacterial and fungal pathogens, viruses or viroids and insect pests (Somssich, 1994; Van de Rhee *et al.*, 1994).

In order to defend themselves against these biotic stresses, plants possess preformed structural physical barriers, such as the cell wall, and have also developed inducible mechanisms which allow them to respond rapidly to external stimuli. This hypersensitive response causes the cells in the immediate vicinity of the infection site to die, making it impossible for the pathogen to spread through the plant. In nearly all of the systems studied to date, transcriptional activation of specific plant genes plays a key role in the defence of plants against pathogens (Dixon and Lamb, 1990). The proteins induced in this way are all involved in defending the plant against attack or preventing the spread of infection, and include enzymes of the phenylpropanoid pathway, enzymes involved in phytoalexin production, structural cell wall proteins, peroxidases, superoxide dismutases, proteinase inhibitors, thionins and the "pathogenesis-related" (PR) proteins (Van de Rhee *et al.*, 1994). PR proteins have been defined as proteins coded for by the host plant but induced by various pathogens as well as under stress situations similar to those provoked by pathogens (Van Loon, 1990). These proteins may also play a role in enhancing the levels of resistance against secondary challenges by pathogens since they not only accumulate locally around the sites of infection, but also appear in the uninfected parts of the plant (Van de Rhee *et al.*, 1994). This implies the phenomenon of induced resistance or systemic acquired resistance (SAR) (Ross, 1966). Other stress conditions that induce specific subsets of PR proteins include wounding and

ultraviolet (UV) light, chemical substances such as salicylic acid, the plant hormone ethylene and fungal elicitors (Van de Rhee *et al.*, 1994).

PR proteins have been divided into groups according to sequence similarities and immunological cross-reactivities. Five PR protein groups have been defined from tobacco, namely PR-1 to PR-5. Each group has been shown to comprise both extracellular, acidic proteins and intracellular, basic counterparts (Van de Rhee *et al.*, 1993). Proteins from PR-2 and PR-3 have been identified as being β-1,3-glucanases and chitinases, respectively, while PR-5 proteins were identified as being thaumatin-like proteins (Somssich, 1994). Glucans and chitins are major constituents of fungal cell walls. Other PR protein groups have been identified in potatoes, tomatoes, parsley, bean and peas (Somssich, 1994).

The regulatory elements involved in PR gene expression have been studied by fusing large fragments of the respective promoters to appropriate heterologous reporter genes and analysing the expression patterns in transgenic plants. Review papers on regulatory elements governing PR gene expression are available (Somssich, 1994; Van de Rhee *et al.*, 1994) and some examples are discussed below.

i) PR-1 Genes

At least 689 bp of 5' flanking sequences of the *pr-1a* promoter are needed for significant induction of GUS activity by salicylic acid (SA) treatment and infection with tobacco mosaic virus (TMV) in transgenic tobacco. An upstream fragment of nucleotides -902 to -625 from the *pr-1a* promoter was shown to contain salicylic acid and TMV-responsive sequences (Van de Rhee *et al.*, 1993). Deletion analysis of the tobacco basic PR-1 protein gene promoter, *prb-1b*, identified sequences between -141 and -213 to be required for ethylene inducibility. This region contains a G-box consensus core sequence, CACGTG, at position -187 (Somssich, 1994).

ii) PR-2 and PR-3 Genes

Gene expression of β-1,3-glucanase (PR-2) and chitinase (PR-3) appears to be

coordinately regulated in several plant species upon induction by bacteria, fungal elicitors, ethylene, SA, wounding, cytokinin and during development (Meins *et al.*, 1992). One common motif, AGCCGCC (AGC box), has been found in nearly all chitinase and glucanase promoters analysed so far (Somssich, 1994). The developmental and stress-induced expression of a GUS construct containing 2 kb of a β-1,3-glucanase promoter from *Nicotiana plumbaginifolia* in transgenic tobacco has been studied (Castresana *et al.*, 1990). In healthy plants, GUS expression was mainly detected in the roots and lower leaves and was tissue-specific. GUS activity was strongly induced by SA, and bacterial infection leading to the hypersensitive response of the transgenic plants resulted in increased GUS activity around the necrotic lesions (Castresana *et al.*, 1990).

Functional analysis of the bean chitinase *ch5b* promoter in transgenic tobacco plants revealed a region between -195 and -422 which is important for ethylene responsiveness (Broglie *et al.*, 1984). Although this region contains one AGC box at position -205 to -211, subsequent transient expression assays using bean protoplasts more precisely defined the ethylene responsive element to reside between -236 and -305 (Roby *et al.*, 1991). Deletion of the AGC box had no effect on overall expression and it did not appear to function as an enhancer in the bean system. A construct of 1.7 kb of the bean chitinase promoter fused to the GUS gene in transgenic tobacco showed that the promoter is activated by infection with several pathogenic fungi (Roby *et al.*, 1990). GUS expression was very strong around the necrotic regions, but was much reduced further from the infection site, indicating that promoter activity was not strongly induced systemically (Roby *et al.*, 1990).

Transient expression studies of various tobacco chitinase *chn50* promoter-GUS constructs in tobacco protoplasts identified a distal region between -345 and -788 containing sequences required for high level expression, whereas the region between -47 and -68 contains a silencer. Two 12 bp direct repeat sequences, which include one AGC box motif, are located at -698 and -637. The silencer region contains an inverted repeat located between -49 and -70 (Fukuda *et al.*, 1991). The region

between positions -539 and -518 contains a direct repeat, GTCAGAAAAGTCAG. Methylation interference experiments revealed that the repeated sequences are core sequences for the binding of nuclear proteins, suggesting that this motif is a candidate for an elicitor-responsive element (ERE) (Fukuda and Shinshi, 1994).

iii) PR-5 Genes

Analysis of transgenic tobacco plants containing acidic PR-5 (thaumatin-like)-E2 promoter-GUS constructs of varying lengths identified a region between -718 and -1364 to be involved in TMV induction of gene expression (Albrecht *et al.*, 1992). GUS was found both locally within TMV-infected leaves, and systemically within virus-free leaf material. Transcriptional activation of a chimeric construct containing 1 630 bp of the basic PR-5 protein, osmotin, promoter and GUS was observed in the presence of ABA and 100-200 mM NaCl. The promoter lacks any consensus ABREs (Nelson *et al.*, 1992).

iv) PR-6 Genes

Promoter studies of the potato proteinase inhibitor *IIK* gene in transgenic tobacco uncovered a 421 bp sequence between -136 and -557 which is required for wound-induced expression. A 10 bp sequence within this region, AAGCGTAAGT, binds to a wound-inducible nuclear protein derived from tomato leaves. This motif lies adjacent to an 8 bp motif, ACCTTGCC, which is present in several elicitor- and light-responsive promoters (Palm *et al.*, 1990). A methyl jasmonate (MJ) responsive element which coincides with the position of a G-box motif at -574 was also identified by promoter deletion analyses (Kim *et al.*, 1992).

v) PR-10 Genes

In a parsley PR1 promoter an elicitor-inducible *in vivo* footprint (Galas and Schmitz, 1978) was found around position -240, suggesting that protein-DNA interactions important for elicitor-induced expression occur at this site. A constitutive footprint was located further downstream at position -130. An 11 bp motif was found in opposite orientation at the sites of both footprints. The sequences did not show homology to

sequences in other elicitor-responsive promoters (Meier *et al.*, 1991).

Regulatory elements involved in elicitor-mediated induction of gene expression were studied for a second group of parsley PR genes (*pr2*). A 116 bp sequence from -52 to -168 was found to be necessary and efficient for responsiveness to fungal elicitor-mediated gene expression (Van de Löcht *et al.*, 1990).

A wound-responsive gene, *AoPR1*, related in sequence to the parsley *PR1* and *PR2* genes, has been isolated from asparagus. Transgenic tobacco plants containing a 982 bp *aopr1* promoter-GUS construct showed strong GUS expression localised to wound sites and in the vicinity of necrotic lesions caused by infection with *Botrytis cinerea* (Warner *et al.*, 1993). This promoter contains sequences very similar to the ACCTGACC motif in the parsley phenylalanine ammonia-lyase gene and to the H-box sequence (Warner *et al.*, 1993).

2.2.2.5 Light-inducible promoters

Plants use light as a source of information about their environment as well as a source of energy for photosynthesis, and can sense both the quantity (fluence) and quality (wavelength) of light (Terzaghi and Cashmore, 1995). Light-induced responses require changes in both nuclear and chloroplast gene expression and provide the basis for much of plant development (Kuhlemeier *et al.*, 1987). Many of the regulatory effects of light on the growth and differentiation of plants are mediated by the control of gene expression for numerous proteins, including those for a variety of enzymes that change in response to irradiation (Gilmartin *et al.*, 1990). The induction of gene expression as well as the degree to which the genes are expressed are not only determined by the quality and quantity of light, but also by the developmental state of the cells intercepting the light (Fluhr and Chua, 1986). Transcriptional regulation became a major focal point after it was found that *in vitro* translation of mRNA from light and dark-grown plants yielded different sets of polypeptides (Apel and Kloppstech, 1978). Several review papers have been written on light-regulated gene expression (Tobin and Silverthorne, 1985; Gilmartin *et al.*,

1990; Terzaghi and Cashmore, 1995), and some important facts are summarised below.

The general paradigm adopted for light-regulated transcription is that upon light perception, photoreceptors generate signals that are transduced via intermediates to activate transcription factors bound to their cognate sequences within regulatory regions of light-regulated genes (Terzaghi and Cashmore, 1995). The most extensively studied light-responsive genes are those encoding the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcS*) and the chlorophyll *a/b*-binding proteins (*cab*) (Gilmartin *et al.*, 1990). Numerous regulatory *cis*-acting sequences have been identified by a combination of deletion and mutagenesis studies, but relatively few have been studied in detail. The following generalisations were made in a review by Terzaghi and Cashmore (1995):

- Similar elements are found in many different promoters.
- Elements that may be the same are assembled differently, and even promoters of the same gene family can be quite different.
- No universal element is found in all light-regulated promoters.
- No element is sufficient to confer light-responsiveness by itself to a non-light-regulated minimal promoter.
- The same element can confer different expression characteristics in different contexts.
- Monocots may use different elements for light regulation than do dicots.
- Light regulation may involve turning genes off as much as turning them on and some light-regulatory sequences may repress rather than activate transcription.

Elements found repeatedly in light-regulated genes include the G-box, the GT-1 site, I-boxes, and AT-rich elements (Terzaghi and Cashmore, 1995).

GT-1 binding sites, or boxes II and III of the pea *rbcs3a* promoter, have the core sequence GGTTAA, but the transcription factor GT-1 will bind to many related sequences such that the consensus site is Gpu(T/A)AA(T/A). Similar sequences are

found in the promoters of light-regulated genes including *rbcs* from many species, *phya* from oats and rice, *cab* from tobacco, *rca* and *peta* from spinach and *chs15* from bean (Terzaghi and Cashmore, 1995). I-boxes, which are defined as GATAA, are regulatory elements that are functionally important in many light-regulated promoters of both monocots and dicots. Related GATA motifs are also found in many other promoters, some that are not regulated by light (Terzaghi and Cashmore, 1995). G-boxes are elements with the core CACGTG, and these are found in the promoters of many genes that respond to a variety of different stimuli. The H-box motif is essential for both light regulation and elicitor induction, and has the consensus sequence CCTACC.

AT-rich sequences that are bound by nuclear proteins have been found in numerous light-regulated promoters but have not been functionally characterised. The *Lemna gibba Lhcb* gene encoding a light-harvesting chlorophyll a/b protein contains two 10 bp regions that are essential for phytochrome-responsiveness. One region contains a CCAAT motif (-125 to -134) and the other a GATA motif (-106 to -114). These motifs are highly conserved in *Lhcb* genes as is shown in Table 2.1 (Kehoe *et al.*, 1994).

GC-rich elements have been identified in many promoters and a protein binding to a 21-bp GC-rich motif from the *cabe* promoter was identified in tobacco nuclear extracts. Although a specific role for the GC-rich elements has not been demonstrated conclusively in dicots, elements identified as important for light regulation in monocots, but that are not recognised by dicots, are often GC-rich (Schäffner and Sheen, 1991).

2.2.3 Tissue-specific expression

Development of a multicellular organism is critically dependent on the proper temporal and spatial cues that regulate gene expression. Thus, in any one cell at a particular time, only a subset of the total genetic information is expressed. Typically, the regulatory elements that are responsible for directing specific transcription

initiation reside within the 5' promoter region of the gene (Lam, 1994). Numerous genes and associated promoters have been described which exhibit a wide range of tissue-specific or developmental expression patterns. Some include genes that are specifically expressed in pollen (Miyoshi *et al.*, 1995), flower (Faktor *et al.*, 1996), phloem (Yang and Russell, 1990; Rhode *et al.*, 1995), root (Depater and Schilperoort, 1992) and seed (Iida *et al.*, 1995; Nunberg *et al.*, 1995; Van der Geest and Hall, 1996).

The specific regions of promoters that are necessary for tissue-specific expression are identified by deletion analysis and reporter gene expression studies. This approach was used, for example, to identify the location within the proximal 295 bp of the β-phaseolin *phas* promoter of sequence motifs that have been implicated in directing seed-specific expression (Van der Geest and Hall, 1996). These include the legumin box (CATGCATG) which has been identified in other seed-specific promoters, for example soybean glycinin (Iida *et al.*, 1995), and endosperm boxes (TGTAAAGT and RTGAGTCAT) (Van der Geest and Hall, 1996).

Deletion analysis of the helianthinin promoter of sunflower revealed regionalised GUS expression in transgenic tobacco seeds. Deletions up to -74 resulted in GUS activity in the cotyledons extending through the shoot apical region but not into the mid zone. Additional sequences up to -116 did not alter the shoot/ cotyledon expression pattern. However, sequences between -116 and -321 extended the tissue range of GUS expression to include most of the mid zone of the seed. Staining of the cotyledons, shoot and entire root apical regions were obtained when the -739 deletion was examined (Nunberg *et al.*, 1995).

Functional dissection of a bean chalcone synthase gene promoter (*chs15*) revealed that maximal floral and root-specific expression required sequence elements located on both sides of the TATA-box. Two adjacent sequence motifs located near the TATA-box, the G-box (CACGTG) and H-box ($CCTACC(N)_7CT$), were both essential for floral expression (Faktor *et al.*, 1996).

One element in the promoter of the *CHS1* gene of white mustard, Unit 1, was found to mediate organ-specific expression in seedlings and flowers. Unit 1 consists of two motifs, Box I (GTCCCTCCAACCTAACC) and Box II (CCACGTGGCC), and is located between -131 and -168 (Kaiser and Batschauer, 1995). Box II includes the G-box, as was observed in the *chs15* promoter (Faktor *et al.*, 1996).

The promoter of maize sucrose synthase-1 (*Sh*) directs phloem cell-specific expression of GUS in transgenic tobacco, but not in xylem parenchyma, vascular cambium or any other cell types of vegetative tissue. No specific sequence motifs were identified in this promoter (Yang and Russell, 1990).

2.2.4 Organelle Promoters

Plastids contain their own genome in the form of multiple, double-stranded, circular DNA molecules coding for the organellar rRNAs and tRNAs as well as for mRNAs specifying almost 100 plastid-localised proteins. The latter can be grouped into proteins involved in photosynthesis and CO_2 fixation, and those involved in plastid gene expression itself, such as ribosomal proteins, translation factors and components of the plastid transcription apparatus (Link, 1994). Despite the high coding capacity of the organelle genome, the majority of plastid proteins are nuclear-encoded and post-translationally imported from the cytoplasm, suggesting a balanced network of regulatory interactions within each plant cell (Link, 1994).

Regulation of plastid gene expression occurs at various levels, ranging from DNA modification and transcription, to RNA maturation, translation and post-translational modifications. By using sequencing and mapping techniques, it has become possible to locate numerous plastid genes and to identify their coding regions and regulatory sequences. It has been shown that -35/-10-like motifs (-35: TTGACA and -10: TATAAT) typical for many *E.coli* promoters (Reznikoff *et al.*, 1985) are present in front of the mapped RNA 5' end of a number of mustard chloroplast genes, for example the *rrn* operon, *psbA*, *trnK* and *trnQ* (for a review see Link, 1994).

A putative blue light-responsive promoter (LRP) upstream of the *psbD-psbC* chloroplast operon has been identified in several plant species. It shows a slight resemblance to the -35/-10 consensus sequence and reveals GATA motifs reminiscent of light-responsive plant nuclear genes (Gilmartin *et al.*, 1990). There are examples of genes that lack the -35-type element, but the TATA-box consensus sequence element (-10) has been shown to be required for faithful transcription in a chloroplast *in vitro* system (Eisermann *et al.*, 1990).

2.3 ISOLATION AND ANALYSIS OF PLANT PROMOTERS

Several approaches have been used to isolate DNA fragments carrying regulatory elements from plants, with the choice of techniques to be used in a study depending on the particular circumstances.

Two distinct groups of techniques can be identified. The first group is used for the identification and isolation of promoters of genes that have not previously been identified as being useful. These techniques are not directed at any specific gene, but rather at a group of genes that are expressed in a certain tissue, at a specific developmental stage, or in response to specific stimuli. The second group of techniques is directed at specific genes, and is used when more information is available about the gene product. All these techniques involve the isolation of cDNA fragments that can be used to screen a genomic library and isolate the promoter sequence. Promoter tagging methods can also be used to detect and isolate regulatory regions directly from the plant genome (An and Kim, 1993).

2.3.1 Non-directed methods for the identification and isolation of useful promoters

Differential or "+/-" screening of cDNA libraries have been successfully used to identify and isolate cDNAs representing genes whose expression is altered by stress. The cDNAs identified in this manner do not represent genes with known functions. During differential screening replicates of a cDNA library are screened by hybridisation to the RNA from which the library was constructed. For example, cDNA

synthesised from RNA isolated from drought-stressed organs can be compared to cDNAs synthesised from RNA isolated from non-stressed organs. cDNAs that hybridise to the source of the drought-stress library and not to the non-stressed comparison are candidates for genes that are induced by stress (Bray, 1994). The differential screening technique has been used in the identification of cDNAs representing genes from maize (Gómez *et al.*, 1988), pea (Guerrero and Mullet, 1988), rice (Mundy and Chua, 1988), *Craterostigma plantagineum* (Bartels *et al.*, 1992), tomato (Cohen and Bray, 1990) and *Arabidopsis* (Yamaguchi-Shinozaki *et al.*, 1992).

A PCR differential screening method for the isolation of clones from a cDNA library has also been developed (Luo *et al.*, 1994). This method involves a primary differential screening step followed by a PCR screening step. From the primary screening step, impure pools of positive cDNA clones are obtained. Each pool of clones is then amplified directly using primers flanking the cloning site in the vector. The PCR products are separated on two duplicate agarose gels and blotted onto filter strips that are subjected to differential Southern hybridisation with different cDNA probes. Each pure positive cDNA band on the gel is identified and selected for further analysis (Luo *et al.*, 1994).

Another variation of this technique is the use of subtractive hybridisation which is based on subtracting away the common transcripts between different cell types or tissues, leaving the specific transcripts (mRNA) for further analysis (Sargent, 1987). A "tracer" cDNA is hybridised to an excess of "driver" mRNA from another cell type. Unhybridised tracer cDNA represents an enriched population of sequences that are expressed in the one cell type only (Lee, *et al.*, 1991). Subtraction can also be performed by hybridisation between mRNA of one cell type and the cDNA synthesised from mRNA from another cell type using an oligo$(dT)_{30}$ primer that is covalently linked to Latex particles in an Eppendorf tube. The mRNA common to both cell types can be removed by a brief centrifugation (Hara *et al.*, 1993).

An improvement of this subtraction approach has been the use of magnetic bead-assisted subtraction (Sharma *et al.*, 1993). First strand cDNA is immobilised onto magnetic beads as a result of the oligo(dT)$_{25}$ which is attached to the beads (Aasheim *et al.*, 1994). This subtraction is also known as "magnet-assisted subtraction technique" or MAST (Schraml *et al.*, 1993). A combination of this technique and a Southern blot screening by means of PCR (MAST-PCR-SBDS) was recently used to identify cDNAs from genes that are differentially expressed during strawberry fruit ripening (Medina-Escobar *et al.*, 1997).

The major limitations of all these techniques are that they can only be used to identify genes that are relatively highly expressed, they are time-consuming and often difficult to perform, and they can only be used to compare two different cell types at a time. The differential display technique (Liang and Pardee, 1992) circumvents some of these limitations and will be discussed in greater detail.

2.3.1.1 Differential display of mRNA by PCR

RNA fingerprinting by the differential display technique has been used to isolate differentially expressed genes in a number of experimental systems (for a review see McClelland *et al.*, 1995). The technique was developed with the goal of identifying differentially expressed genes by detecting individual mRNA species that are altered in mammalian cells and then recovering and cloning the cDNA (Liang and Pardee, 1992; Liang *et al.*, 1993).

This approach involves PCR amplification of cDNA fragments and polyacrylamide gel electrophoresis for the separation of amplified products. The basic principle is to reverse transcribe and systematically amplify the 3' termini of mRNAs with a set of anchored oligo(dT) primers and a random decamer. Four sets of degenerate anchored oligo(dT) primers, (T_{12}MN), where M can be G, A or C and N is G, A, T or C, are used to prime reverse transcription by binding to the start of the poly(A) tails of the mRNA. Each primer set is dictated by the 3' base (N), with degeneracy in the penultimate (M) position. The resulting cDNA population is PCR-amplified using the

degenerate primer set, an arbitrary decamer, and a radioactive nucleotide. The radioactively labelled PCR products that represent a subpopulation of mRNAs defined by the given primer set are separated in a denaturing polyacrylamide gel. By changing primer combinations, most of the mRNA species in the cell may be represented. Multiple different RNA samples are compared to allow the identification and isolation of differentially expressed genes (Liang *et al.*, 1993). The procedure depends on the amplification of an mRNA subpopulation without prior knowledge of sequence information.

The differential display technique has been used with varying degrees of success. Big drawbacks of the technique are the high incidence of false-positives and the fact that the technique often shows a strong bias towards high copy number mRNAs (Bertioli *et al.*, 1995). Several changes to the original method have been made and certain critical parameters have been identified since the technique was first developed. These parameters are discussed below and changes that have been made by other research groups are included.

i) RNA template for RT-PCR

One of the most important factors for successful differential display is to use DNA-free RNA samples (Liang *et al.*, 1993). One way of determining whether an RNA sample contains contaminating DNA is to perform the PCR without reverse transcription. If the sample is free of DNA, the display should be totally dependent on the reverse transcription step (Liang *et al.*, 1993).

Because the technique relies on the reverse transcription of mRNA from an oligo(dT) primer, both total RNA (Bauer *et al.*, 1993; Zimmermann and Schultz, 1994; Zhao *et al.*, 1995) or purified poly(A)mRNA (Liang *et al.*, 1993; Sokolov and Prockop, 1994) can be used. The use of cytoplasmic rather than total RNA eliminates false positives due to nuclear RNA that is not transported to the cytoplasm (Sompayrac *et al.*, 1995).

Several investigators optimised the RNA template concentration for RT-PCR. Welsh,

et al. (1992) varied the concentration of input RNA, and serial dilutions of the RNA ranging from 0.012 to 2.5 µg per 20 µl reaction before cDNA synthesis revealed a broad concentration optimum. They concluded that the method would be reproducible over a wide range of RNA concentrations (Welsh, *et al.*, 1992). In another series of reactions that were done to determine the sensitivity limits of the differential display method in terms of input RNA concentration, total RNA was varied from 0,002 to 2 µg per 20 µl RT reaction (Liang *et al.*, 1993). Results indicated that the amount of input RNA can be as low as 0.02 µg which is equivalent to RNA from about 200 cells, but that the banding pattern was much sparser when 0.002 µg RNA was used (Liang *et al.*, 1993).

The possibility of using very small RNA samples is a major advantage of the differential display technique because in some cases only very small tissue samples can be obtained. An example of this was the analysis of differential expression during preimplantation embryogenesis in mouse embryos. Reverse transcription was successfully performed on RNA obtained from a number of embryos that corresponded to about 10 pg of poly(A)+mRNA (Zimmermann and Schultz, 1994).

ii) Primers used for RT-PCR

Twelve anchored oligo(dT) primers consisting of 11 or 12 T's plus two additional 3' bases were initially used for reverse transcription in twelve separate reactions (Liang and Pardee, 1992). The number of primers have since been reduced to four to improve the efficiency and specificity of the technique, each primer being degenerate at the 3' penultimate position (T_{12}MN where M may be G, A or C and N may be G, A, T or C) (Liang *et al.*, 1993; Sompayrac *et al.*, 1995).

Results of an investigation into the specificity of anchored oligo(dT) primers suggested that the two 3' bases, MN, are absolutely essential to provide the primers with specificity for anchoring to the beginning of the poly(A) tails. The penultimate base, M, can exhibit considerable degeneracy because it is the last base on the 3' end that provides most of the specificity (Liang *et al.*, 1993). Because most of the

fragments obtained from reverse transcription using oligo(dT) primers are situated on the 3' end of the mRNAs, some groups found it more advantageous to use random hexamers for reverse transcription. This ensured that fragments with internal sequences of the mRNAs were obtained instead of the less-informative 3' sequences (Sokolov and Prockop, 1994). In another case an oligo$(dT)_{12-18}$ primer with no anchoring bases was used for the reverse transcription, while anchored oligo(dT) primers were used in subsequent amplification reactions (Doss, 1996).

For the amplification of the synthesised cDNA fragments, random decamer primers were used in combination with the oligo(dT) primer that was used for reverse transcription. Generally, any decamer primer with a GC-content of 50% to 70% can be used as long as it does not contain palindromic sequences. Since it has been shown that the random decamer primers can have up to 4 bp mismatches with the original cDNA template, and that these mismatches are often clustered on the 5' end of the primers (Liang *et al.*, 1993), the primers can be designed in such a way that the 3' sequences are maximally randomised while the 5' bases (up to 4 bases) are fixed.

Several other investigations were done involving the upstream PCR primers (Bauer *et al.*, 1993; Sokolov and Prockop, 1994; Bertioli *et al.*, 1995; Zhao *et al.*, 1995), and one of the conclusions that was made was that under the low stringency PCR conditions that are used in the differential display technique, mismatches in the priming site can occur and lead to the amplification of false-positive fragments and irreproducible PCR displays (Bauer *et al.*, 1993; Zhao *et al.*, 1995). The use of longer primers and elevated annealing temperatures was therefore suggested to minimise false positives. In an enhanced differential display technique (EDD), the stringency was increased by increasing the length of the primers to 22 nucleotides and using low annealing temperatures for only the initial few PCR cycles. Under these conditions the primers behave in a similar way to the short 10 nucleotide primers. For the remaining PCR cycles, the annealing temperature was raised during which the products made in the initial cycles were efficiently and specifically replicated

(Linskens *et al.*, 1995). Because random decamer primers are too short to prime a sequencing reaction, the fragments have to be cloned if the sequence is to be determined. The primers can be extended and used for direct sequencing of cDNA fragments (Wang and Feuerstein, 1995; Reeves *et al.*, 1995) which will overcome the problem of some fragments being too short for efficient cloning or labelling (Sokolov and Prockop, 1994). Longer primers can also be designed to contain restriction enzyme sites which will simplify the cloning of the fragments (Linskens *et al.*, 1995).

The number of primer combinations will depend on the application and the extent to which the cell types differ from each other. Liang and Pardee (1992) suggested that 20 random decamer primers must be used to obtain all possible mRNA sequences upstream of the 12 anchored oligo(dT) primers, because the decamer primers act like 6- to 7-mers under low stringency reaction conditions. Results obtained by Bauer *et al.* (1993) confirmed this and showed that PCR with decamer primers generates about 120 fragments after 40 cycles using an annealing temperature of 40°C. This corresponds to an effective primer length of 6 bases. In order to reach a confidence level of 0.95 for displaying any messenger as a cDNA fragment in a gel, one should use at least 25 upstream primers (Bauer *et al.*, 1993).

iii) Display of amplified cDNA fragments

Initially, the differential display amplification products were separated in denaturing polyacrylamide gels which were dried after electrophoresis. Because a radioactive nucleotide was included in the amplification reaction mixture, the banding profile could be visualised by autoradiography (Liang and Pardee, 1992). The dried gel was oriented with the autoradiogram, and cDNA fragments of interest could be cut from the gel. Several variations of this method for the display of cDNA products have been developed. Non-denaturing polyacrylamide gels were used to reduce an observed artificial complexity of the banding pattern (Bauer *et al.*, 1993; Bertioli *et al.*, 1995), while silver staining of the polyacrylamide gels eliminated the need for radioactivity (Lohmann *et al.*, 1995; Doss, 1996).

The separation of amplification products in agarose gels, followed by ethidium bromide staining, was used by Sokolov and Prockop (1994) and proved to be sensitive enough to detect differences in mRNA expression that appeared after infection of plant leaves with pathogens (Rompf and Kahl, 1997). Although the resolution of agarose gels is much lower than that of polyacrylamide gels and fewer amplified fragments can be resolved, this can be overcome by increasing the number of primer combinations used.

More recently, the use of digoxigenin-conjugated or biotinylated oligo(dT) primers and colorimetric or chemiluminescence-based detection of PCR products has been described (An *et al.*, 1996). A fluorescent detection procedure, using a fluorescently labelled oligonucleotide has also been developed. This has the advantage that the amplification products can be analysed using an automated gene sequencer, which facilitates a high throughput of samples, easier data capture, and storage of data for future profile comparison (Smith *et al.*, 1997). Several investigators have found that even with the availability of very sensitive detection methods, the differential display should be used as a qualitative rather than a quantitative technique (Liang *et al.*, 1993).

iv) Isolation of differentially expressed cDNA fragments

After the banding profiles have been analysed, the differentially expressed sequences have to be isolated. Where denaturing polyacrylamide gel electrophoresis has been used for the separation of products, the gels are dried and after autoradiography the fragments of interest are excised, eluted from the gel, and reamplified prior to their analysis (Liang *et al.*, 1993).

Several problems have occurred in the isolation of specific fragments, and many investigators have reported the isolation of more than one fragment from the same position in the gel (Welsh *et al.*, 1992; Callard *et al.*, 1994; Sokolov and Prockop, 1994; Hadman *et al.*, 1995). After cloning an isolated fragment, sequencing of several different clones of the expected size revealed that the sequence of the insert

was heterogeneous (Callard *et al.*, 1994). Hybridisation techniques have therefore been developed to identify the clones that contain the true differential cDNA fragment (Callard *et al.*, 1994).

Gery and Lavi (1997) have also described a method to improve the efficiency of the isolation of differentially expressed fragments. Reamplification of a fragment can be done in the presence of an isotope, after which the reamplified product is run in parallel with the original PCR products to confirm that the correct fragment has been reamplified. Raising the annealing temperature of the reamplification reaction may also reduce contaminating background (Gery and Lavi, 1997).

2.3.2 Directed techniques for the isolation of useful promoters

When more information is available about the gene product to be studied, directed methods can be used to isolate the desired cDNA. The directed techniques include heterologous hybridisation, hybridisation with degenerate primers after partial amino acid sequence determination, PCR-based screening, and functional complementation to identify specific cDNAs (Bray, 1994).

If a similar cDNA has been isolated from a different species it can be used to screen a cDNA library from the desired species. This method has, for example, been successfully used in the identification of an osmotin-like gene from *Atriplex* (Casas *et al.*, 1992). A salt- and drought-induced gene was also isolated from rice using a degenerate oligonucleotide (Claes *et al.*, 1990). The expressed salt-induced protein was identified by two dimensional polyacrylamide gel electrophoresis, and the amino-terminal amino acid sequence of the tryptic peptide was determined after transfer from the 2-D gel onto nitrocellulose. An oligonucleotide was synthesised based on the amino acid sequence and was used to screen a cDNA library (Claes *et al.*, 1990).

In cases where a number of related proteins have been identified, regions of conservation among these proteins can be used to identify a specific mRNA. PCR primers can be designed and a probe can be synthesised by PCR that can be used

to screen a cDNA library. This PCR-based method has been used to isolate a drought- and abscisic acid (ABA)-induced protein kinase from wheat (Anderberg and Walker-Simmons, 1992). Degenerate oligonucleotides corresponding to highly conserved regions in serine / threonine protein kinases were designed for PCR amplification. Consequently, the cDNA synthesised from poly(A+)mRNA isolated from dormant wheat embryos was able to be amplified (Anderberg and Walker-Simmons, 1992).

If a bacterial mutant is available, functional complementation of that mutant may be used to obtain the desired cDNA. Many plant genes can be isolated by their ability to complement mutations in bacteria and yeast (Gibson and Somerville, 1993). For example, each of eight different auxotrophic mutants of yeast were functionally complemented by a cDNA library from *Arabidopsis*. Two genes involved in higher plant proline biosynthesis have also been cloned by functional complementation of *E.coli* mutants (Delauney and Verma, 1990; Hu *et al.*, 1992). A phagemid library produced in Lambda Zap II (Stratagene) and excised *in vivo* with a helper phage was transformed into an *E.coli proC* mutant. This mutant has a defective Δ^1-pyrroline-5-carboxylate reductase gene and is unable to grow in media lacking proline. The complemented mutant was able to grow on media lacking proline, indicating that the gene product functions in the proline biosynthetic pathway (Delauney and Verma, 1990).

2.3.3 Isolation of tissue-specific promoters from sugarcane

Several options for isolating gene regulatory sequences with desired patterns of expression in sugarcane have been discussed (Birch *et al.*, 1996). The first approach involves the use of characterised promoters from other plant species. These promoters can be used either directly to drive introduced gene expression, or indirectly as the basis for probes or primers used to isolate corresponding promoters from sugarcane. Limiting factors of this approach are the availability of suitable foreign promoters and in some cases, patent considerations (Birch *et al.*, 1996).

Introduction of promoterless reporter genes to tag sugarcane promoters is another possible technique to use (Koncz *et al.*, 1989). The relatively low efficiency of promoter tagging by direct gene transfer into plants and difficulty in recovering the tagged promoters have limited the use of this approach. Differential hybridisation approaches to isolate tissue-specific cDNA fragments and then to use these to recover corresponding gene and promoter sequences from genomic libraries, have been used in other plant systems. Constraints include the requirement for large amounts of RNA, technical difficulties in cDNA library construction, and a bias toward strongly expressed genes (Watson and Margulies, 1993).

The differential display technique discussed previously can be used to isolate tissue-specific cDNA fragments from sugarcane. These fragments can then be used as a starting point for isolating corresponding gene promoters by PCR or hybridisation techniques (Liang *et al.*, 1993). The complex polyploid genome of sugarcane may create additional difficulties in isolating the correct promoter sequence driving an observed useful pattern of expression in differential hybridisation and display techniques (Birch *et al.*, 1996).

Preliminary work has been done to isolate wound- and root-specific promoters from sugarcane. A differential hybridisation approach was used to isolate a full-length root-specific cDNA clone. Northern hybridisation analysis indicated that the gene is expressed in roots under various stress conditions, but not in leaves, stems or healthy field-grown roots. A corresponding 770 bp putative root-specific promoter region was isolated from a genomic library and fused to both GUS and *luc* reporter genes. Transient expression analysis has been done and expression levels in transformed roots were low. Insertion of an upstream enhancer (OCS) increased overall expression levels, but not root-specificity. This promoter is currently being characterised in transgenic plants (Birch *et al.*, 1996).

A wound-induced peroxidase cDNA fragment from buffel grass has been used to isolate the corresponding gene from a sugarcane genomic library. The putative

promoter region is currently being characterised in transgenic sugarcane plants (Birch *et al.*, 1996).

Culm-specific cDNA fragments have been isolated from a sugarcane cDNA library by differential hybridisation and are being characterised (Potier and Birch, 1997a). Two fragments were specifically expressed in the meristematic region of the culm, one was expressed in the young culm only, and another fragment was fully culm-specific with its expression peaking in the top and in the mature parts of the culm (Potier and Birch, 1997a). These fragments have been sequenced enabling the identification of a likely functional role for some of them. Based on expression patterns, copy number and gene identity, some of the cDNA fragments have been selected for the recovery of the corresponding promoter sequence by an inverse PCR (iPCR) technique. One of the isolated promoters has been used to make a fusion construct to the GUS reporter gene, and sugarcane and tobacco have been transformed with this construct. Further analysis remains to be done (Potier and Birch, 1997b).

2.4 PLANT TRANSFORMATION

Genetic transformation can be defined as the transfer of foreign genetic material isolated from plants, viruses, bacteria or animals into a new genetic background, resulting in chromosomal (nuclear or organellar) integration and the stable heritability of that material through meiosis (Webb and Morris, 1992; Gruber and Crosby, 1993). This definition distinguishes transformation from transient expression, where genetic material is introduced through physical uptake approaches and may exhibit short-lived mitotic stability in cells (Gruber and Crosby, 1993).

In plants, successful genetic transformation requires the production of normal, fertile plant which express the newly inserted genes (Webb and Morris, 1992). Transformation can utilise gametophytic tissues, prior to fertilisation, or somatic cells, such as explants, cells and protoplasts, which can be stimulated to regenerate plants *de novo* in culture (Webb and Morris, 1992).

Conceptually there are two reasons for transforming a plant. Firstly, it is used as an experimental tool for plant physiology where the capacity to introduce and express or inactivate specific genes allows the elucidation of certain biological processes. Secondly, it is used as a practical tool to confer a trait to a plant that will improve its agricultural, horticultural or ornamental value (Greenberg and Glick, 1993; Birch, 1997). Some of the traits that have been "engineered" into plants by the addition of a single gene or a small cluster of genes include insecticidal activity, tolerance to viral or fungal infection, herbicide resistance, altered fruit ripening, improved nutritional quality of seed proteins and self-incompatibility (Gasser and Fraley, 1989; Gatehouse *et al.*, 1992; Peferoen, 1992; Glick and Thompson, 1993; Smith *et al.*, 1993; Birch, 1997).

There are a number of reviews of the development of transformation systems including *Agrobacterium*-mediated transformation, direct gene transfer into protoplasts and particle bombardment (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995; Birch and Franks, 1991; Christou, 1992), and their potential for practical application (Gatehouse *et al.*, 1992; Setlow, 1997). The methods involved have also been discussed in detail (Gatehouse *et al.*, 1992; Glick and Thompson, 1993). In recent reviews, key problems remaining in the development of plant transformation systems, key issues to be resolved in the practical application of these systems, and strategies to overcome these limitations have been addressed (Christou, 1996; Birch, 1997).

2.4.1 Transformation via particle bombardment

Monocotyledonous plants, including important grain crops, have been difficult to transform. One reason is that these plants are not susceptible to infection by *Agrobacterium tumefaciens*. Consequently, the delivery of DNA to the cells using particle bombardment has become the method of choice for the transformation of these plants (Greenberg and Glick, 1993; Morrish *et al.*, 1993). With this technique, microprojectile particles (e.g. 1.2 µm tungsten or gold beads) coated with DNA are accelerated at high speeds into plant tissue (Christou, 1993). A number of the cells

usually express the introduced gene transiently, while some are capable of stably integrating the foreign DNA into the genome. This approach has the distinct advantage of applicability to any intact plant tissue or region of the plant, and has been used to transform organellar DNA (Svab *et al.*, 1990).

Since the first report of transformation of living cells using high velocity microprojectiles (Klein *et al.*, 1987), several crop species that were not previously accessible to transformation have been successfully transformed (Sautter *et al.*, 1991). Crop plants that have been transformed using this technique include maize (Klein *et al.*, 1988; Oard *et al.*, 1990; Vain *et al.*, 1993a; Rasmussen *et al.*, 1994; Kemper *et al.*, 1996), wheat (Wang *et al.*, 1988; Lonsdale *et al.*, 1990; Chibbar *et al.*, 1991; Vasil *et al.*, 1991), barley (Mendel *et al.*, 1989; Koprek *et al.*, 1996), cotton (Finer and McMullen, 1990), tobacco (Klein *et al.*, 1988; Tomes *et al.*, 1990; Iida *et al.*, 1991), soybean (Christou *et al.*, 1990; McCabe *et al.*, 1988; Sato *et al.*, 1993), rice (Christou *et al.*, 1991; Li *et al.*, 1993) and sugarcane (Franks and Birch, 1991; Bower and Birch, 1992; Gambley *et al.*, 1993). Microprojectile bombardment has also been used as an effective method to wound plant tissues to promote *Agrobacterium*-mediated transformation (Bidney *et al.*, 1992).

Several different types of bombardment devices have been developed, including an electrically triggered discharge gun (McCabe and Christou, 1993), pneumatic particle guns (Kikkert, 1993; Oard, 1993), helium, nitrogen and carbon dioxide powered devices (Finer *et al.*, 1992; Vain *et al.*, 1993b) and a micro-targeting gun (Sautter *et al.*, 1991). Most of these devices are based on the same basic concept for particle acceleration and delivery: a force provided by either an explosion or expansion of compressed gas propels a macrocarrier holding particles towards an immobile object which retains the macrocarrier but permits the particles to pass (Finer *et al.*, 1992).

The particle inflow gun (PIG)(Finer *et al.*, 1992) is based on the acceleration of DNA-coated tungsten particles using pressurised helium in combination with a partial vacuum. The particles are accelerated directly in a helium stream rather than being

supported by a macrocarrier (Finer *et al.*, 1992).

There are a number of features of the PIG that make it easy to construct and operate. Expansion of compressed helium is used since helium is inert, leaves no residue, and gives reproducible acceleration. A timer relay-driven solenoid is used to release the helium and can be used at low helium pressure, which allows good particle penetration and less damage to the target tissue. The particles are accelerated directly in the helium stream and no macrocarriers are necessary, saving costs and time in between bombardments. A vacuum chamber is used to hold the target tissue. The vacuum reduces the aerodynamic drag on the particles, thereby increasing particle velocities, and lessening tissue damage by dispersion of the helium gas prior to impact (Finer *et al.*, 1992).

Parameters that have to be optimised for the PIG are helium pressure, distance of the target tissue from the syringe filter, packed cell volume of target cells and volume of particle preparation to be used. Other particle accelerating devices have been developed with various modifications on the original PIG, and these are commercially available (Brown *et al.*, 1994; Buckley *et al.*, 1995).

2.4.2 Vectors for plant transformation

Once a gene of interest is isolated, it is introduced into a plant transformation vector. The vector DNA facilitates manipulation of the gene in *Escherichia coli* prior to plant transformation, as well as transfer of the gene to the host plant. An ideal vector contains a multiple cloning site, an antibiotic resistance gene allowing for selection in bacteria (e.g. ampicillin resistance), a broad-host bacterial origin of replication, and an antibiotic resistance gene for selection of the foreign DNA in transformed plants (e.g. kanamycin resistance) (Walden *et al.*, 1990; Greenberg and Glick, 1993).

Agrobacterium plasmids have been exploited as natural vectors for biological delivery of foreign DNA to plants and this has become the most widespread transformation strategy in use (Gruber and Crosby, 1993). *Agrobacterium tumefaciens* carries the

tumour-inducing or Ti plasmid whereas *Agrobacterium rhizogenes* contains the root-inducing or Ri plasmid. Two types of vector systems have been developed, namely cointegrating and binary (Walden and Schell, 1990).

The cointegrating system features two independent plasmids: a Ti plasmid in *Agrobacterium* and an intermediate vector in *E.coli*. Both plasmids have a region of homology which undergoes recombination to form a large, cointegrated plasmid after conjugation between *Agrobacterium* and *E.coli* (Van Haute *et al.*, 1983). Genes which are to be introduced into plants are cloned and manipulated in *E.coli* and, after recombination with the Ti plasmid in *Agrobacterium*, are situated between two T-DNA border repeats. The *E.coli* plasmid has no origin of replication for maintenance in *Agrobacterium* and is not retained without the recombination step (Shaw *et al.*, 1983).

The binary system features two plasmids which coexist autonomously in *Agrobacterium* after conjugation, a shuttle plasmid and a Ti plasmid (Hoekema *et al.*, 1983). Shuttle plasmids contain one or both T-DNA border repeats as well as broad-host-range replication and mobilisation functions. They encode plant selectable markers, expression signals and polylinkers for the subcloning of foreign genes within the two T-DNA borders. The *Agrobacterium* plasmid in a binary system is an engineered Ti plasmid with plasmid *vir* genes, an *Agrobacterium* origin of replication (*oriA*), but no T-DNA borders. After conjugation, the two plasmid partners coexist autonomously with selective pressure in *Agrobacterium*. When *Agrobacterium* infects a wounded plant, the *vir* genes on the Ti plasmid interact with the right border on the shuttle plasmid in *trans* to transfer the T-DNA into the plant genome. These two latter features define the binary vector strategy (Hoekema *et al.*, 1983).

The gene cassettes within the T-DNA region can be arranged for optimum transfer and plant expression (Velten and Schell, 1985). Genes of interest should be situated directly adjacent to the right border where integration is faithful, leaving the plant selectable marker closer to the left border where incorrect integration has been known to occur. Dual promoters can also be subcloned in opposite orientation in the

centre of the T-DNA, so that there is less influence on the expression of introduced genes from flanking plant genomic DNA (Klee and Rogers, 1989).

Methods for physical delivery of DNA into plants include the direct uptake of DNA into protoplasts using polyethylene glycol, electroporation or microprojectile bombardment delivery to tissues using a biolistic device. Physical vectors are usually 4 to 7 kb in size although vectors of up to 11 kb have been used without compromising transgene stability (Vasil *et al.*, 1991). DNA conformation plays a role in the frequency of physical transformation and both linear as well as supercoiled plasmids are used as vectors (Gruber and Crosby, 1993). Plant expression cassettes in vectors include multiple selection markers (Tomes *et al.*, 1990), tandem promoters (Hain *et al.*, 1985) and transcription enhancing elements (Callis *et al.*, 1987).

The expression of intron-containing genes can be strongly influenced by the sequence content and the presence of functional gene regulatory sequences in an intron (Gruber and Crosby, 1993). Several examples have been described where a plant intron may contain a position-dependent enhancer sequence, for example in the maize *Adh*1 and rice *Act* 1 structural genes (Mascarenhas *et al.*, 1990; McElroy *et al.*, 1990). The Inclusion of an intron between the promoter of maize *Sh1* and reporter gene in a chimeric construct has enhanced reporter gene expression in rice and maize protoplasts by 100-fold (Maas *et al.*, 1991).

Organelle transformation through particle bombardment is also possible (Butow and Fox, 1990). The vectors used for these studies include genes providing resistance to antibiotics which only affect the chloroplast ribosome, such as streptomycin, spectinomycin, chloramphenicol and erythromycin to select chloroplast transformants (Haring and De Block, 1990). Chloroplast-specific replication sequences and chloroplast-driven selectable markers can also be included in an *E.coli* plasmid framework (Gruber and Crosby, 1993).

2.4.2.1 Selectable marker genes

Selection of transformed cells is a key factor in developing successful methods for genetic transformation (Webb and Morris, 1992). The choice of marker is dictated by the antimetabolite chosen as counterselection agent, which is used in preventing growth of untransformed cells. Other factors must be considered in choosing a marker gene, including that the expression of the marker gene should not metabolically disrupt the transformed host cell, expression of the marker gene must effectively protect the host cell form the growth-inhibitory properties of the selection agent, and exposure to the antimetabolite should have minimal effects in subsequent growth and development of the transformed plant (Gruber and Crosby, 1993).

The gene *nptII*, encoding neomycin phosphotransferase (NPT), is most commonly used as a selectable marker and has proven particularly effective in transformation experiments involving potato, tobacco and tomato. However, *nptII* has proven less successful with legumes and some monocot species (Hauptmann *et al.*, 1988; Gruber and Crosby, 1993). The *nptII* gene product acts by enzymatically phosphorylating and inactivating aminoglycoside antibiotics such as kanamycin, neomycin and geneticin (G418).

Several selectable marker gene products function by detoxifying antimetabolites such as hygromycin (Waldron *et al.*, 1985) and gentamycin (Hayford *et al.*, 1988), while other markers encode an altered target which is insensitive to the inhibitor (Etzold *et al.*, 1987). Marker genes encoding tolerance to herbicides have also been used. For example, the *bar* gene isolated from *Streptomyces hygroscopicus* codes for phosphinothricin acetyltransferase (PAT), which inactivates phosphinotricin (PTT), the active ingredient of Bialaphos and Basta herbicides and an irreversible inhibitor of glutamine synthase (Webb and Morris, 1992).

2.4.2.2 5' Regulatory sequences in plant expression vectors

The expression of a transgene is dependent on the presence of transcriptionally-efficient 5' regulatory regions, a 5' untranslated leader sequence, a translational start

sequence (AUG) with plant favourable context, and a 3' transcription termination/polyadenylation sequence (Gruber and Crosby, 1993). These and other determinants contribute to mRNA and protein production, stability and turnover and are all important components to consider in a strategy to develop a plant vector (Webb and Morris, 1992; Gruber and Crosby, 1993).

The choice of a promoter for plant transformation will depend on the circumstances under which the transgene should be expressed. Constitutive promoters will drive expression in all developmental stages in all tissues, whereas tissue-specific promoters will only be active in certain tissues.

The 5' regions of the maize polyubiquitin gene (Christensen *et al.*, 1992) and the rice actin 1 gene (Zhang *et al.*, 1991) have been used to drive constitutive gene expression in transgenic plant studies. Another commonly used constitutive promoter is the CaMV *35S* promoter (Guilley *et al.*, 1982).

Examples of tissue-specific promoters which have been used to drive expression in particular areas of the plant, include the maize sucrose synthase-I (*Sh*) promoter suitable for phloem-specific expression (Yang and Russell, 1990), the pea and bean vicilin and phytohemaglutinin promoters suitable for seed-specific expression, and the α-amylase promoter for driving expression in the aleurone of cereal grains (Christou, 1996).

Promoters which are induced by certain chemical (Gatz, 1997) or environmental stimuli such as light (Terzaghi and Cashmore, 1995) or heat (Czarnecka-Verner *et al.*, 1994) can also be used in transformation studies. In some cases the use of promoter cassettes will be necessary to enhance gene expression. Several combinations of promoters and terminator sequences have been tested in both dicotyledonous and monocotyledonous plants (Mitsuhara *et al.*, 1996).

The large differences between regulatory elements from prokaryotes and eukaryotes

mean that sequences from bacteria may not function in plant cells. Plant viruses, which depend on the plant transcription and translation factors, have been used a sources of regulatory elements. The most common of these is the promoter of the *35S* RNA gene of CaMV (Guilley *et al.*, 1982). This promoter is active in all cell types, although a certain degree of cell- and tissue-specificity has been observed (Benfey and Chua, 1990; Lam, 1994).

Regulatory elements in certain genes of *Agrobacterium tumefaciens* and *A.rhizogenes* are not expressed in *Agrobacterium*, but become active on transfer to plant cells. The *nos* (nopaline synthase), *ocs* (octapine synthase) and *mas* (mannopine synthase) gene promoters belong in this category, and have been used successfully to direct expression of genes in plant cells (Webb and Morris, 1992). These promoters have been studied intensively (Singh *et al.*, 1990; Kim *et al.*, 1994). One example of a bacterial promoter that is being used to direct tissue-specific transgene expression in plants is the *rolC* promoter of *A.rhizogenes.* The expression pattern of this promoter in sugarcane was evaluated in the present study.

i) The rolC promoter of Agrobacterium rhizogenes

The *rolA, B, C* and *D* genes of *A.rhizogenes* are located on the TL-DNA of the Ri plasmid A4 and are involved in the pathogenesis of hairy root disease (Moore *et al.*, 1979; White and Nester, 1980; Vilaine *et al.*, 1987; Nilsson and Olsson, 1997). When expressed separately in transgenic tobacco plants, these genes established phenotypic alterations characteristic for each gene (Oono *et al.*, 1987; Schmülling *et al.*, 1988; Sinkar *et al.*, 1988).

Transgenic tobacco plants expressing a single *rolA* gene from its endogenous promoter displayed severe wrinkling of leaves and the plants were stunted with extremely short internodes and small rounded leaves (Schmülling *et al.*, 1988). The *rolB* gene displayed alterations in leaf and flower morphology and adventitious root formation on the stem in transgenic tobacco plants (Schmülling *et al.*, 1988). Transgenic tobacco plants expressing *rolC* from its endogenous promoter had

increased ratio of leaf length to leaf width. The plants were also shorter, more highly branched, flowered earlier and had smaller flowers and a reduced pollen production compared to the wild type (Oono *et al.*, 1987; Scmülling *et al.*, 1988). When *rolC* was expressed from the strong *35S* promoter, these characteristics were exaggerated, giving dwarfed plants with very short internodes and an increased number of lanceolate light-green leaves. Apical dominance was reduced, resulting in bushy plants (Scmülling *et al.*, 1988; Nilsson *et al.*, 1993). Because of the dominant pleiotropic nature of the *rolC* gene and the fact that it causes a reduction in the chlorophyll content in leaves of transgenic plants when placed under control of the CaMV *35S* promoter, it has been used as a visible cell-autonomous marker in aspen (*Populus)* (Fladung and Ahuju, 1997). The expression of *rolD* in transgenic tobacco plants caused early flowering and explants showed earlier and enhanced organogenesis of flowers (Mauro *et al.*, 1996).

Several groups have studied *rol* gene regulation by means of promoter fusions to the reporter gene *uidA*, and subsequent analysis of GUS activity in transgenic tobacco, hybrid aspen and rice plants (Matsuki *et al.*, 1989; Scmülling *et al.*, 1989; Sugaya *et al.*, 1989; Maurel *et al.*, 1990, Altamura *et al.*, 1991, Capone *et al.*, 1991, Guivarc'h *et al.*, 1996; Nilsson *et al.*, 1996). These studies showed that the *rolD* gene differs from the others, because it does not exhibit tissue-specific expression. Instead, it appears to be developmentally regulated, being expressed during the elongation and maturation phases of certain tissues. The other *rol* genes have a similar tissue-specific expression pattern, being mainly confined to root meristems and the phloem (Nilsson *et al.*, 1996).

The intergenic region separating the *rolB* and *rolC* genes represents a bidirectional promoter (Scmülling *et al.*, 1989). Detailed analysis showed that the *rolB* promoter seems to be generally expressed in phloem, phloem parenchyma and ray cells (Altamura *et al.*, 1991; Nilsson *et al.*, 1997) while the *rolC* promoter is specifically expressed in the phloem companion cells (Guivarc'h *et al.*, 1996; Nilsson *et al.*, 1996).

In expression studies of the *rolC* gene in transgenic tobacco plants, it was shown that the transcriptional level of ORF12 (*rolC*) differed greatly among organs, the order being roots>stems>leaves (Nakamura *et al.*, 1988). This organ-related activation of the GUS gene under control of the *rolC* promoter was not observed in rice plants, although phloem-specific expression was observed (Matsuki *et al.*, 1989). In hybrid aspen a cell-specific localisation of the *rolC* promoter was reported. Within the phloem at the shoot base GUS activity was always detected in companion cells and occasionally in ray and axial parenchyma, but never in sieve tubes (Nilsson *et al.*, 1996). Expression was also highly specific in groups of pericycle cells prior to and during lateral root initiation (Nilsson *et al.*, 1997).

The *rolC* promoter was found to be activated by sucrose in phloem cells of transgenic tobacco plants bearing the *rolC* promoter-*uidA* chimeric fusion gene (Yokoyama *et al.*, 1994). The highest GUS activity was recorded when 400 mM sucrose was exogenously supplied to young seedlings, and a time course analysis showed that the activity was highest after 40 - 48 h incubation. The *cis*-acting, sucrose-responsive region of the *rolC* promoter was shown to be located between positions -135 and -94 (Yokoyama *et al.*, 1994). An AT-rich palindromic sequence in this sucrose-responsive region was found to be homologous to the *cis*-acting, sucrose-responsive region of the class I patatin gene, suggesting that the AT-rich sequence may be important for sucrose-responsiveness (Yokoyama *et al.*, 1994).

The site of initiation of transcription of the *rolC* gene is located 27 bp upstream from the first ATG codon. A CCAAT-box and TATA-box were found at -57 and -35, respectively (Kanaya *et al.*, 1990). DNA sequences responsible for the phloem-specificity of the *rolC* promoter were found within the -153 region, whereas a minimum region needed for the expression in the seed embryo was located at position -120 (Sugaya and Uchimiya, 1992). In the same study an enhancer element with consensus sequence CAAACCAC was found between -298 and -230, while an AT-box (AATATTTTTATT) which is a consensus sequence in the promoters of light-regulated genes, was found at -77 to -66 (Kanaya *et al.*, 1990; Sugaya and Uchimiya,

1992). In transgenic carrot cells containing the *rolC*-GUS fusion gene, suppression of the *rolC* promoter was seen when embryos were cultured in the presence of 2,4-dichlorophenoxyacetic acid. It was assumed that the developmental switch leading to the onset of embryogenesis could be responsible for the activation of the *rolC* promoter (Fujii and Uchimiya, 1991).

The regulatory functions of the *rolB* and *rolC* genes were shown to be conserved in the homologous genes (*Ngrol*) of *Nicotiana glauca* in tobacco genetic tumors (Nagata *et al.*, 1995). The *rolC* promoter has also been used in studies where phloem-specific expression of genes were required. In a study involving the investigation of the role of inorganic phosphate in phloem metabolism, the *ppa* gene from *E.coli* was expressed using the *rolC* promoter (Lerchl *et al.*, 1995). In another study, the *rolC* promoter was used together with the phloem-specific *Sh* promoter of maize sucrose synthase-1 to engineer potato leafroll virus (PLRV)-resistant plants (Graham *et al.*, 1997). The authors showed that the *rolC* promoter can be used to create PLRV-resistant line of potato and that it is likely to be effective for engineering resistance to other phloem-limited viruses. It was, however, not useful for engineering resistance to viruses that replicate in non-vascular tissues (Reimann-Phillip and Beachy, 1993).

2.4.2.3 Reporter genes

The expression of a foreign gene by transformed plant cells can best be assessed by determining the abundance or activity of its product. The coding sequences of genes for bacterial enzymes, which are easily assayed and whose activity is not normally found in plants, form the basis of many reporter genes (Webb and Morris, 1992).

Reporter genes are used for the assessment of promoter expression characteristics and the analysis of subcellular compartmentation studies. An ideal plant reporter gene should have a number of inherent characteristics, including that its product should be unique and should not be toxic to the host plant; the marker enzyme should exhibit a high degree of post-translational stability; a convenient and sensitive

enzyme assay for the reporter gene product should be available; and it should be amenable to translational fusions with extraneous polypeptides while retaining enzymatic activity (Jefferson *et al.*, 1987).

Examples of reporter genes are the *uidA* gene product of *E.coli* (β-glucuronidase or GUS) (Jefferson *et al.*, 1987), the *lacZ* gene product of *E.coli* (β-galactosidase or *lac*) (Herrera-Estrella *et al.*, 1990), chloramphenicol acyltransferase (*cat)* (Boutry *et al.*, 1987), neomycin phosphotransferase (*nptII)* (McDonnell *et al.*, 1987) and luciferase (*luc)* (Riggs and Chrispeels, 1987). Recently the jellyfish *Aequorea victoria* green fluorescent protein (GFP) has become a popular reporter gene due to its non-invasive detection method (Hu and Cheng, 1995). The two reporter genes that are used in this study are GUS and GFP, and these are discussed below.

i) β-glucuronidase (GUS)

The *E.coli* β-Glucuronidase (GUS, EC 3.2.1.31) encoded by the *uidA* locus is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides, many of which are available as spectrophotometric, fluorometric and histochemical substrates (Jefferson, 1987). The GUS gene has been cloned and sequenced, and encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Jefferson *et al.*, 1987). The blue staining indicative of GUS activity, using 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-Gluc) as substrate in transgenic plants, is usually easy to localise and gives visually striking results (Beeckman and Engler, 1994). It is not the product of the glucuronidase action on X-Gluc that is colored; instead the indoxyl derivative produced must undergo an oxidative dimerisation to form an insoluble and highly coloured indigo dye. The dimerisation is stimulated by atmospheric oxygen and can be greatly enhanced by using an oxidation catalyst such as potassium ferricyanide/ferrocyanide mixture. The relative degree of staining may not necessarily reflect the concentrations of β-Glucuronidase (Draper *et al.*, 1988). The GUS fusion system has successfully been used in numerous studies involving the development of transformation protocols and the analysis of promoter activity.

In many cases, as in whole-mount preparations and thick hand-cut sections, the pigments present in most plant tissues can partially or even totally mask the sites of GUS activity and consequently hinder the detection of it (Beeckman and Engler, 1994). A technique to clear all pigments, and even the brown phenolics that are formed due to oxidative polymerisation, has been developed. The clearing agent contains a 2:1:1 mixture of chloral hydrate, lactic acid and phenol, and the tissue is cleared after staining in X-Gluc solution (Beeckman and Engler, 1994).

The commonly used histochemical protocols for the detection of GUS activity are destructive, as they involve fixation, vacuum infiltration and prolonged incubation of the plant material in solutions containing X-Gluc, detergents, and organic solvents. A non-destructive assay system for roots using short-term incubations in X-Gluc and the spraying of 4-methylumbelliferyl-β-glucuronide (4-MUG) on the leaves was developed to follow both the quantitative and qualitative expression of chimeric GUS genes in most plant tissues without destroying the plants (Martin *et al.*, 1992).

ii) Green fluorescent protein (GFP)

Green fluorescent protein from the Pacific jellyfish (cnidarian) *Aequorea victoria* is a 27 kD monomer that absorbs violet/blue light (λ_{max}=395 nm with a minor peak at 470nm) and fluoresces green (λ_{max}=508 nm)(Hu and Cheng, 1995). The GFP chromophore is formed through intramolecular polypeptide cyclisation and oxidation (Heim *et al.*, 1994) and is derived from the primary amino acid sequence of the protein by post-translational modification (Cody *et al.*, 1993).

The structure and fluorescence mechanism (Youvan and Michel-Beyerle, 1996) as well as the physical and chemical properties of GFP (Yang *et al.*, 1996) have been investigated and it was found that the full length GFP, consisting of 238 amino acids, is required for fluorescence. The minimal chromophore responsible for light emission can, however, be assigned to a hexapeptide within the protein. This region contains a Ser-Tyr-Gly trimer at amino acid positions 65 to 67, which cyclises and yields a fluorophore that emits light (Cody *et al.*, 1993). The overall shape of the protein

consists of eleven strands of β-sheet forming the shape of a cylinder, capped with α-helices on the top and bottom. The α-helices form a scaffold for the chromophore, which is located near the geometric centre of the protein. This structure represents a protein fold nicknamed the β-can (Yang *et al.*, 1996).

The GFP is rather thermosensitive with the yield of fluorescently active to total GFP protein decreasing at temperatures greater than 30°C (Yang *et al.*, 1996). Besides light, no exogenous substrate or cofactor is required for fluorescence and GFP is extremely resistant to photobleaching, allowing detection over extended periods of time (Chalfie *et al.*, 1994). It maintains fluorescence even after prolonged incubation in strong denaturing agents such as 6 M guanidine HCl, 8 M urea or 1% (w/v) sodium dodecyl sulphate (SDS) (Bokman and Ward, 1981). GFP also has a broad range of pH stability, retaining conformation from pH 5.5 - 12.0 (Bokman and Ward, 1981).

GFP is expressed in both prokaryotic as well as eukaryotic cells (Kain *et al.*, 1995), and has been used as a reporter gene in a variety of systems including *E.coli*, *Caenorhabditis elegans* (Chalfie *et al.*, 1994), mosquitoes (Higgs *et al.*, 1996), *Drosophila melanogaster* (Wang and Hazelrigg, 1994; Brand, 1995), mammalian cells (Moss and Rosenthal, 1994; Pines, 1995; Garamszegi *et al.*, 1997), *Dictyostelium* (Hodgkinson, 1995), fungi (Flach *et al.*, 1994) and plants (Haseloff and Amos, 1995, Baulcombe *et al.*, 1995; Niedz *et al.*, 1995; Köhler *et al.*, 1997; Nagatani *et al.*, 1997). In a recent review on the applications of green fluorescent protein in plants, this reporter gene was described as the first truly *in vivo* reporter system useful in whole plants (Leffel *et al.*, 1997)

There are two major uses for GFP in plants: monitoring gene expression and protein localisation at high resolution, and providing an easily scored genetic marker in living plants (Haseloff and Amos, 1995). To employ GFP successfully in plants, the GFP apoprotein must be produced in suitable amounts in the plant cells, it must undergo efficient post-translational cyclisation and oxidation to produce the mature GFP, and the fluorescent protein may need to be suitably targeted within the cell to allow

efficient post-translational processing, safe accumulation to high levels, or to facilitate detection of expressing cells (Haseloff and Amos, 1995). It proved to be difficult to regenerate fertile *Arabidopsis* plants from the brightest GFP transformants, indicating the possibility that high levels of GFP are mildly toxic or interfere with regeneration, perhaps due to the fluorescent or catalytic properties of the protein (Haseloff and Amos, 1995).

Several modified versions of GFP have been developed to improve fluorescence. In *Arabidopsis thaliana* the expression of the wild-type GFP cDNA was curtailed by aberrant splicing, with an 84 nucleotide intron being excised efficiently from within the GFP coding sequence between nucleotides 400 and 483 (Haseloff *et al.*, 1997). A modified version of the GFP has been constructed with altered codon usage, to mutate the cryptic splice sites and to decrease the AU content of the mRNA (Haseloff *et al.*, 1997). This modified GFP was named mGFP4 and has been shown to result in increased fluorescence when compared directly to the wild-type GFP in *Arabidopsis* (Haseloff *et al.*, 1997) and conifer tissues (Tian *et al.*, 1997). Similar modifications were made to the GFP coding sequence and this modified protein, *synGFP*, also exhibited improved fluorescence in plants (Rouwendal *et al.*, 1997). The wild-type GFP has, however, been successfully expressed in *Citrus sinensis* protoplasts (Niedz *et al.*, 1995) and in maize protoplasts (Hu and Cheng, 1995), suggesting that the cryptic intron may not be recognised with equal efficiency in different plant species or perhaps during transient expression studies (Haseloff and Amos, 1995).

A synthetic GFP was also constructed with improved codon usage and by recursive cycles of DNA shuffling, a whole cell fluorescence signal that was 45-fold greater than the standard was obtained (Crameri *et al.*, 1996). In another investigation, a synthetic GFP gene was constructed to improve GFP expression in plants (Pang *et al.*, 1996). Replacement of the serine at position 65 with a threonine (*S65Tpgfp*) or a cysteine (*S65Cpgfp*) yielded 100- to 120-fold brighter fluorescence than wild-type GFP upon excitation with 490 nm light (Pang *et al.*, 1996; Reichel *et al.*, 1996).

Incorporation of a plant intron into the coding region yielded an additional 1.4-fold improvement (Pang *et al.*, 1996). When the tyrosine at position 66 was exchanged with a histidine (*Y66H*), a blue fluorescence was observed (Reichel *et al.*, 1996).

Several practical problems present themselves in visualising stably transformed plants for GFP and engineering a transgenic tracking system. The question whether GFP fluorescence is stable across generations was addressed by Leffel and coworkers (1997). Over 200 independent lines of GFP-containing transgenic tobacco was produced and the analysis of approximately 20 of these showed that the transgene was stably expressed. Fourth generation plants were still fluorescent (Leffel *et al.*, 1997). Protein analysis was conducted on transgenic plants to quantify GFP expression and to determine how much GFP must be produced for it to be unambiguously visualised in whole plants. The results indicated that approximately 0.1% mGFP4 expression is sufficient to yield highly fluorescent plants, and that fluorescence is positively correlated with protein expression ($P>0.05$)(Leffel *et al.*, 1997). A third important aspect that was looked at was the relationship between light quality and light quantity that plants use for growth and fluorescence. They found that irradiance can cause an apparent switch on/switch off of fluorescence. Under prolonged periods of cloudiness, previously highly fluorescent plants were scored as low-to-no fluorescence (Leffel *et al.*, 1997). Field experiments will have to be performed to test whether GFP decreases plant growth, seed yield and photosynthesis.

2.4.3 Transformation of sugarcane

Sugarcane (*Saccharum* spp. hybrids, Gramineae) was the first grass species to be grown in tissue culture (Sun *et al.*, 1993). Attempts to transform sugarcane using *Agrobacterium tumefaciens* have not been successful so far. Stably transformed sugarcane callus has been regenerated following electroporation and polyethylene glycol (PEG) treatment of protoplasts (Chen *et al.*, 1987) but the regeneration of plants from protoplasts has been extremely difficult (Chen *et al.*, 1988). The method of choice for the transformation of sugarcane is particle bombardment, and

suspension cultures (Franks and Birch, 1991; Sun *et al.*, 1993), embryogenic callus (Bower and Birch, 1992) and meristems (Gambley *et al.*, 1993) have been used as target tissues. The progress in molecular biology, and advances in the transformation of sugarcane have recently been reviewed (Birch *et al.*, 1996; Moore, 1996).

The success of microprojectile bombardment-mediated transformation of sugarcane callus is variety dependent, and the most critical parameter is the ability of the variety to produce embryogenic callus (Bower and Birch, 1992). Twelve sugarcane varieties grown commercially in South Africa were compared for both their ability to form white, embryogenic callus and their transformation potential (Snyman *et al.*, 1996). The plasmid pDP687 (Pioneer Hi-Bred International), which contains two anthocyanin transcriptional activators, each under control of a double CaMV *35S* promoter, was used to monitor transient expression after particle bombardment. Varieties N11, N21, N22, N23 and N24 produced little or no white, embryogenic callus while NCo310, NCo376, N12, N16 and N20 produced the highest proportion of white callus. Of these, NCo310 gave the best results. The highest transient anthocyanin production was observed in varieties NCo310 and Nco376 (Snyman *et al.*, 1996).

The relative expression strengths obtained from various promoter regions used in the transformation of sugarcane cells have been determined (Rathus *et al.*, 1993; Gallo-Meagher and Irvine, 1993). The cultivar NCo310 was used in a study to determine the effects of tissue type and promoter strength on transient GUS expression following particle bombardment (Gallo-Meagher and Irvine, 1993). Four promoters were tested in this study and the constructs including the GUS reporter gene were as follows: 1) pAHC27 (Christensen *et al.*, 1992) containing the promoter, first exon and first intron of the maize ubiquitin 1 gene (*Ubi-1*); 2) pEmuGN (Last *et al.*, 1991) containing the recombinant *Emu* promoter consisting of six copies of the anaerobic responsive element of the maize *Adh-1* gene, four copies of the *Agrobacterium tumefaciens* octopine synthase gene enhancer element, and a truncated maize Adh1 promoter plus first intron; 3) pAct1-D (Zhang *et al.*, 1991) containing the promoter, first exon, first intron and a portion of the second exon of the rice actin 1 gene (*Act1*)

in pBlueScript and 4) pBI221, a derivative of pUC19 containing the cauliflower mosaic virus 35S promoter (*35S*)(Jefferson *et al.*, 1987). The physical parameters used in this study had previously been optimised for the transformation of sugarcane. These included helium pressure regulator setting, target tissue distance, microprojectile type and size, DNA precipitation method and DNA load per bombardment. The results indicated that the *Ubi-1-GUS* construct (pAHC27) gave the highest mean number of GUS foci which was approximately 2, 4 and 12 times higher than *Emu-GUS* (pEmuGN), *Actl-GUS* (pAct1-D) and *35S-GUS* (pBI221) respectively. The size and intensity of the blue foci were also different with each construct, and when fluorometric GUS analysis was done, the same expression pattern was observed (Gallo-Meagher and Irvine, 1993).

The effects of promoter, intron and enhancer elements on transient gene expression in sugarcane and carrot protoplasts have been investigated (Rathus *et al.*, 1993). Several constructs were made containing the nopaline synthase promoter (*nos*), the CaMV 35S promoter (*35S*), the *ocs* enhancer, and intron 1 from maize *Adh1*, arranged upstream of the GUS-coding region. Levels of expression in electroporated sugarcane and carrot protoplasts were compared. *nos* and *35S* are weak but approximately equivalent strength promoters in sugarcane and result in 100-fold to 500-fold lower expression in sugarcane than in carrot. When two *35S* promoters were used back to back, they resulted in almost doubled expression in sugarcane, but caused no change in carrot. The inclusion of a 180 bp fragment including the *ocs* enhancer immediately upstream of *35S* increased expression 5-fold in sugarcane and 2-fold in carrot. Inclusion of intron 1 from maize *Adh1* between the promoter and coding region decreased expression from both *nos* and *35S* in sugarcane. The *Emu* promoter, with multiple enhancers close to the core promoter region, is more strongly enhanced relative to *35S* in sugarcane (400-fold) than in cereals (10- to 50-fold). This promoter is equivalent in strength to *35S* in carrot (Rathus *et al.*, 1993).

A lot of emphasis is currently being placed on the identification of gene regulatory elements which provide tissue and developmental specificity in sugarcane. Because

the first few sugarcane promoters have been silenced upon reintroduction by transformation, it is very important to extend the range of promoters tested for activity with heterologous genes following transformation (Potier and Birch, 1997a). These regulatory elements will be utilised in the transformation of sugarcane with the aims of improving crop traits and breeding higher yielding or better quality varieties (Moore, 1996).

In South Africa the major reasons for the production of transgenic sugarcane include disease and pest resistance and the increase of sucrose yield. Damage caused to cane plants by pests and diseases have a big effect on cane yield, with subsequent economical implications. The industry is also looking toward the manipulation of sucrose metabolism in order to increase sucrose production in the plant.

A major area of the sugarcane plant where transgene expression is required is the culm. This is not only the area where the stalkborer, *Eldana saccharina,* enters the plant and lives, but is also the region of the plant where sucrose is stored. Regulation of specific expression of transgenes in the culm will require culm-specific or culm-preferential promoter elements. The possible value of using regulatory elements which direct expression specifically to the vascular bundles has become apparent from a study of the anatomy of the sugarcane culm (Jacobsen *et al.*, 1992).

Sugarcane culm is composed of storage parenchyma tissue permeated by numerous vascular bundles. These bundles are surrounded by a sheath of two or more layers of thick-walled, lignified sclerenchyma cells (Jacobsen *et al.*, 1992). About 1 500 vascular bundles are scattered throughout sugarcane internodes. The bundles on the periphery of the culm are both smaller and more densely packed than those closer to the centre so that 50% of the total number are within the outer 1 mm and 75% are within the outer 3 mm of the culm (Jacobsen *et al.*, 1992).

The complicated arrangement of vascular bundles in the monocotyledonous stem, and especially in the Gramineae, is associated with the position of the various

bundles of each leaf in the stem (Fahn, 1990). Three types of leaf trace bundles are present in the culm. The first type is represented by those bundles derived from mid-ribs and the lateral veins. These penetrate deep into the interior of the stem and then pass to the periphery in lower nodes. The second type is represented by leaf trace bundles derived from smaller lateral veins which, immediately on entering the culm, occupy the position of the outermost peripheral bundles with which they fuse. The third type is represented by bundles that are very thin and which soon fade out within the cortex close to the level of the node. In the Gramineae there are special horizontal bundles at the nodes which connect the leaf bundles together (Fahn, 1990).

A consequence of the arrangement of the sugarcane vascular system is that products of transgenes that are expressed only in the vascular bundles will be concentrated on the periphery of the culm and only in the "veins" of the leaves. This is particularly advantageous for targeting aphids and leafhoppers, which are phloem feeders, viruses that replicate in the vascular bundles, and stalkborers. Because of the tissue-specificity, the expression of a foreign gene will not take place at the expense of other processes in the plant.

Genes that are expressed in a tissue-specific manner in sugarcane have to be identified. One candidate gene was identified in a study of sucrose metabolism where the enzyme activity patterns in the sink tissues of the sugarcane plant were investigated (Whittaker and Botha, 1996). This gene, pyrophosphate-dependent phosphofructokinase (PFP), has not previously been isolated from sugarcane.

2.5 PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE (PFP)

Two enzymes in the cytosol of plant cells catalyse the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. The first of these is ATP-dependent phosphofructokinase (PFK, EC 2.7.1.11) which utilises ATP, catalyses an irreversible reaction, and is found in the majority of organisms (for a review see Dennis and Greyson, 1987). The second, PFP (EC 2.7.1.90), catalyses the reversible

interconversion between fructose 6-phosphate (Fru-6-P) and fructose 1,6-bisphosphate (Fru-1,6-P_2), using pyrophosphate (PPi) as a phosphoryl donor in the forward reaction and inorganic phosphate (Pi) as a phosphoryl acceptor in the reverse direction:

$$\text{Fru-6-P} + \text{PPi} \leftrightarrows \text{Fru-1,6-P}_2 + \text{Pi}$$

A pH optimum of 7.5 - 8.0 and magnesium is essential for the interconversion reaction. PFP activity has been investigated in a number of plant species, for example pineapple (Carnal and Black, 1979), *Citrillis lanatus* (Botha and Botha, 1991), *Phaseolus vulgaris* (Botha and Small, 1987), *Brassica nigra* (Duff *et al.*, 1989), potatoes (Kruger and Dennis, 1987), soybean (Macdonald and Preiss, 1986), carrots (Wong *et al.*, 1990) and wheat (Yan and Tao, 1984), as well as in the specialised microorganisms *Entamoeba histolytica* (Reeves *et al.*, 1976) and *Propionibacterium shermanii* (O'Brien *et al.*, 1975).

In most of the plants examined, PFP is composed of two immunologically unrelated subunits (Kruger and Dennis, 1987), namely the larger α-subunit (65 - 68 kD) and the smaller β-subunit (60 - 64 kD). In wheat tissue the PFP protein is present as two isoforms, a heterotetramer ($\alpha_2\beta_2$) and a homodimer (β_2) (Yan and Tao, 1984). In carrot (Wong *et al.*, 1988) and *Citrillis lanatus* (Botha and Botha, 1991), isoforms were detected with either an α- and β-subunit or only a β-subunit composition. The amount of α- and β-subunit also varies in different plant tissues (Botha and Botha, 1991) and in some cases, correlates with the phosphate status of the tissue (Theodorou and Plaxton, 1992). Although there is an approximate 60% homology between the deduced amino acid sequence of the two subunits, they are immunologically distinct (Carlisle *et al.*, 1990).

The sequences of cDNA clones indicate that the β-subunit is catalytic, since it has putative Fru-6-P/Fru-1,6-P_2 binding sites (Yan and Tao, 1984) and the catalytic residues, Asp-127 and Arg-171, were shown to be conserved in this subunit (Carlisle *et al.*, 1990). The α-subunit may be involved in the regulation of the enzyme by binding the allosteric activator fructose 2,6-bisphosphate (Fru-2,6-P_2)(Cheng and

Tao, 1990). Fru-2,6-P_2 activates PFP in both the forward (glycolytic) and reverse (gluconeogenic) directions by increasing V_{max} and increasing the affinity for Fru-6-P, Fru-1,6-P_2 and PPi. Fru-2,6-P_2 also relieves the inhibition of PFP by high levels of PPi (Sabularse and Anderson, 1981).

Although the kinetic properties of PFP have been studied in detail, the physiological role of the enzyme is still unknown. PFP is suggested to be involved in both the regulation of sucrose accumulation and the biosynthetic activity in plant cells (Dennis and Greyson, 1987; Huber and Akazawa, 1986; Nakamura *et al.*, 1992). Possible functions that have been suggested for PFP include the regulation of cytosolic PPi (ap Rees *et al.*, 1985), maintenance of the equilibrium of the hexose/triose phosphate pools (Dennis and Greyson, 1987), and an adenylate bypass for glycolysis during periods of phosphate limitation or starvation (Duff *et al.*, 1989).

The regulation of the cytosolic PPi levels by PFP is consistent with the involvement of the enzyme during sucrose cleavage (Dancer and ap Rees, 1989). In certain tissues engaged in starch biosynthesis, PFP is proposed to generate PPi as an energy source to stimulate the PPi-dependent sucrose cleavage pathway by sucrose synthase and uridine 5'-diphosphate glucose (UDPGlc) pyrophosphorylase (Dancer and ap Rees, 1989). PFP and sucrose synthase activity have been suggested to play a role in controlling sink strength (Black *et al.*, 1995), which can be defined as the ability of the sink organ to import assimilate (Ho *et al.*, 1989). Sucrose may act as a signal molecule to induce expression of both sucrose synthase and PFP (Black *et al.*, 1995). It has been concluded that PFP is an adaptive enzyme with kinetic and molecular properties that are responsive to a variety of environmental, developmental and tissue-specific cues (Plaxton ,1996).

Full-length cDNA clones for the α- and β-subunits of PFP have been isolated from a cDNA expression library derived from potato tuber mRNA (Carlisle *et al.*, 1990). The nucleotide sequences of the subunits were determined and when compared, it was shown that the subunits were related with approximately 40% of the amino acids

being identical. It was suggested that the genes for these polypeptides may have been formed by gene duplication (Carlisle *et al.*, 1990). However, the genes for the two subunits are complex and different with the α- and β-genes containing 19 and 16 exons, respectively, and only two introns showing alignment (Todd *et al.*, 1995). Near full-length cDNA clones for both subunits from a developing castor seed endosperm cDNA library have also been isolated and these were similar in sequence to those described for potato (Blakeley *et al.*, 1992).

2.5.1 PFP in sugarcane

Sucrose accumulation in sugarcane is suggested to be primarily regulated within the translocation system and/or the level of the sink (Moore, 1995). Significant differences in the levels of sucrose and fiber occur among the *Saccharum* species. In sugarcane sink tissue there is likely to be substantial competition for incoming sucrose for storage and biosynthetic activity, including structural polysaccharide (fiber) synthesis (Whittaker and Botha, 1996).

In a recent study to investigate whether PFP is linked to sucrose accumulation and fiber content in sugarcane, the activity patterns of PFP were measured in the top ten internodes of five different cane varieties (Whittaker and Botha, 1996). PFP activity increased from internode 3, reaching optimal activity at internode 6 and 7, after which the enzyme declined to internode 10. An inverse correlation between peak PFP activity/expression levels and the prevailing sucrose content of internodal tissue, and a positive relationship between PFP activity and the insoluble matter content was observed (Whittaker and Botha, 1996). Differences in PFP activity both within the developing sugarcane stalk and between different varieties, were reflected by PFP protein concentrations.

To investigate the distribution of sucrose, fiber and PFP activity within an internode, representative sections of an internode were cut into three concentric rings (each constituting 30% of the total area), from the core to the periphery. PFP activity correlated positively with the increase in fiber deposition from the core to the

periphery. There was an inverse trend in sucrose accumulation, which correlated negatively with PFP activity (Whittaker and Botha, 1996).

CHAPTER 3

USE OF A DIFFERENTIAL DISPLAY TECHNIQUE TO ISOLATE DIFFERENTIALLY EXPRESSED GENE SEQUENCES FROM SUGARCANE CULM TISSUE

3.1 ABSTRACT

An mRNA differential display technique was evaluated for the identification and isolation of differentially expressed gene sequences in the sugarcane culm. RNA was isolated from leafroll, leaf, mature culm and young culm tissues and reverse transcribed to cDNA. The two original methods developed by Liang and Pardee (1992) and Welsh *et al.* (1992) were combined and modified in this study. After reverse transcription polymerase chain reaction (RT-PCR), amplification products were separated in agarose gels and the fragments that appeared to be unique to the culm tissues were isolated and reamplified. An RT-PCR Southern blot technique was used to verify the tissue specificity of isolated fragments, and to determine whether those fragments were members of multigene families or if they were present in several isoforms. Fragments that were possibly culm-preferential were cloned and their sequences determined.

A series of 120 random decamer primers was used to obtain 1 767 fragments, resulting in an average of 15 fragments per primer. Thirty five (2%) of these fragments were possible culm-specific sequences. Twenty one of the fragments were characterised using a hybridisation technique and four of these were thus identified as putative culm-preferential rather than culm-specific fragments. The complete sequences of three of the four fragments and a partial sequence of the other one were determined, and no significant homology to known differentially expressed sequences in the international databases were found. One of the fragments, SA11, was analysed further by RT-PCR using longer sequence-specific primers. Amplification products obtained from cDNA and genomic DNA templates using these primers were of identical size. Results of a series of control reactions showed that the

synthesis of the SA11 fragment from RNA was reverse transcription-dependent, and was not a product of genomic DNA contamination. Although the fragment appeared to be culm-preferential, it could not be used to obtain a culm-specific promoter. The other three fragments were not analysed further because, as in the case of SA11, no conclusive evidence that these fragments were indeed culm-preferential could be obtained from either the northern blots or the sequencing analysis. Further modifications and refinements of the mRNA differential display technique have to be made before it can be routinely applied to sugarcane.

3.2 INTRODUCTION

The development of techniques to manipulate specific DNA fragments has led to advances in the ability to selectively alter the genetic make-up of plants and other organisms. Physiological function can be investigated by altering gene expression (ap Rees, 1995), and genes that can confer resistance to pests (Peferoen, 1992), diseases (Sturtevant and Beachy, 1993; Shah *et al.*, 1995) or environmental conditions (Vierling and Kimpel, 1992) can be introduced. In addition, crop quality can also be improved for specific purposes, e.g. taste and processing characteristics (Hiatt, 1993), color (Fraley, 1992; Mol *et al.*, 1995), shelf life (Oeller *et al.*, 1991), etc. Many useful modifications involve the alteration of the amount of desirable or undesirable compounds by up- or down-regulating the gene expression. For example, the insertion of an antisense form of the polygalacturonase (PG) gene into tomato fruit, led to a reduction in the PG levels and an improvement of the post-harvest fruit quality, due largely to enhanced firmness and increased resistance to post-harvest fungal disease (Hiatt, 1993; Smith *et al.*, 1993). Plants can also be used for the production of novel compounds (for reviews see Groenewald *et al.*, 1995; Goddijn and Pen, 1995) or for the overproduction or bioconversion of natural compounds in the plant (Pen *et al.*,1993).

The most important aspect of the genetic manipulation of plants, is the isolation of relevant genes and regulatory elements (Fraley and Schell, 1992; Gibson and Somerville, 1993). By using appropriate promoter elements, the desired response of

the transgenes in the recipient plant to environmental stimuli, or the synthesis of the product in a specific tissue can be ensured. Furthermore, a strong promoter is the prerequisite of a high transcription frequency (Walden and Wingender, 1995).

For some applications the constitutive expression of transgenes will be required, while in other cases the use of an inducible or tissue-specific promoter sequence would be more advantageous. Targeted gene expression is currently being used in studies involving the elucidation of specific metabolic pathways. For example, the phloem-specific expression of the *E.coli* inorganic pyrophosphatase gene led to the removal of pyrophosphate (PPi) from the phloem and the elucidation of the role of PPi in phloem metabolism (Lerchl *et al.*, 1995).

The first step in the search for tissue-specific promoters is the identification and isolation of differentially expressed genes. General approaches that are followed to identify and isolate gene sequences that are differentially expressed, i.e. in a regionally, temporally or environmentally specific way, include "+/-" screening and subtraction approaches (Sargent, 1987). These methods have several disadvantages which have been discussed in Chapter 2.

The differential display technique was developed to identify and characterise differentially expressed genes by detecting individual mRNA species, and then isolating and analysing the cDNA (Liang and Pardee, 1992; Liang *et al.*, 1993). The method uses the polymerase chain reaction (PCR) to amplify even rare cDNAs, making the identification of differentially expressed genes of low abundance possible. This method can replace subtractive hybridisation techniques because it is less time consuming, and allows the simultaneous handling of numerous samples. Comparison of RNA samples from different cells allows the identification and cloning of differentially expressed genes. A similar technique utilising a single randomly selected primer at low stringency for first and second strand cDNA synthesis, was also developed (Welsh *et al.*, 1992).

The differential display technique was originally developed for the detection of altered gene expression in mammalian cell populations (Liang and Pardee, 1992). Numerous refinements and optimisations have since been made (Liang *et al.*, 1993; Bauer *et al.*, 1993; Callard *et al.*, 1994; Hadman *et al.*, 1995; Liu and Shearn, 1995; Zhao *et al.*, 1995; Doss, 1996), and these have been discussed in Chapter 2. The method has been used in different studies with various degrees of success. Applications range from the analysis of gene expression in pre-implantation mouse embryos (Zimmermann and Schultz, 1994) to the isolation of senescence-specific genes from *Arabidopsis* plants (Callard *et al.*, 1994).

In sugarcane, the culm is the major area of sucrose accumulation, and it is also the point of entry for the stalkborer, *Eldana saccharina*. The culm is therefore one of the main target areas for transgene expression in both borer resistance programmes as well as metabolic manipulation studies in South Africa. After positive results were obtained by other groups using the differential display technique, this method was investigated as a tool for the isolation of culm-specific genes from sugarcane. The aim was to simplify the method and yet still identify as many culm-specific cDNA sequences as possible, and then to use them to isolate the genes and subsequently the promoters.

The working hypothesis was as follows: the use of a simple oligo$(dT)_{15}$ primer instead of a group of anchored dT-primers to prime reverse transcription, will reduce the number of reverse transcription reactions from four to one, but will still target the poly(A)-tails of the mRNA. Secondly, since no sequence information of sugarcane genes is available, the cDNA can be amplified using the reverse transcription primer together with random primers. Thirdly, the separation of the amplification products in agarose gels instead of denaturing polyacrylamide gels can make the method easier and faster. Although less fragments can be resolved in agarose gels, more primer combinations can be used to overcome this drawback. The use of this method should thus allow the rapid screening of cDNA populations in sugarcane with large numbers of primers.

3.3 MATERIALS AND METHODS

3.3.1 RNA Preparation

Total RNA was isolated from mature (6 to 12 month old) field-grown sugarcane plants (variety N19) using a modified acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). In sugarcane, the internode attached to the leaf with the uppermost visible dewlap was defined as internode no. 1, according to the system of Kuijper (Van Dillewijn, 1952). All solutions were prepared with water that had been treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC). Glassware was autoclaved and baked at 200°C prior to use, and plastic ware was used directly from sterile unopened packs. Five grams of tissue from leafroll, leaf, young culm (internode 2) or mature culm (internode 7) was ground in liquid nitrogen and homogenised in 5 ml denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH7.0; 0.5% (w/v) sarkosyl; 100 mM β-mercaptoethanol). After the addition of 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml phenol and 1 ml chloroform:isoamylalcohol (24:1), the suspension was shaken vigorously and left on ice for 15 min. After centrifugation at 10 000 *g* for 20 min at 4°C, the aqueous phase was removed and the RNA was precipitated with an equal volume of isopropanol at -20°C for at least 1 h. RNA was collected by centrifugation at 10 000 *g* for 20 min at 0°C. The pellet was dissolved in 1.5 ml denaturing solution and the precipitation was repeated, after which the resulting pellet was washed with 70% ethanol (room temperature) and dried under vacuum. The RNA was resuspended in 250 µl DEPC-treated water at 65°C for 15 min. Isolated RNA was quantified spectrophotometrically and the integrity was verified by examining the 18S and 28S ribosomal fragments after separation in a formaldehyde-containing 1.5% (w/v) agarose gel. RNA preparations were stored at -80°C.

3.3.2 Reverse transcription

cDNA was synthesised in 20 µl volumes containing 1 µg total RNA, 20 pmol oligo$(dT)_{15}$ primer, 10 units placental ribonuclease inhibitor, 0.1 mM of each deoxynucleoside triphosphate (dNTP) and 4 units M-MuLV Reverse Transcriptase (Boehringer Mannheim). The reaction mixture was incubated at 37°C for 1 h.

3.3.3 Amplification of cDNA

Prior to amplification the cDNA was purified through a QIAquick-spin PCR purification column (QIAGEN). Single random decamer primers were used for PCR (Operon Technologies). The PCR reaction mixture contained 5 µl of cDNA template, 10 mM Tris-HCl, pH 8.3; 10 mM KCl; 3.8 mM $MgCl_2$; 0.2 µM random primer; 0.1 mM of each dNTP and 1 unit of Ampli*Taq* Stoffel fragment (Perkin Elmer) in a volume of 15 µl. The samples were overlaid with 30 µl mineral oil and subjected to 41 cycles of PCR using the following cycling parameters: 1 cycle of 94°C for 3 min, 35°C for 1 min and 72°C for 2 min with a 2.4°C s^{-1} ramp; 40 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 2 min using a 2.4°C s^{-1} ramp. The last cycle was followed by a 5 min extension at 72°C. PCR amplification products were separated in 2% (w/v) agarose gels in 1x TAE buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8.0). After staining with ethidium bromide (0.01 mg ml^{-1}), the gels were photographed and the banding patterns analysed for the presence of tissue-specific fragments.

3.3.4. Preparation of molecular weight markers for agarose gel electrophoresis

The numbers of the following molecular weight markers are those numbers that were assigned by Boehringer Mannheim.

Marker 2: 20 µg of bacteriophage lambda DNA was treated with 20 units of the restriction endonuclease *Hind* III for 3 h at 37°C in a 100 µl reaction volume. A volume of 200 µl TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) was added to the restricted product, together with 30 µl loading buffer (40% (v/v) glycerol; 1 mM EDTA; 0.02% (w/v) bromophenol blue; 0.02% (w/v) xylene cyanol). Ten microlitres of this mixture was loaded per lane of the agarose gels. The sizes of the fragments obtained after electrophoresis in a 0.8% (w/v) agarose gel are shown in Figure 3.1(A).

Marker 3: 20 µg of bacteriophage lambda DNA was treated with 20 units of each of the restriction endonucleases *Eco*R I and *Hind* III for 3 h at 37°C in a 100 µl reaction volume. The mixture was then treated the same as Marker 2. The sizes of the fragments obtained after electrophoresis in a 0.8% (w/v) agarose gel are shown in Figure 3.1(B).

Marker 5: 20 µg of pBR322 plasmid DNA was treated with the restriction

endonuclease *Hae* III for 3 h at 37°C in a reaction volume of 100 µl. The mixture was then treated the same as Marker 2. The sizes of the fragments obtained after electrophoresis in a 2% (w/v) agarose gel are shown in Figure 3.1(C).

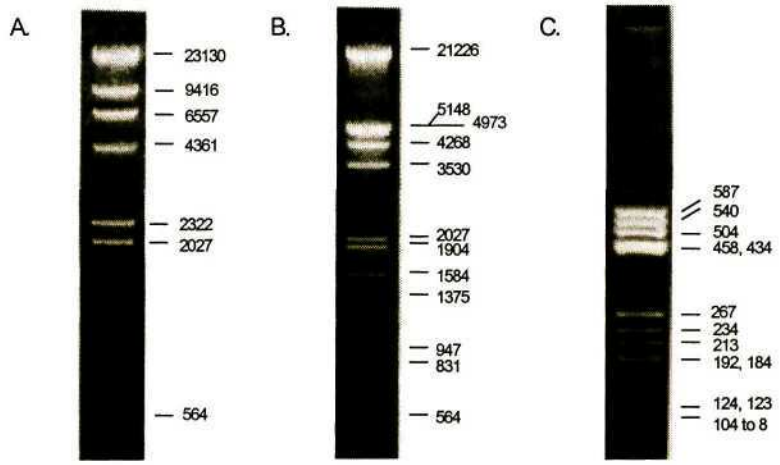

Figure 3.1 Molecular weight markers used in this study. A.) Marker 2. Fragments obtained after lambda DNA was treated with *Hind* III and separated in a 0.8% (w/v) agarose gel. B.) Marker 3. Fragments obtained after lambda DNA was treated with *Eco*R I and Hind III and separated in a 0.8% (w/v) agarose gel. C.) Marker 5. Fragments obtained after pBR322 DNA was treated with *Hae* III and separated in a 2% (w/v) agarose gel. Sizes of fragments are indicated in base pairs (bp).

3.3.5 Isolation and reamplification of possible tissue-specific fragments

Fragments that appeared to be unique to the culm tissue were punched out of the gel using a Pasteur pipette, and were placed in 100 µl TE buffer. Samples were incubated at 65°C for 10 min, vortexed and stored at 4°C. For reamplification of the fragments, 5 µl of this solution was used. PCR conditions were as described before (3.3.3) and after reamplification, the size of the product was verified by loading 1 µl of the reaction mixture in a 2% (w/v) agarose gel. In cases where more than one reamplification product was observed, the fragment of expected size was isolated from the gel using the QIAquick Gel Extraction Kit (QIAGEN). These isolated fragments were stored at -20°C.

3.3.6 Characterisation of isolated fragments by RT-PCR Southern blotting

The amplification products in the differential display gels showing putative tissue-specific fragments were transferred to a nylon membrane (MagnaGraph, MSI). A downward capillary blotting technique as described by Chomzcynski and Mackey (1994) was used. After overnight transfer the damp membrane was exposed to ultraviolet (UV) light for 2.5 min at 120 mJ cm^{-1} to link the DNA to the nylon. The membrane was then probed with the specific isolated fragment, which was radioactively labelled using a random primer labelling kit (Prime-It II; Stratagene) in the presence of [α-^{32}P]dCTP (Amersham; 3000 Ci $mmol^{-1}$). Prehybridisation and hybridisation were performed in a solution containing 5x SSC (150 mM NaCl; 15 mM tri-sodium citrate, pH 6.8), 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.5% (w/v) SDS, 5x Denhardt's solution (0.02% (w/v) of each bovine serum albumin (BSA), polyvinylpyrrolidone (PVP) and ficoll), and 100 µg ml^{-1} sheared herring sperm DNA. Prehybridisation of 4 h at 55°C was followed by overnight hybridisation at 55°C after the addition of the probe that was denatured by boiling for 5 min. The membrane was washed twice at room temperature for 20 min and once at 50°C for 20 min in a solution containing 2x SSC and 0.1% (w/v) SDS. This was followed by a 15 min wash at 50°C in 0.2x SSC and 0.1% (w/v) SDS. The membrane was exposed to X-ray film for 24 to 48 h.

3.3.7 Cloning of cDNA fragments

Because of the presence of the same primer on both ends of the fragment, sequencing could not be done directly using the random decamer primer. The fragments were therefore cloned using the pCR-Script (SK+) cloning kit (Promega). The manufacturer's instructions were followed, except for the use of *E.coli* JM83 cells instead of *Epicurian coli* supercompetent cells for transformation. Clones were identified using blue/white selection on X-Gal-containing Luria plates (1% (w/v) NaCl; 1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1.5% (w/v) agar). Insert sizes were determined by PCR amplification of the inserted fragments using the vector-specific T7 promoter and pUC18/M13 reverse primers.

3.3.8 Sequence determination

The *fmol*™ DNA sequencing system (Promega) was used together with the vector-specific T7 promoter primer and the M13/pUC18 reverse primer. A cycle sequencing protocol using direct incorporation of [α-^{35}S]dATP (Amersham) was followed, after which the sequencing products were separated in 6% (w/v) denaturing polyacrylamide gels. The gels were dried and exposed to X-ray film for 24 to 48 h. Sequences were read manually from the film.

3.3.9 Genomic Southern blot analysis

Genomic DNA from sugarcane variety N19 (extracted using the method of Honeycutt *et al.*,1992) was treated separately with each of the restriction endonucleases *Bam*HI, *Eco*R I and *Hind* III. The three restriction reactions were incubated for 24 h at 37°C using 20 µg of DNA and 60 units of each enzyme in a 200 µl reaction volume. After ethanol precipitation, the samples were dried and resuspended in 20 µl TE buffer. Elecrophoresis in a 0.8% (w/v) agarose gel was followed by ethidium bromide staining (0.01 mg ml^{-1}) to confirm the satisfactory restriction of the DNA. The restricted DNA was then transferred to a nylon membrane (MagnaGraph; MSI). Downward capillary blotting was done as described (3.3.6), and the DNA was linked to the nylon membrane by exposing it to UV light for 2.5 min at 120 mJ cm^{-1}. As a positive control for the labelling of the culm-preferential cDNA fragments, 0.5 ng of each fragment was also included in the gel.

Twenty five nanogram of each fragment was radioactively labelled using the Prime-It II random primer labelling kit (Stratagene) in the presence of [α-^{32}P]dCTP (Amersham; 3000 Ci mmol^{-1}). After 4 h prehybridisation in RapidHyb buffer (Amersham) at 55°C, the probe was denatured for 5 min in boiling water and added to the prehybridisation buffer. Hybridisation was done for 16 h at 55°C, after which the membrane was washed twice at 55°C for 20 min in a solution containing 2x SSC and 0.1% (w/v) SDS, once at 60°C for 15 min in a solution containing 2x SSC and 0.1% (w/v) SDS and once at 60°C for 20 min in a solution containing 0.2x SSC and 0.1% (w/v) SDS. The membrane was sealed in a plastic bag and exposed to X-ray

film for 48 h. After the film was developed, the radioactive probe was stripped from the membrane by washing in a solution containing 50% (v/v) formamide and 2x SSC at 65°C for 1 h. The membrane was rinsed in 0.1x SSC before it was prehybridised again and probed with the next fragment. The membrane was kept moist between hybridisations and was stripped three times before being discarded.

3.3.10 Northern blot analysis of isolated cDNA fragments

Twenty microgram of total RNA from leafroll, leaf, young culm (internode 2) and mature culm (internode 7) was separated in a 1.2% (w/v) agarose gel. Samples were prepared by adding 50% (v/v) formamide, 6% (v/v) formaldehyde and 1x MOPS buffer (200 mM 3-[*N*-Morpholino]propanesulphonic acid (MOPS); 50 mM NaOAc; 5 mM EDTA) and heating to 65°C for 10 minutes before loading. One sample was loaded separately together with the 0.24 - 9.5 kb RNA Ladder (GibcoBRL), which was treated the same as the samples. After separation in the gel, these two lanes were cut off and stained in ethidium bromide (0.1 mg ml^{-1}). The sizes of the fragments were verified using the RNA Ladder. The rest of the samples were transferred overnight to a positively charged nylon membrane (Boehringer Mannheim) by downward capillary blotting in 10x SSC. The RNA was linked to the nylon membrane by exposing it to UV light for 2.5 min at 120 mJ cm^{-1}.

Prehybridisation was done in a solution containing 250 mM phosphate buffer (pH 7.0), 7% SDS and 1 mM EDTA at 65°C for at least 4 h. Labelling of fragments was done as described in 3.3.6, and the specific activities ranged from 1.2 x 10^9 to 2.3 x 10^9 cpm μg^{-1}. Hybridisation followed in the same buffer for 20 h at 65°C. The membrane was washed twice at 65°C in a solution containing 0.2x SSC and 0.1% (w/v) SDS for 20 min after which it was sealed in a plastic bag and exposed to X-ray film. The film was developed after 24 h, but a signal could only be observed after 6 days. Radioactive probes were stripped from the nylon membrane by washing 3 times in a solution containing 0.1x SSC, 1% (w/v) SDS and 40 mM Tris-HCl (pH 7.5) at 80°C.

3.3.11 *Characterisation of the culm-preferential fragment, SA11, by specific amplification*

Sequence specific amplification primers were designed to amplify the SA11 fragment. The primers were both 20-mers with a GC-content of 50 -55%: SA11-F: 5'-ATC GCC GTC AAT GAG CAC TC-3' and SA11-R: 5'-AGA GGT TGT AGT GAC ATC GG-3'. Specific amplification reactions were done in 15 µl volumes using 1 µM of each primer. Different templates were used in these reactions and were as follows: cDNA from internode 7 tissue: 5 µl of purified reverse transcription mixture as described in 3.3.3, RNA from leafroll and internode 7 tissues: 1 µg of total RNA, genomic DNA from sugarcane varieties NCo310 and 84F3097: 100 ng, and the cloned SA11 fragment as positive control: 15 ng. The rest of the PCR mixture was the same as described in 3.3.3, except that it contained 2 units of *Taq* DNA Polymerase (Boehringer Mannheim) instead of the Ampli*Taq* Stoffel fragment. The cycling parameters were as follows: 1 cycle of 94°C for 2 min, 39 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min. The last cycle was followed by a 7 min extension at 72°C. Amplification products were separated in 2% (w/v) agarose gels.

3.3.12 *Characterisation of the culm-preferential fragment, SA11, by specific RT-PCR*

The GeneAmp RNA PCR Kit (Perkin Elmer) was used for the specific RT-PCR of SA11. The reverse transcription reaction was done at 42°C for 15 min in a volume of 10 µl containing 5 mM $MgCl_2$, 1x PCR buffer II (supplied in the kit), 1 mM of each dNTP, 2.5 units of RNase inhibitor, 2.5 µM oligo$(dT)_{16}$ primer or 0.75 µM of the specific primer; 1 µg of total RNA and 5 units of M-MuLV Reverse Transcriptase, and was overlaid with 30 µl of mineral oil. The reaction was terminated by heating to 99°C for 5 minutes and then cooling to 5°C. The amplification reaction mixture was added directly to the cDNA after reverse transcription and contained 2 mM $MgCl_2$, 1x PCR buffer II, 2.5 units of AmpliTaq DNA Polymerase and 0.15 µM of each primer. This mixture was spun through the oil of the RT mixture and was subjected to 42 cycles of PCR using the following cycling parameters: 1 cycle of 94°C for 2 min, 40 cycles of 94°C for 30 s, 45°C for 45 s and 72°C for 3 min. The last cycle was followed by

an 8 min extension at 72°C. The amplification products were separated in 2% (w/v) agarose gels.

3.4 RESULTS

3.4.1 Optimisation of PCR template concentration

The RNA isolation technique that was used yielded intact RNA averaging between 40 µg g^{-1} fresh mass (fm) tissue from the mature culm to 150 µg g^{-1} fm tissue from the leaf. Initially, aliquots of 2.5 µl and 5.0 µl of the reverse transcription reaction mixture were used in amplification reactions, and the oligo$(dT)_{15}$ primer was used together with a random decamer primer.

Figure 3.2 Amplification of cDNA using the oligo$(dT)_{15}$ and a random decamer primer. Reverse transcription was done using an oligo$(dT)_{15}$ primer after which 2.5 µl (lane1) and 5.0 µl (lane 2) of the RT mixture was used in amplification reactions. Both the dT primer as well as random primer OPA02 were used in this PCR. Molecular weight marker 3 is shown in lane 3.

Only smears could be seen after the amplification reactions. The possibility that the template concentration was too high was investigated by using a dilution series of the reverse transcription reaction mixture ranging from 1:5 to 1:1 000 (results not shown). When amplification of these dilutions also yielded smeared profiles, the interference of the reverse transcription primer was investigated. The oligo$(dT)_{15}$ primer was removed by the purification of the reverse transcription reaction mixture through an affinity column. Initially, undiluted purified cDNA was used in amplification reactions, but only a few fragments were formed (results not shown). The possibility of an excess of template was investigated. A dilution series was prepared from mature culm (internode 7) cDNA ranging from 1:50 to 1:5 000 to determine the optimal concentration for amplification, and the amplification was done using a single random decamer primer, OPF12. Four reactions were done with each template concentration

to verify reproducibility. In all the amplification reactions, a volume of 5 µl of the template was used. The banding profiles are shown in Figure 3.3.

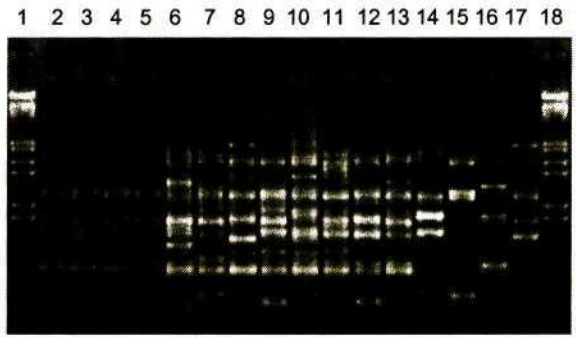

Figure 3.3 Optimisation of the PCR template concentration for differential display. A dilution series of the reverse transcribed cDNA from internode 7 was prepared. Four identical samples were amplified from each concentration to test the reproducibility of the amplification profiles. Primer OPF12 was used in these reactions. Lanes 1 and 18: molecular weight marker 3, lanes 2 - 5: cDNA dilution 1:50, lanes 6 - 9: cDNA dilution 1:500, lanes 10 - 13: cDNA dilution 1:1000, lanes 14 - 17: cDNA dilution 1:5000.

The only reproducible banding pattern was produced when the 1:50 dilution (Figure 3.3 lanes 2 - 5) was used. At lower template concentrations (higher dilutions) the banding patterns were highly variable between supposedly identical samples, and therefore these template concentrations could not be used for differential display. A second dilution series was prepared for finer optimisation of template concentration, and this ranged from 1:50 to 1:500. These dilutions were amplified using primer OPF06. Four identical samples were used from each dilution to ensure reproducibility (Figure 3.4).

A reproducible banding pattern was produced when the 1:50 (Figure 3.4 lanes 2 - 5) and 1:100 (Figure 3.4 lanes 6 - 9) dilutions were used. With lower template concentrations, the banding patterns were once again very variable. The 1:100 dilution of the purified reverse transcription reaction mixture, in which 1 µg of total RNA was reverse transcribed, was used as template in the series of amplifications that followed. The concentration of the cDNA was not determined.

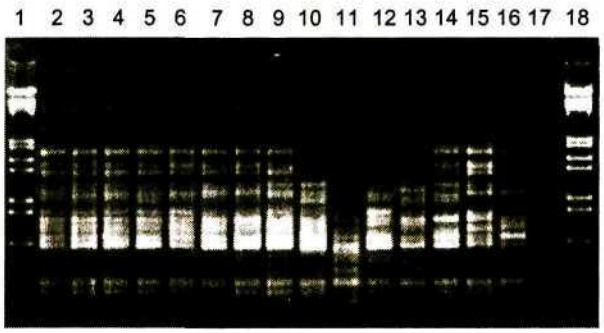

Figure 3.4 Optimisation of the PCR template concentration for differential display. A dilution series of the reverse transcribed cDNA from internode 7 was prepared. Four identical samples of each dilution were amplified to test the reproducibility of the amplification profiles. In these reactions primer OPF06 was used. Lanes 1 and 18: Molecular weight marker 3, lanes 2 - 5: cDNA dilution 1:50, lanes 6 - 9: cDNA dilution 1:100, lanes 10 - 13: cDNA dilution 1:250, lanes 14 - 17: cDNA dilution 1:500.

3.4.2 Differential display: the comparison of random amplified fragments from different sugarcane tissues

A series of 120 random decamer primers was used in amplification reactions and cDNA fragments from leafroll, leaf, young culm (internode 2) and mature culm (internode 7) were compared. A total of 1767 fragments were amplified, resulting in an average of 15 fragments per primer. The size of amplified fragments ranged from 100 to 2 000 bp. Thirty five (2%) of these fragments were identified as possible culm-specific fragments. Results obtained with some of the primers, for example OPF18 in Figure 3.5 (A), showed the presence of many polymorphisms, even in the two internode 7 samples, while with others, for example OPB10 in Figure 3.5 (B), the banding pattern was identical in all tissues.

To minimise false positives due to the synthesis of spurious fragments, two identical internode 7 samples were included in each reaction as suggested by Liang *et al.* (1993). This was not only used to verify banding profile reproducibility, but it also facilitated the characterisation of the isolated fragments (discussed in 3.4.3).

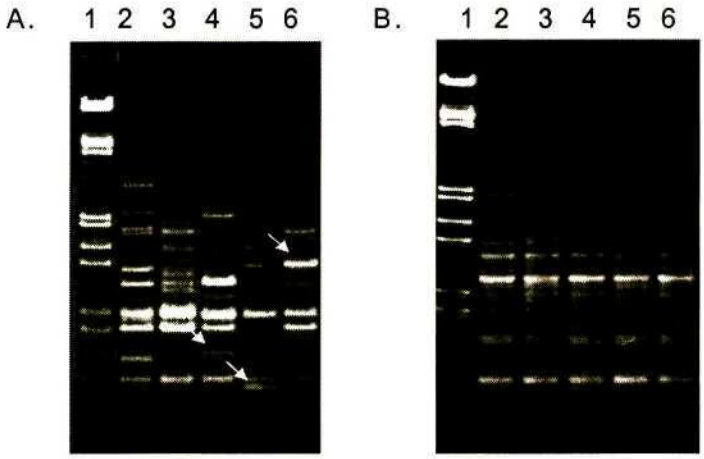

Figure 3.5 Amplification profiles of four sugarcane tissues using different random primers. A.) Amplification profiles obtained using primer OPF18 where numerous polymorphisms were detected. Some polymorphic fragments are indicated with arrows. B.) Amplification profiles obtained using primer OPB10 where no polymorphisms were detected. Lanes 1: molecular weight marker 3, lanes 2: leafroll, lanes 3: leaf, lanes 4: young culm (internode 2), lanes 5 and 6: identical samples from mature culm (internode 7).

Since this method relies on the amplification of DNA, very low concentrations of contaminating DNA can skew the display of amplified expressed fragments. After each RNA isolation that was done, a control reaction was included in the first set of amplifications where the RNA was amplified without prior reverse transcription. This ensured that any genomic DNA contamination that might be present in the template would be detected. No amplification products were ever seen after these reactions (results not shown). Further confirmation that genomic DNA was not amplified during the differential display reactions was obtained by comparing the banding profiles to those obtained from random amplification of polymorphic DNA (RAPD) analysis of sugarcane genomic DNA using the same random primer. These profiles were very different (results not shown).

3.4.3 Characterisation of isolated fragments using RT-PCR Southern blots

To determine whether the specific fragments that were isolated were present in more than one isoform or whether they possibly belonged to multi-gene families, the fragments were characterised by hybridisation analysis. Twenty one of the possible

35 culm-specific fragments were characterised using a RT-PCR Southern blot technique. These fragments were chosen because they could be successfully reamplified or could be labelled directly without prior reamplification. Fragments that did not reamplify were not characterised further. After a seemingly unique fragment was identified from the culm tissue, it was removed from the gel and used as a probe. A range of different results was obtained. Some fragments that were not tissue-specific hybridised strongly to the same size fragments in all tissues, while others hybridised to fragments of different sizes in the same and other tissues indicating the possibility of the presence of isoforms or multi-gene families. In four cases the isolated fragment hybridised to the expected size fragment in the culm and a weaker signal could be seen in other tissues. These fragments were termed culm-preferential. None of the characterised fragments were culm-specific.

The four culm-preferential fragments were designated 2A08 (667 bp), SA11 (640 bp), 7C16 (589 bp) and 7F18 (~1100 bp). The first part of the name identifies the tissue from which the fragments were isolated, i.e. internode 2, internode 7, or both of the culm tissues(S), while the second part indicates the primer that was used to amplify the fragment, e.g. OPA08. The differential display gel as well as the hybridisation result after RT-PCR Southern blot analysis of each of the culm-preferential fragments are shown in Figures 3.6 to 3.9.

3.4.4 Sequence determination of the cloned fragments

The complete sequences of 2A08, SA11 and 7C16 and a partial sequence of 7F18 were determined. Due to limitations in the length of sequences that could be read from polyacrylamide gels, only a partial sequence could be obtained of 7F18. Sequences are shown in Figures 3.10 to 3.13 .

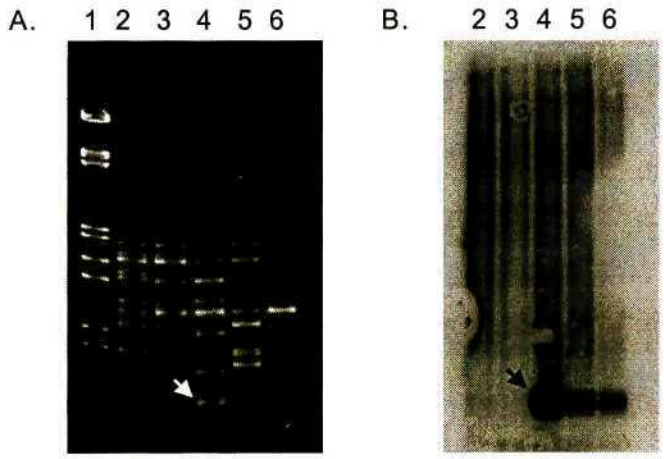

Figure 3.6 Identification and characterisation of culm-preferential fragment 2A08. A.) Amplification profile of sugarcane tissues produced by primer OPA08. The 667 bp fragment (2A08) isolated from lane 4 is indicated with an arrow. B.) Southern blot of the amplified fragments in (A) probed with isolated fragment 2A08. The hybridisation of the fragment to the same size fragments in the culm lanes (4, 5 and 6) is indicated with an arrow. Lane 1: molecular weight marker 3, lanes 2: leafroll, lanes 3: leaf, lanes 4: young culm (internode 2), lanes 5 and 6: mature culm (internode 7).

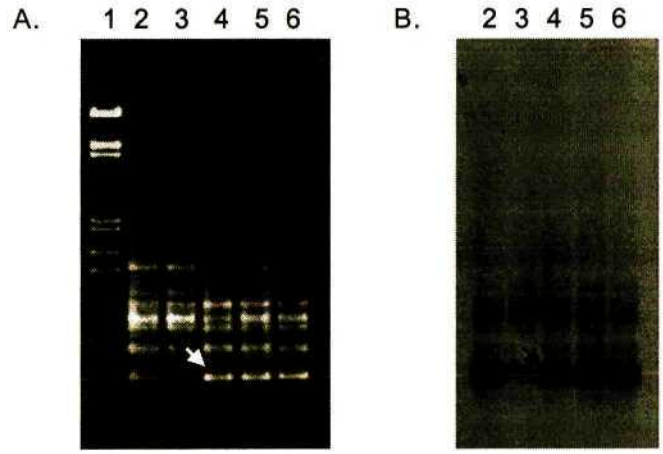

Figure 3.7 Identification and characterisation of culm-preferential fragment SA11. A.) Amplification profile of sugarcane tissues produced by primer OPA11. The 640 bp fragment (SA11) visible in lanes 4, 5 and 6 is indicated with an arrow. B.) Southern blot of the amplified fragments in (A) probed with isolated fragment SA11. The preferential hybridisation of the fragment to the same size fragments in the culm lanes (4, 5 and 6) is indicated with an arrow. Lane 1: molecular weight marker 3, lanes 2: leafroll, lanes 3: leaf, lanes 4: young culm (internode 2), lanes 5 and 6: mature culm (internode 7).

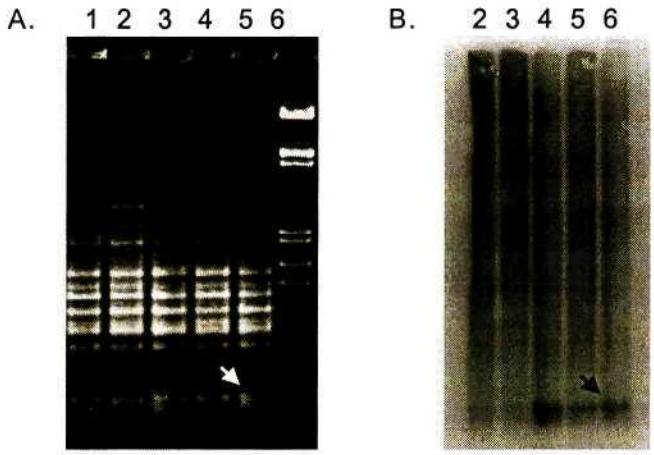

Figure 3.8 Identification and characterisation of culm-preferential fragment 7C16. A.) Amplification profile of sugarcane tissues produced by primer OPC16. The 589 bp fragment (7C16) isolated from lane 6 is indicated with an arrow. B.) Southern blot of the amplified fragments in (A) probed with isolated fragment 7C16. The hybridisation of the fragment to the same size fragments in the culm lanes (4, 5 and 6) is indicated with an arrow. Lane 1: molecular weight marker 3, lanes 2: leafroll, lanes 3: leaf, lanes 4: young culm (internode 2), lanes 5 and 6: mature culm (internode 7).

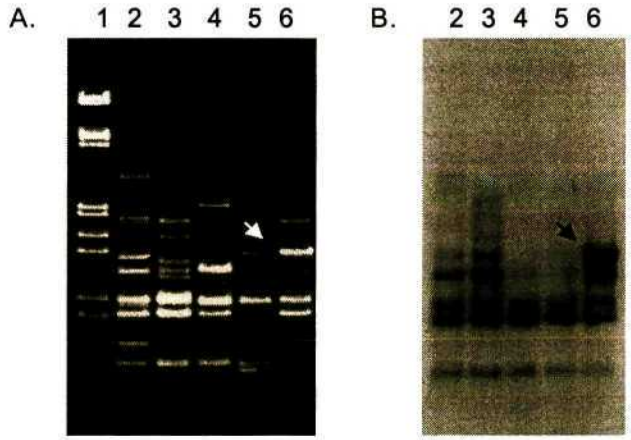

Figure 3.9 Identification and characterisation of culm-preferential fragment 7F18. A.) Amplification profile of sugarcane tissues produced by primer OPF18. The 1 100 bp fragment (7F18) visible in lanes 5 and 6 is indicated with an arrow. B.) Southern blot of the amplified fragments in (A) probed with isolated fragment 7F18. The specific hybridisation of the fragment to the same size fragment in one of the two mature culm lanes (6) is indicated with an arrow. Lane 1: molecular weight marker 3, lanes 2: leafroll, lanes 3: leaf, lanes 4: young culm (internode 2), lanes 5 and 6: mature culm (internode 7).

5'GTGACGTAGGCATAGGTTTCATAGTTCAGTTGCTTTGATAGGTTATATTGCCACTGTTTGGCCTATGAGTATGAGAACA
ACAATTCCTATTTTTTTGGTTAGTGAAACTCGATGTGTCATGTGTCCGTGCAGCTCTAAGTACACCTGTGTTCGTTAATGG
AAAATATTTTCCTAAATGCGTGCAAATCATTCTATTAGTTCCTCGATCANCATGTTGAGAAACATTGTACATTGTTAAACA
TTGCCTATTGCGACAACACAGATTCATTTGTGTAGCCTATTNGACACACAGATTCATGTTGTTTAACAGAACACTAGTCAT
GATTTGTGACTTTGATCGAATTCATCCATGTTCTCTTTTCTTCAAATTTAGTTCGTTGTAAACAATTATAGATACTGATTG
CTGGCAGTCCATGCCATGCTCAATTGTCTAATTCAAAAACCAAGATAGTAGTACCTACTGAACTGACACTGGCTATATATT
GTGCCTTTGTGGTATGCTTAAATCGATCACCCACCATGCCTCACCCGAAAACACCTACTAATCTACTAATCTCTATTTTCA
GAGTAAATATGCTAGCTGTCTTACGTATATGTTTTCTATTAAACTCCTAACACTTCAATATATCTATCATGGTTAATTTCC
TGCGCTGTTTACCTACGTCAC 3'

Figure 3.10 Complete nucleotide sequence of cloned fragment 2A08. Sequences determined using T7 and pUC18/M13 reverse primers were overlapped and a 667 bp sequence was obtained. Bases that could not be clearly read from the sequencing gel, are indicated with an N.

5'GACCCGCCCATCGCCGTCAATGAGCACTCTATCAAATTGTACTTTATGATACAAGGGATGAGACAAGAGGCGAAATGAG
CTGGTTCAGGGATGGTGGCCCATTGATCCGCCCTGCTAAAGTTGATTTCGCGGTGTGACCAGAGGGGTAGTCTTGGATCTG
CGATGAGGTCGTCTACCTTGGCCATGTTTAAGCAAGCGCGGGCGAGCAGCTTCCGCTCGTGTTTACTTTCAACAGACACCT
TACCCCCGACAATGGTTGAAACATGTCTCACAGGTTCCTAATACTGGTGTTTGGGGTTTCTTGTTAGGATCATCCTTGTCA
TCATCCTTGTGGTTATGCTGGTCTGTCGCATCGTTAGTCTTGTTGGCATTTTCTGAAGCCTGTTTGCGGCGGTAGATTTCC
TTGCGGACCTTGCAATTTTCCAACATGTGGTTGGACTTAGGATGGGCTAGGCAAGGCCCCTTCAAGGCTTTAGTGTAGTCC
TCTTCATAATTGTGATGCCCCCCAAATTTCTTCACGGCGTTGACCTCTCTGTCGTCGTCGTGAGCACGCTTGCCCTTGAAG
TTGTCACGGCGATCGCCATGCTAATTATTTTGTCGCGTCGATCACGATTGTTATTACGACGGTCCCCGATGTCACTACAAC
CTCTAAAGTCATCA 3'

Figure 3.11 Complete nucleotide sequence of fragment SA11. Sequences determined using T7 and pUC18/M13 reverse primers were overlapped and a 641 bp sequence was obtained. The specific primers, SA11-F on the 5' end and SA11-R on the 3' end, are underlined in the sequence.

5'CACACTCCAGCATTATCATGATTAAAAAGGTAAGATCAATTGTCATTAGGCATTCATCGACAAGGGAACAGCTGAAGAT
TGTACACTCACTCAACCAATCGGTTAGCAAGAGAAAAGTTCACTGATGGCGCACCCGTGCTGAAATGAAACAACAAATTCT
GTACTGTACCCCGTACTGTGTACTCCCTATTCGTACCCAGTTCGCTGTCAAGCAAGAACAAGTACAAATTCAACGCGCAAC
AAGTGATTCCGTGCGCAAATCCTGCACAATCGCAGTCGACATGATCATGCAACCTATAACTGACAAACCCAGACCAAGAAA
GTACAGCGACTGAAACTGAAGCAGCAGCCAAGCAATTCGAAGCAAGGACAAGAAAGCAAGACAAGACAGCCCAGCTACTCT
GACTTGAGCGACGCTCGCAATAATAGCCTGAATCACAAAAGCACCAGGTAACCGTGCGACCGATCCTACACCTGAACTTTT
GCTTCGTAACAAGACTGCTCGACAACACCCCAAGAGTCCATGCGCGAGCCGAGCAGGGGAGGGAGGAAACGAAGGGGGAGT
GAAGAGAGAGGGACCTGGAGTGTG 3'

Figure 3.12 Complete nucleotide sequence of fragment 7C16. Sequences determined using T7 and pUC18/M13 reverse primers were overlapped. A sequence of 589 bp was obtained.

5'CCCGGGTTCTTTCGGGGCGAACTGAGTTCTTTTCCAATTAGACTCTGGAACACAGCATCAACTCTACAAGAGATGAATT
CAAATCTACTTCAGGTTTCTTCCAAGAAGTTGAGTCGCCTCCCAACCAGACTTAACAACATGGCAAAGACGTATCAAGATT
TACTCTGAAATACGGCTGGTCGAGCGTGGAGTCTACATCTTACGTAGGCACAAGAAGGTCTCGTATTAACCGTAACATCTA
AAGATTGCCTAGTCCACTGACCGGGTACTTGTCCGCAGAACAATAATGCCGACTAGTTGAAGAAAATATCACTCTACCTAC
CAAGAAGGTCGTGCACTCGCGATGCACACTCACTAAGTATCAGCAGCTATAGCAGCAGCTGTGCAGTCGGAG---------
----
ACGGANAGACAGTGNGTGCGGATACAACAGNGNATTAGGAAGAAGCCAAGGAAAGGAGCGCAGAGACACGGAGTCCTTAGA
CAGATACGGGAGCACTAGAGAACTGAAGCATNTCGCATCCCAACGAGAACAAGGATAACTNTGAGAAGTNTCCGGACAACA
GCTTTGAAGGCATATGGCGGAAGGACGCATTTCAGGTTCCTGNTAGAACGTCATCGCATAAACAAGACGCGCAAAGTCAAA
GGAGGTCTGCCTTCGAAAAACTAGGACTAAACGGAGCCAGCATCAAGAGAAAAGAAGAAACCATGCCTAGAGTAATCAAGT
TGAGCATCCAAGAGAAGACAGGAGCAGAGCATCTTACCACTCATCCCCAAGATTTATTCCTAGGCTAAATGACAGCTGGCA
GAAAGAAGGAGCTCAGTCAGAACACAGAGAAGCCAGAACGCATGATAGGTTTTCCTGTTTTTTAAGGCGACTTGCTTCGGT
ACGGCTGTCTCACAAGTTTAAGCCAAACCACTCCAAATATGATGGCAAGACCGAACCAAAGTAATGGCTCAGAATCTACTC
GCAATCAATCGAACTAGCCTGCAGAGATGACGATATCAAGACATTATTCTTTCCCATGTCCCTAGAAAGCATGCCCCTCCA
ATAGTTCGATAAATGAACGNNGGG 3'

Figure 3.13 Partial nucleotide sequence of fragment 7F18. Sequences were determined using T7 (394 bp) and pUC18/M13 reverse primers (673 bp). A sequence of ~100 bp is missing (-----).

The sequences were compared to both the non-redundant (NR) nucleotide sequence as well as the expressed sequence tag database (dbEST) by BLASTN, and to the deduced amino acid database by BLASTX (Altschul *et al.*, 1990). No significant homologies were found, and only very short pieces of the fragments were aligned in the analyses by the basic local alignment search tool (BLAST) programmes, as can be seen in Table 3.1.

Table 3.1 Summary of results obtained from sequence similarity BLAST searches of the culm-preferential fragments. Only the highest percentage homology results obtained with an "expected" value of 5.0 are shown in the table. The latest results that were obtained on 8/1/98 are included in the table.

	Sequence	%	Length	Sequence	%	Length
	2A08			**SA11**		
BLASTN NR	1.Human BAC clone RG072E11 from 7q21-7q22	73	49 bp	1. *S.cerevisiae* chromosome XIV reading frame ORF YNR075w	75	52 bp
	2. *Methanococcus* small extra-chromosomal element	72	58bp	2. *P.falciparum* mRNA for AARP1 protein	64	73 bp
	3. Yeast DNA cut14 protein	69	56 bp			
BLASTX	None			1. *Orgyia pseudotsugata* hypothetical protein	35	53aa
				2. *Nicotiana tabacum* DNA-binding protein	34	29aa
BLASTN dbEST	None			None		
	7C16			**7F18**		
BLASTN NR	1. *H.sapiens* ETS-2 gene promoter region	86	38 bp	1. Maize mRNA for phosphoenolpyruvate carboxylase	75	49bp
BLASTX	1.E8 Hypothetical protein [Bovine papillomavirus]	70	10aa	1. *Arabidopsis thaliana* unnamed protein product	32	25aa
	2. ORF2 *Trypanosoma brucei*	40	27aa			
BLASTN dbEST	1. *C.elegans* cDNA clone yk281b7	75	44 bp	1. *Mus musculus* cDNA clone 1181395	69	59bp

Sequence: The sequences in the databases to which the highest percentage homology were recorded.
%: The percentage similarity recorded for the specific fragment that was compared in the search.
Length: The number of base pairs (bp) or amino acids (aa) that were compared in the BLAST search.
NR: Non-redundant databases of Genbank and EMBL sequences.
EST: Non-redundant database of Genbank EST division.

3.4.5 Genomic Southern blot analysis of culm-preferential fragments

Southern blot analysis of all 4 fragments showed positive signals. The number of bands observed ranged from 2 to 11, and the results are summarised in Table 3.2. With each of the 4 fragments, the positive control gave a very strong signal which confirmed that the probe labelling was satisfactory.

Table 3.2 Characterisation of culm-preferential fragments by genomic Southern blot analysis.

	Number of bands observed after hybridisation			
	2A08	SA11	7C16	7F18
*Bam*H I	6	6	3	3
*Eco*R I	4	4	2	11
Hind III	10	11	6	7

3.4.6 Northern blot analysis of culm-preferential fragments

No clear signals were obtained from the northern blot analysis. Even after an exposure time of 6 days, only the following faint signals were observed:

2A08: no signal whatsoever was visible on the film; SA11: faint bands at 650 bp in the leafroll and internode 7 lanes were visible in smears; 7C16: 2 faint bands at 1400 and 900 bp, respectively, were visible in the leaf, internode 2 and internode 7 lanes; 7F18: a very faint band at approximately 1 500 bp was seen in smears in the leafroll and internode 7 lanes. No strong culm-specific signals were obtained (results not shown).

3.4.7 Characterisation of the culm-preferential fragment, SA11

Based on the result of the northern blot, and the fact that it was the first fragment from which the complete sequence was obtained, the SA11 fragment was characterised further. Sequence-specific 20-mer primers were designed for the amplification of this fragment. The sequence and location of these primers are indicated in the sequence of SA11 (Figure 3.11) The forward primer, SA11-F, contained eight of the ten bases of the OPA11 random primer on the 5' end. Because of sequence ambiguity on the 3' end of the SA11 sequence, the reverse primer, SA11-R, did not contain any part of the random primer sequence. PCR using the 20-mer primers was done using cDNA from internode 7 and a genomic DNA template. The results are shown in Figure 3.14. The amplification products from both templates were of identical size (Figure 3.14 lanes 2 and 8).

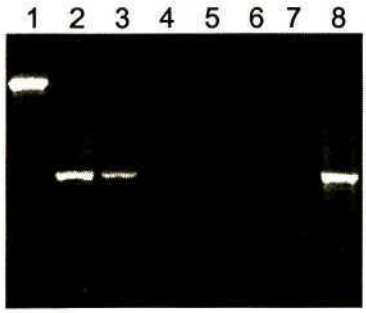

Figure 3.14 Specific amplification of fragment SA11 from cDNA and genomic DNA. A concentration series of internode 7 cDNA was prepared after the RT mixture was purified. Lane 1: molecular weight marker 3, lane 2: cDNA undiluted, lane 3: cDNA 1:10 dilution, lane 4: cDNA 1:50 dilution, lane 5: cDNA 1:100 dilution, lane 6:cDNA 1:250 dilution, lane 7: cDNA 1:500 dilution, lane 8: 120 ng genomic DNA (variety N19).

The presence of genomic DNA contamination in the RNA samples was investigated by an amplification reaction where RNA from leafroll and internode 7 tissues, without prior reverse transcription, was used as template. Genomic DNA from two sugarcane varieties, NCo310 and 84F3097, was also amplified and the cloned SA11 fragment was included as positive control for the reaction. The two SA11-specific primers were used for the amplification of all these templates. Resuts are shown in Figure 3.15.

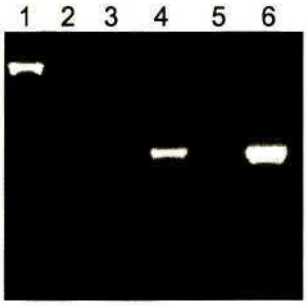

Figure 3.15 Determining the origin of fragment SA11. RNA from leafroll (lane 2) and internode 7 (lane 3) was amplified without prior reverse transcription. Genomic DNA from variety NCo310 (lane 4) and 84F3097 (lane 5) was amplified using the same primers. The cloned SA11 fragment was used as a positive amplification control (lane 6). Molecular weight marker 3 (lane 1) was used to determine the size of the amplified products.

No amplification products were formed from the RNA templates (Figure 3.15 lanes 2 and 3), but a 640 bp fragment was amplified from both the genomic DNA samples (Figure 3.15 lanes 4 and 5). In variety NCo310 (lane 4) a high concentration of the fragment was amplified, but in variety 84F3097 (lane 5), only a very faint product could be seen in the gel. In the positive control reaction (lane 6), the expected 640 bp fragment was amplified.

The dependence of the synthesis of SA11 on the reverse transcription reaction was investigated by a series of RT-PCR reactions.

Firstly, reverse transcription of RNA from leafroll and internode 7 tissues was primed with either the forward specific primer, SA11-F, the reverse specific primer, SA11-R, or the oligo(dT)$_{16}$ primer. Amplification of the reverse transcribed cDNA was done using the SA11-F and SA11-R primers, and the results are shown in Figure 3.16(A). In all six reactions, the 640 bp SA11 fragment was synthesised. The only difference that was observed was that the fragments were amplified at different concentrations. Secondly, the RT-PCR was done using either SA11-R or SA11-F primers to prime reverse transcription, but no reverse transcriptase enzyme was added to the reaction. Amplification was then done using SA11-F and SA11-R primers and the results that were obtained are shown in Figure 3.16(B). No amplification products were formed. In the third RT-PCR, no primers were used in the reverse transcription reaction, but after amplification with SA11-F and SA11-R, the 640 bp SA11 fragment had been synthesised as can be seen in Figure 3.16(C).

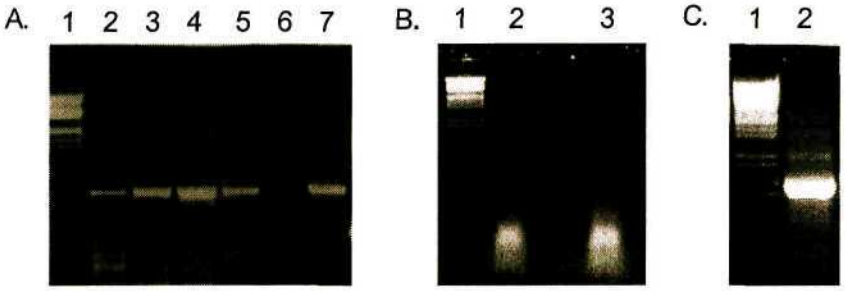

Figure 3.16 Dependence of the formation of fragment SA11 on reverse transcription. A.) Reverse transcription of RNA from leafroll (lanes 2 - 4) and internode 7 (lanes 5 - 7) was primed with 3 different primers: SA11-F (lanes 2 and 5), SA11-R (lanes 3 and 6) and oligo(dT)$_{16}$ (lanes 4 and 7). Amplification of the reverse transcribed cDNA was done with SA11-F and SA11-R in all cases. B.) Reverse transcription of RNA from leafroll using primers SA11-F (lane 2) and SA11-R (lane 3) without the addition of the reverse transcriptase enzyme. Subsequent amplification was done using SA11-F and SA11-R. C.) Reverse transcription of RNA from leafroll using reverse transcriptase without the addition of a primer. Amplification was done using SA11-F and SA11-R (lane 2). In each case, the sizes of amplified fragments were determined with molecular weight marker 3 (lane 1).

3.5 DISCUSSION

The differential display methods of Liang and Pardee (1992) and Welsh *et al.* (1992) were combined and simplified to generate discrete cDNA bands from different sugarcane tissues with ease. In each step of the procedure the conditions had to be optimised for use with sugarcane.

Instead of the sets of anchored dT primers that were used in the original method, a simple oligo$(dT)_{15}$ was used for reverse transcription to produce cDNA in a single reaction. Random hexanucleotides have sometimes been used for reverse transcription to ensure that the amplified products were not all situated on the 3' end of the mRNA (Sokolov and Prockop, 1994). However, an oligo$(dT)_{15}$ primer targets mRNA and was therefore used in this study to reduce the possibility of obtaining ribosomal and transfer RNA. The influence of this oligo$(dT)_{15}$ primer in the amplification reaction following reverse transcription did, however, have to be determined.

Where the oligo$(dT)_{15}$ as well as a random decamer primer were used for the amplification after reverse transcription, smears were observed in the gel, as have also been observed by other groups (Liang *et al.*, 1993; Hadman *et al.*, 1995). This can be due to the oligo$(dT)_{15}$ primer, without anchoring 3' bases, annealing to any part of the poly-A tail of a given mRNA. Subsequent reverse transcription will thus result in a series of amplified cDNA products of different sizes from the same mRNA. Supporting evidence for this was obtained from the experiment where the oligo$(dT)_{15}$ primer was removed on completion of the reverse transcription reaction, and amplification was done using a single random decamer primer. Discrete amplification products were observed in the gel.

The importance of template concentration to obtain reproducible amplification profiles when using the differential display technique was investigated. The purified template cDNA was initially used without prior dilution, and only a few fragments could be seen in the gels. This may have been due to an excess of template in the amplification

reactions. Results obtained after several dilutions of the template cDNA had been used in PCR confirmed this speculation, and a 1:100 dilution of the cDNA gave the best reproducible banding pattern. The observation reported by Liang and co-workers (Liang *et al.*, 1993) that some fragments were expressed at different concentrations or were differentially amplified, was also made in this study. The conclusions that can be made from this part of the investigation are that cDNA template concentration for RT-PCR is very important for the generation of reproducible amplification profiles and furthermore, the differential display method is not quantitative under the PCR conditions that are used.

Results obtained from RT-PCR using different random primers proved to be variable for the numbers of amplification products, the concentration of fragments amplified, the number of polymorphisms, and the clarity of fragments in the agarose gel after staining. An average of 15 clear fragments per primer were detectable in an agarose gel after RT-PCR, which is comparable to the 10 to 20 amplified fragments that were detected by Welsh *et al.* (1992), and the 15 to 24 fragments that were amplified by Sokolov and Prockop (1994). Two percent of the 1767 fragments that were scored appeared to be culm-specific. This is higher than the 1% differentially expressed fragments that were amplified by Liang *et al.* (1993). This may be due to the fact that they compared different developmental stages of the same tissue, and more variation can be expected when different tissues are compared.

A different approach to the original method (Liang and Pardee, 1992) was followed for the analysis of the differential display profiles after RT-PCR. Instead of denaturing polyacrylamide gels to separate the amplification products after PCR, agarose gels and ethidium bromide staining was used, thereby eliminating the need for radioactivity. Although modified, nonradioactive differential display techniques have been described since this study was started, and silver staining of the acrylamide gels can be done (Lohmann *et al.*, 1995; Doss, 1996), agarose gelelectrophoresis combined with ethidium bromide staining has the advantages of being quicker and simpler to perform. Although the resolution of agarose gels is lower than that of

polyacrylamide gels, the number of primers that were used for the amplification of cDNA fragments was increased to overcome this drawback. The use of agarose gels also facilitated the characterisation of isolated fragments since the complete amplification profile could be transferred to a nylon membrane for Southern blot analysis.

The RT-PCR Southern blot technique was developed to allow the immediate characterisation of isolated fragments that appeared to be unique to mature culm tissue. Apart from cases where a fragment that appeared to be culm-specific was actually present in the other tissues, but was not amplified from that particular cDNA sample because of incomplete cDNA synthesis or priming mismatches (Bauer *et al.*,1993), this hybridisation technique eliminated obvious false positives. From the different results that were obtained from the Southern blot analyses, it was clear that not all the amplified fragments could be visualised in an agarose gel. In some cases, fragments of the same size as the isolated fragment were seen in the other tissues after hybridisation. These fragments were, however, not visible in the gel. In other cases the probe hybridised to fragments of different sizes in the same and in the other tissues. This could mean that the primer found multiple priming sites on the same mRNA, which can be explained by the fact that mismatches in the priming site were tolerated at the low stringency PCR conditions that were used (annealing temperature of 35°C and $MgCl_2$ concentration of 3.8 mM) (Bauer *et al.*, 1993). Another possible explanation for this is that those mRNAs could be present in multiple isoforms that may be tissue-specific, or that they may be members of a multi-gene family.

In contrast to other groups that reported some heterogeneity in the purified PCR products obtained by differential display (Bauer *et al.*, 1993; Callard *et al.*, 1994), none of the four culm-preferential fragments that were cloned and sequenced in the present study contained contaminating sequences. Although the results of RT-PCR Southern blot analyses indicated that four of the isolated fragments were culm-preferential, northern blot analyses of fragments 7C16 and 7F18 revealed that they

were expressed in other tissues as well. Fragment SA11 showed a faint signal in leafroll and a slightly stronger signal in internode 7, which may indicate that the fragment could be culm-preferential. The northern blot results also indicated that these fragments were not abundant since the signals were very low, even after using 20 µg of total RNA per sample lane, and probe specific activities of 10^9 cpm μg^{-1} as suggested by Zebrowski *et al.* (1994). No signal was observed on the blot probed with fragment 2A08. Similar results have been reported by Liang *et al.* (1993) where 6 of 15 isolated fragments failed to give a signal with northern blotting. Five of these fragments were confirmed to be tissue-specific and the rest were false positives.

Sequence analysis of the fragments showed that no significant homology to differentially expressed sequences in the international database could be found. Only three plant genes showed a degree of homology to these sequences. The database search results may indicate that the isolated fragments are novel sequences that have not yet been characterised. Genomic Southern blot analysis showed that these fragments are indeed part of the sugarcane genome. Judging from the number of bands that were observed after hybridisation, these fragments could either be present as multiple copies in the genome, or located in repetitive DNA regions.

Specific characterisation of the SA11 fragment led to the conclusion that the formation of the 640 bp product was dependent on the reverse transcription reaction, but independent of the primers used in the reaction. The fragment was also not a result of genomic DNA contamination in the RNA samples. This phenomenon could not be explained.

Results of northern blot and sequence analyses indicated that the isolated fragments were not tissue-specific and could thus not be used in this study to obtain a culm-specific promoter sequence.

3.6 CONCLUSIONS

The original differential display method was simplified to facilitate the identification and isolation of differentially expressed gene sequences in four different sugarcane tissues. Although the results that were obtained compared favourably to other groups who have successfully used the same technique, the fragments that were isolated proved to be false positives. The modified differential display technique could not be used successfully in this study to obtain differentially expressed gene sequences from sugarcane mature culm tissue.

During the course of this study, several modifications of the differential display technique were reported in the literature. The following suggestions to minimise false positives were summarised and may be useful in future optimisation of this technique for use in sugarcane:

- The use of long composite primers and higher annealing temperatures during PCR may increase the sensitivity of the technique (Zhao *et al.*, 1995).
- A combination of mRNA isolation and DNase I treatment of RNA prior to reverse transcription can reduce the level of contaminating DNA to a minimum (Doss, 1996). Although this was not a problem in the present study, it may be included as part of the method when other tissues are analysed.
- Reverse transcription primed with a simple oligo$(dT)_n$ primer and subsequent amplification reactions using anchored dT primers together with random decamers, can make the technique more specific while still maintaining its ease of operation (Doss, 1996).

CHAPTER 4

EXPRESSION OF REPORTER GENES UNDER CONTROL OF THE *rolC* PROMOTER IN SUGARCANE

4.1 ABSTRACT

The expression patterns of GUS and GFP reporter genes driven by the *Agrobacterium rhizogenes rolC* promoter were investigated for the first time in transgenic sugarcane culm tissue. The constructs were introduced into sugarcane callus via particle bombardment and stable transformants were selected on the basis of resistance to geneticin, conferred by the cotransferred plasmid. Seven GUS transformants and four GFP transformants were analysed. Results of DNA and RNA analyses confirmed the stable integration of the foreign DNA into the genome and mRNA transcription. Histochemical analysis revealed that reporter gene expression was localised in the vascular bundles and specifically in the phloem cells. The expression pattern in the culm was directly comparable to the sucrose gradient in mature sugarcane plants. Expression was higher in the mature parts of the plant and increased from the peripheral region towards to core of the culm.

These results demonstrate that, as in the cases of tobacco, rice and hybrid aspen, the *rolC* promoter drives phloem-specific expression in sugarcane. Furthermore, it can be concluded that sucrose may activate the phloem-specific expression of the *rolC* promoter in sugarcane plants.

4.2 INTRODUCTION

The *rolA, B and C* genes that are located on the TL-DNA regions of the Ri plasmid of *Agrobacterium rhizogenes* (Chilton *et al.*, 1982; Cardarelli *et al.*, 1987) are involved in the pathogenesis of the hairy-root syndrome upon infection in most dicotyledonous plants. Several investigators have shown that these genes establish phenotypic alterations characteristic for each gene when expressed separately in transgenic tobacco plants (Oono *et al.*, 1987; Schmülling *et al.*, 1988; Sinkar *et al.*, 1988).

The intergenic region separating the *rolB* and *rolC* genes was found to represent a bidirectional promoter that regulates transcription in a similar fashion for both genes in the aerial organs of the plant, but in different ways in the roots. In the leaves expression is mainly limited to the vascular system. In the roots *rolB* is active mainly in the meristematic regions, while *rolC* is active in the phloem (Schmülling *et al.*, 1988). Expression of the *rolC* promoter has been found to be phloem-specific in transgenic tobacco plants (Schmülling *et al.*, 1988), rice (Matsuki *et al.*, 1989) and hybrid aspen (Nilsson *et al.*, 1996).

In an investigation to find the signals related to cell-specific and developmentally regulated expression of the *rolC* gene, the effects of various external stresses and molecules on the *rolC* promoter were analysed (Yokoyama *et al.*, 1994). The results indicated that the *rolC* promoter is activated by sucrose in phloem cells of transgenic tobacco seedlings bearing the *rolC* promoter-*uidA* chimeric fusion gene (Yokoyama *et al.*, 1994).

The *rolC* promoter has already been used in studies where phloem-specific expression of genes were required. In a study involving the investigation of the role of inorganic phosphate in phloem metabolism, the *ppa* gene from *E.coli* was expressed using the *rolC* promoter (Lerchl *et al.*, 1995). The *rolC* promoter was also used together with the phloem-specific *Sh* promoter of maize sucrose synthase-1 to engineer potato leafroll virus (PLRV)-resistant plants (Graham *et al.*, 1997).

For the present study it was postulated that the *rolC* promoter will allow phloem-specific expression in sugarcane. Since the vascular bundles in the sugarcane culm are concentrated mainly on the periphery, the use of such a promoter will ensure preferential expression, with significant enrichment of the transgene product in the outer peripheral area of the culm. This could be of great importance in transgenic plants where phloem-feeders such as aphids and leafhoppers that carry viruses, and borer insects are targeted.

Phloem-specific expression is also advantageous for engineering viral resistance (Graham *et al.*, 1997). A number of plant viruses, such as luteoviruses, reoviruses and most geminiviruses, replicate exclusively in phloem-associated tissue. For such viruses, phloem-specific expression of virus resistance genes is desirable since constitutive expression might unnecessarily increase the risks of transcapsidation or viral recombination in non-vascular tissues (Green and Alison, 1994). Furthermore, phloem-specific gene expression may impose a decreased metabolic load on the plant in comparison to constitutive expression.

To evaluate the activity of the *rolC* promoter in transgenic sugarcane plants, two reporter genes were used. The first of these was the bacterial *uidA* gene coding for β-glucuronidase (GUS) (Jefferson *et al.*, 1987). This is the most popular reporter gene in plant histochemical research since the blue staining is easy to localise, and gives visually striking results.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994) is the second reporter gene that was used. The use of GFP allows direct imaging of the fluorescent protein in living cells without the need for prolonged and lethal histochemical staining procedures. The wild-type GFP is mis-spliced when expressed in *Arabidopsis*, resulting in little or no fluorescence in transgenic plants (Haseloff and Amos, 1995). However, a modified version of the gene was recently constructed which rectified the splicing problem and therefore resulted in improved fluorescence (Haseloff *et al.*, 1997). Since the performance of the wild-type gene has not been determined in sugarcane cells, the modified gene (mGFP4) was used as reporter gene in the present study.

4.3 MATERIALS AND METHODS

4.3.1 Construction of expression vectors

Standard techniques were used to construct the expression vectors (Sambrook *et al.*, 1989; Ausubel *et al.*, 1997). The *rolC* promoter was derived from the plasmid pBIN*rolC* (provided by Dr. J.Schell, Max Planck Institute, Köln, Germany) and was subcloned into pBluescript (SK+) to form pRolC100.

pGUS800 was constructed as follows: an 1120 bp *Eco*R I-*Bam*H I fragment containing the *rolC* promoter was isolated from pRolC100. The promoter, first exon, first intron and a part of the second exon of rice actin was removed from the plasmid p*Act1*-D (Zhang *et al.*, 1991) by an *Eco*R I-*Bam*H I digestion and the *rolC* promoter was inserted in its place upstream of the *uidA* gene coding for β-glucuronidase (GUS)(Jefferson *et al.*, 1987). Restriction characterisation of the vector was done. Because of the presence of a *Hind* III site in the *rolC* promoter, this enzyme could be used to confirm the inclusion of the promoter in the vector. The *rolC* promoter-containing fragment was excised by an *Eco*R I-*Bam*H I double digestion, and the correct size of the construct was verified by a *Bam*H I digestion. Although β-glucuronidase is encoded by the *uidA* gene, this gene was denoted as GUS for the present study.

pGFP800 was constructed as follows: a green fluorescent protein gene (mGFP4) that was modified for expression in plants was obtained from J. Haseloff (Division of Cell Biology, MRC, Cambridge) (Haseloff *et al.*, 1997) in the plasmid pBIN35S-mGFP4. The mGFP4 gene together with the nopaline synthase terminator sequence (NOS) were subcloned into pBluescript (SK+). A 1240 bp *Xba* I-*Xho* I fragment containing the mGFP4 gene and the NOS terminator was cloned into the *Xba* I-*Xho* I site of the plasmid pRolC100 downstream of the *rolC* promoter. Characterisation of the vector by restriction analysis was done. The size of the vector was confirmed by a *Bam*H I digestion, and the inserted mGFP4-NOS terminator sequence was excised by a *Xba* I-*Xho* I digestion to confirm both the presence and size of the insert.

4.3.2 Sugarcane tissue culture

Embryogenic (Type III; Taylor *et al.*, 1992) sugarcane callus was produced and maintained as described from mature, field grown plants of variety NCo310 (Bower and Birch, 1992; Snyman *et al.*,1996).

4.3.3 Sugarcane transformation

Transformation of embryogenic callus by particle bombardment was done using a particle inflow gun (PIG) and DNA-coated tungsten particles. Precipitation of plasmid DNA onto tungsten particles (M17; Bio-Rad Laboratories, CA) was done as described by Snyman *et al.* (1996). The conditions used for microprojectile bombardment using a PIG (Finer *et al.*, 1992) had previously been optimised for sugarcane transformation (Bower and Birch, 1992; Snyman *et al.*, 1996). The callus was placed onto osmoticum medium (MS basal medium (Sigma)(Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 0.1% (w/v) casein hydrolysate, 0.5% (w/v) agargel, 3.64% (w/v) D-mannitol and 3.64% (w/v) D-sorbitol) for at least 4 h before and 1 h after bombardment (Vain *et al.*, 1993a). Cotransformation was done using the pEmuGN plasmid (Last *et al.*, 1991) containing the neomycin resistance gene (2.4.2.1), and either pGUS800 or pGFP800. The ratio of the two plasmids were 1:1 and the total amount of DNA was kept constant at 5 µg. Transient gene expression was monitored by using 2 plasmids:

A. pDP687 (Ludwig *et al.*, 1990), which contains the maize anthocyanin transcriptional activation factors. Anthocyanin is produced after successful transformation, and can be visualised as red foci (Figure 4.1 A).

B. pAHC27 (Christensen *et al.*, 1992), which contains the bacterial *uidA* gene coding for β-glucuronidase (GUS) under control of the maize polyubiquitin (*Ubi-1*) promoter. Incubation in the substrate-containing GUS assay solution (100 mM sodium phosphate buffer (pH 7.0); 5 mM potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$); 5 mM potassium ferricyanide ($K_2Fe(CN)_6$); 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronic acid); 0.3% (v/v) Triton X-100) results in the production of blue foci after incubation at 37°C (Jefferson *et al.*, 1987) (Figure 4.1 B).

A. B.

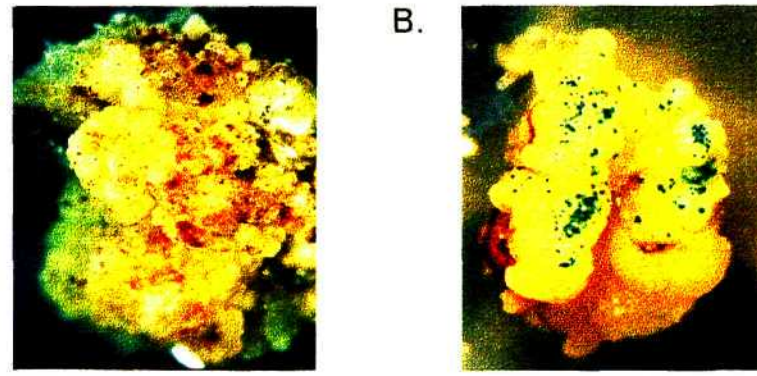

Figure 4.1 Expression of reporter genes in sugarcane callus. A.) Red foci as a result of anthocyanin production in callus 48 h after particle bombardment using pDP687. B.) Blue foci as a result of GUS expression in callus 16 h after particle bombardment using pAHC27. The callus was incubated in GUS assay solution.

Stable transformants were selected on medium containing 45 mg l^{-1} geneticin (G418). Selection was performed for 2 to 3 weeks in the dark on callus maintenance medium (MS basal medium supplemented with 3% (w/v) sucrose, 0.1% (w/v) casein hydrolysate, 0.5% (w/v) agargel and 3 mg l^{-1} 2,4-dichloro-phenoxyacetic acid (2,4-D)) and was continued for several weeks in the light on regeneration medium (MS basal medium supplemented with 3% (w/v) sucrose, 0.1% (w/v) casein hydrolysate and 0.5% (w/v) agargel) until the plantlets that regenerated started rooting. The plants were then grown on regeneration medium without antibiotic until they could be hardened off.

4.3.4 Characterisation of transformed plants: DNA analyses

DNA was isolated from 9 month old transformed plants using the modified method of Dellaporte *et al.* (1983). Specific primers for the amplification of the reporter genes were used to verify their presence in the genome of transformed sugarcane plants. Two 22-mer primers were used for the amplification of the GUS reporter gene: GUS-F: 5'-ATG TTA CGT CCT GTA GAA ACC C-3' and GUS-R: 5'-TCA TTG TTT GCC TCC CTG CTG C-3'. These primers should produce an amplification product of 1 812 bp in length. The amplification of mGFP4 was primed with GFP-F: 5'-AGA TGG TGA TGT TAA TGG GC-3' and GFP-R: 5'-GAT TGT GTG GAC AGG TAA

TGG-3'. These primers should produce an amplification product of 562 bp in length. The PCR mixture was the same as described for the specific amplification of a DNA fragment (3.3.11), and the cycling parameters were as follows: 1 cycle of 94°C for 2 min; 10 cycles of 94°C for 30 s, 50°C for 1 min, 72°C for 1.5 min; 25 cycles of 94°C for 30 s, 45°C for 45 s, 72°C for 1 min. The last cycle was followed by a 5 min extension at 72°C. Amplification products were separated in 1.5% (w/v) agarose gels.

The DNA of transformed plants was analysed by Southern blot hybridisation. Fifteen micrograms of genomic DNA from each transformed plant was treated with the restriction endonuclease, *Eco*R V. In addition, DNA from the GUS transformants was treated with the restriction endonuclease *Alu* I, and the GFP transformants with *Sac*I. These enzymes were chosen because of the presence of their recognition sites inside the regions that were targeted with the probes. The rest of the method was as described in 3.3.9, except for the use of either the GUS or the GFP coding sequence as the probe.

4.3.5 Characterisation of transformed plants: RNA analysis

RNA was isolated from the leaves of 9 month old transformed plants using the RNeasy Plant RNA isolation kit (QIAGEN). The presence of transgene mRNA was verified by RT-PCR using the Titan RT-PCR kit (Boehringer Mannheim) and the sequence-specific primers described in 4.3.4. The method was followed as recommended by the manufacturer and the annealing temperature during the amplification steps was 52°C. The amplification products were separated in a 1.5% (w/v) agarose gel.

4.3.6 Characterisation of transformed plants: Histochemical analysis

To assay GUS activity in the mature culm internodal and nodal tissues, a hand microtome was used to cut 0.5 - 0.8 mm transverse sections. The sections were incubated for 48 h at 37°C in GUS assay solution (4.3.3) which was introduced into the cells by a brief (~1 min) vacuum infiltration. After staining the sections were incubated in chlorallactophenol (CLP, 2:1:1 chloral hydrate: lactic acid: phenol) for

10 min to 1 h to clear the pigments from the tissue (Beeckman and Engler, 1994). The cellular location and intensity of GUS staining were assessed using a Zeiss Axioskope microscope and the photographs were taken using AGFA HDC 100 film.

Fluorescence microscopy was done to detect GFP expression in the culm. Sections of 0.5 mm of the internodal tissue were cut using a hand microtome and were immediately mounted on microscope slides in water. A Zeiss Axiophot microscope was used with the following filter set (487909): excitation filter BP450 - 490 nm, chromatic beam splitter FT510, barrier filter LP520. The light source was an HBO 50 W mercury vapour short arc lamp. Photography was done using AGFA HDC100 film.

4.4 RESULTS

4.4.1 Construction of the expression vectors, pGUS800 and pGFP800

Characterisation of the two expression vectors by restriction analysis was done using several different combinations of restriction enzymes. The expected and obtained sizes of restriction fragments of both constructs are summarised in Table 4.1. The results of the restriction analyses are shown in Figure 4.2 (A) and (B). Maps of the constructs are shown in Figure 4.3.

Table 4.1 Restriction analysis of the reporter gene expression vectors, pGUS800 and pGFP800.

Enzyme	Expected size (bp)[1]	Actual size (bp) in gel[2]
pGUS800		
*Bam*H I	6710	6700
Hind III	6311, 405	6300, 400
*Eco*R I- *Bam*H I	5590, 1120	5600, 1100
pGFP800		
*Bam*H I	5321	5300
Hind III	3356, 1965	3300, 2000
Xba I-*Xho* I	4081, 1240	4000, 1250

[1] The sizes of the fragments as determined by computer analysis of the sequences.

[2] The sizes of the fragments as determined by comparison to molecular weight markers.

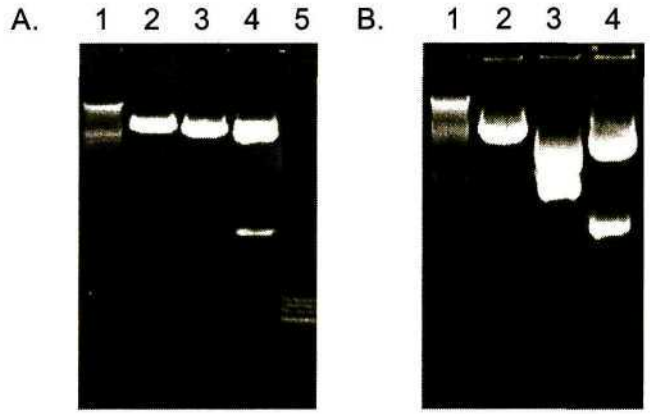

Figure 4.2 Restriction analysis of the reporter gene expression vectors, pGUS800 and pGFP800. The expected and obtained results of these analyses are summarised in Table 4.1. A.) pGUS800 was treated with *Bam*H I (lane 2), *Hind* III (lane 3) and *Eco*R I-*Bam*H I (lane 4). Sizes of the resulting fragments were determined using molecular weight markers 3 (lane 1) and 5 (lane 5). B.) pGFP800 was treated with *Bam*H I (lane 2), *Hind* III (lane 3) and *Xba* I-*Xho* I (lane 4). Sizes of the fragments were determined by comparing them to molecular weight marker 3 (lane 1).

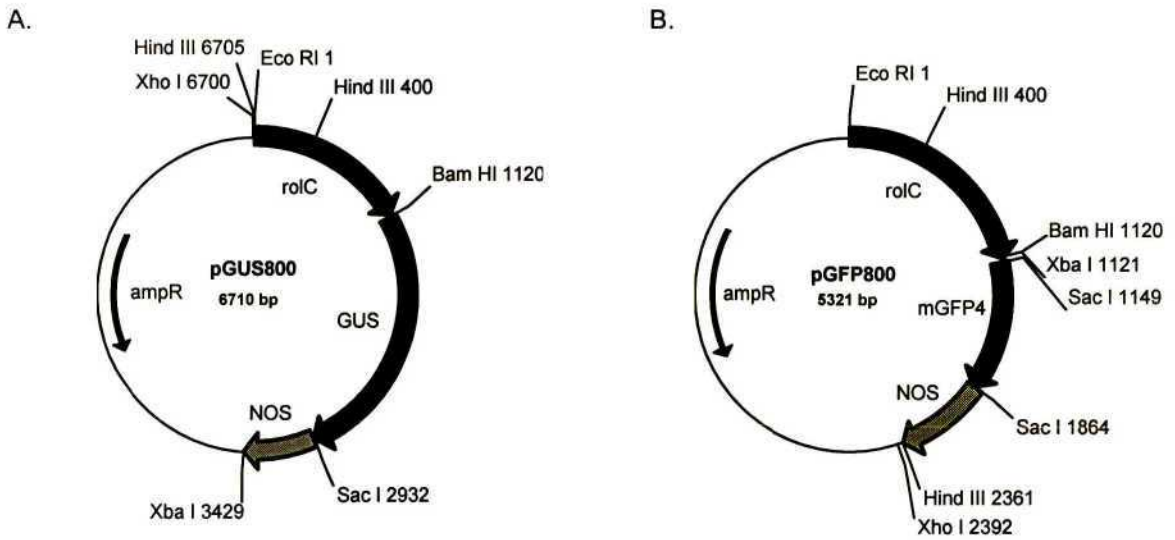

Figure 4.3 Schematic representation of the two reporter gene expression vectors, pGUS800 and pGFP800. A.) pGUS800. The *rolC* promoter-containing *Eco*R I-*Bam*H I fragment was removed from pBIN*rolC* and inserted upstream of the GUS reporter gene after the rice actin promoter was removed from p*Act1*-D. The resulting chimeric construct was 6710 bp in length. B.) pGFP800. An *Xba* I-*Xho* I fragment containing mGFP4 and the NOS terminator sequence was removed from the subclone pBluescript-mGFP4(SK+). This fragments was inserted downstream from the *rolC* promoter in pBIN*rolC* to replace the OCS terminator sequence. The resulting chimeric construct was 5321 bp in length.

4.4.2 Sugarcane transformation

A total of 11 plants survived the selection on geneticin-containing medium. These included 7 plants from a single bombardment with the pGUS800 construct and 4 plants from 2 separate bombardments with the pGFP800 construct.

4.4.3 Characterisation of transformed plants: DNA analyses

Amplification of the inserted GUS and GFP reporter genes from DNA isolated from the transformed plants showed that all 7 of the GUS transformants and 3 of the 4 GFP transformants contained the foreign DNA. All the GFP transformants that showed positive results were obtained from the same bombardment. The same amplification reactions using DNA from the control untransformed sugarcane did not produce the expected size fragments (Figure 4.4).

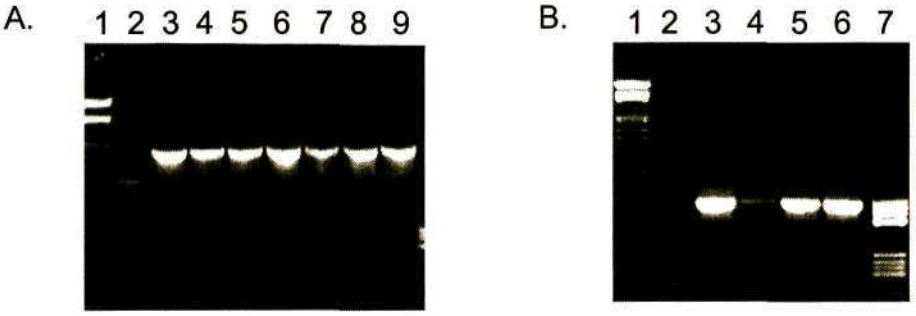

Figure 4.4 PCR amplification of reporter genes from transformed plants. Reporter gene coding regions were amplified from genomic DNA isolated from transformed plants using sequence-specific primers. A.) Amplification of the GUS coding region from the untransformed NCo310 control (lane 2), transformants GUS 4.2.1 (lane 3), 4.2.2 (lane 4), 4.2.3 (lane 5), 4.2.4 (lane 6), 4.3E (lane 7) 4.3.1 (lane 8) and 4.3.2 (lane 9). B.) Amplification of the GFP coding region from the untransformed NCo310 control (lane 2), transformants GFP 1.1E (lane 3), 1.1 (lane 4), 1.2 (lane 5) and 1.3 (lane 6). The sizes of amplified fragments were verified using markers 3 (lane 1) and 5 (lane 7).

Southern blot analysis confirmed the PCR results. Positive signals were obtained from all GUS transformants and were as follows: samples treated with *Eco*R V had two bands of sizes 3400 and 1400 bp, while two bands of sizes 1150 and 700 bp were observed when samples were treated with *Alu* I. No hybridisation signal was

seen in the untransformed control sample (Figure 4.5 A).

Positive signals were obtained from the same 3 GFP transformants that produced positive results with the PCR, namely GFP1.1E, 1.2 and 1.3, but not GFP1.1. A single band of 2 000 bp was seen in the positive samples when the DNA was treated with *Eco*R V, and three bands of 3000, 1200 and 750 bp, respectively, were seen when the DNA was treated with *Sac* I. No bands were seen in the untransformed control sample (Figure 4.5 B).

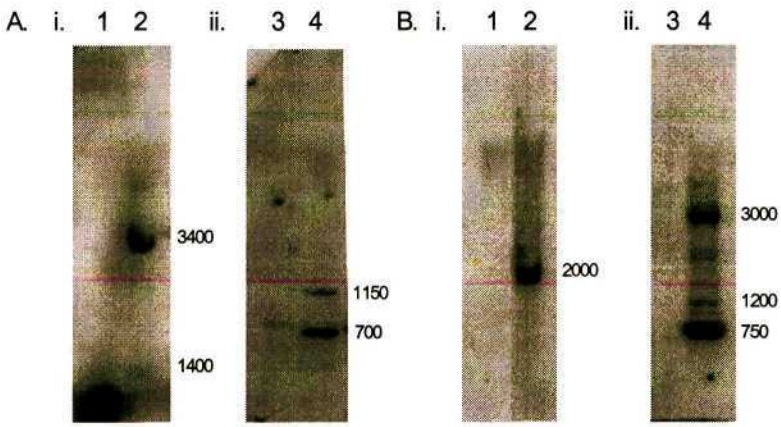

Figure 4.5 Southern blot analysis of transformed plants to confirm the presence of the reporter genes. 15 µg of genomic DNA from transformed plants and an untransformed control plant were treated with endonucleases, separated in 0.8% agarose gels and transferred to nylon membranes. A.) i) DNA treated with *Eco*R V and probed with a radioactively labelled GUS fragment. Untransformed NCo310 control (lane 1), GUS 4.2.1 (lane 2). ii) DNA treated with *Alu* I and probed with a radioactively labelled GUS fragment. Untransformed NCo310 control (lane 3), GUS 4.2.1 (lane 4). The other transformants showed exactly the same hybridisation pattern as observed for GUS 4.2.1. B.) i) DNA treated with *Eco*R V and probed with a radioactively labelled GFP fragment. Untransformed NCo310 control (lane 1), GFP 1.1E (lane 2). ii) DNA treated with *Sac* I and probed with a radioactively labelled GFP fragment. Untransformed NCo310 control (lane 3), GFP 1.1E (lane 4). The other transformants showed exactly the same hybridisation pattern as observed for GFP 1.1E.

4.4.4 Characterisation of transformed plants: RNA analysis

RT-PCR of the RNA isolated from the GUS transformed plants produced the expected 1 812 bp fragment, which was absent from the untransformed control plant,

when the GUS-specific primers were used (Figure 4.6 A). When the GFP-specific primers were used, the expected 526 bp GFP fragment was formed in 3 of the 4 GFP transformants, but not in the untransformed control plant (Figure 4.6 B).

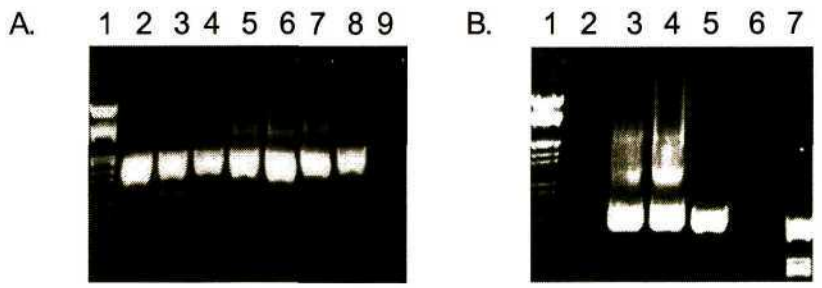

Figure 4.6 RT-PCR of transformed plant RNA to confirm the presence of the reporter gene transcripts. RT-PCR was done using sequence-specific primers and amplification products were separated in 1.5% (w/v) agarose gels. A.) RT-PCR of the GUS transcript from RNA isolated from the untransformed control plant and the GUS transformants. GUS 4.2.1 (lane 2), 4.2.2 (lane 3), 4.2.3 (lane 4), 4.2.4 (lane 5), 4.3E (lane 6), 4.3.1 (lane 7), 4.3.2 (lane 8) and control (lane 9). B.) RT-PCR of the GFP transcript from RNA isolated from the untransformed control plants and the GFP transformants. GFP 1.1 (lane 2), 1.1E (lane 3), 1.2 (lane 4), 1.3 (lane 5) and control (lane 6). The sizes of amplified fragments were verified using marker 3 (lane 1) and marker 5 (lane 7).

4.4.5 Characterisation of transformed plants: Histochemical analysis

GUS expression could be visualised in all GUS transformants after staining with the assay solution for 48 h. In the thicker (0.8 mm) sections, staining was clearer. Blue coloration was limited to the vascular bundles and specifically to the phloem (Figure 4.7 B). Due to the thickness of the sections it was difficult to focus the microscope. In transverse sections of the nodal region of young culm, horizontal vascular bundles also stained intensely (Figure 4.7 D). No specific staining was observed in the untransformed control tissue as shown in Figure 4.7 (A,C).

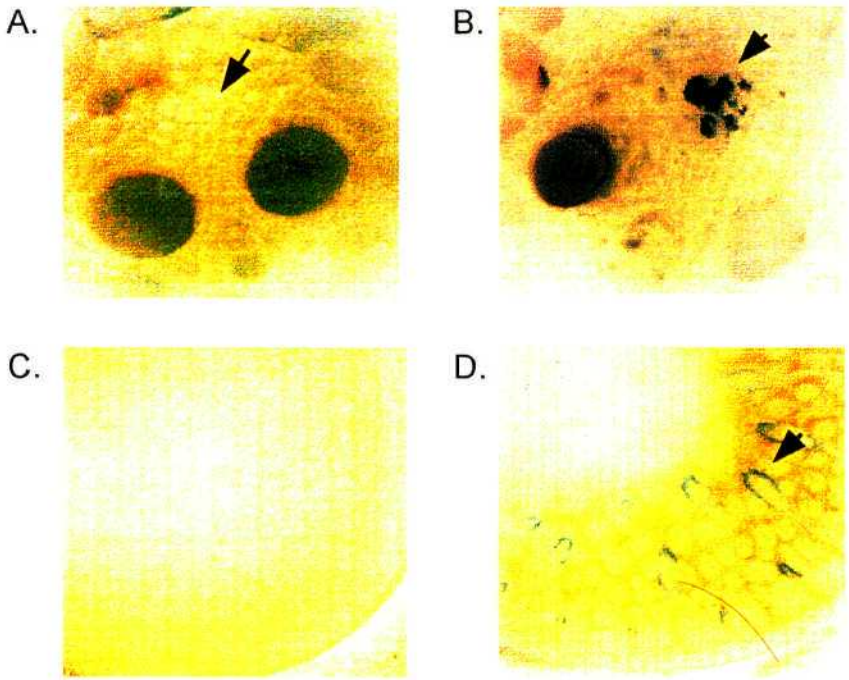

Figure 4.7 Histochemical analysis of GUS expression in sugarcane cells. Culm sections (0.5 - 0.8 mm) of transformed plant GUS 4.2.1 and an untransformed control plant were stained in assay solution for 48 h and cleared in CLP for 1 h. All sections were visualised with brightfield illumination. A.) A single vascular bundle in the culm of untransformed NCo310 internodal region (400 x). B.) A single vascular bundle in the culm of GUS 4.2.1 internodal region (400 x). The position of the phloem is indicated with an arrow. C.) Section of mature node of untransformed NCo310 (16 x). D.) Section of mature node of GUS 4.2.1 (16 x). A horizontal vascular bundle is indicated with an arrow.

Because plant material contains various autofluorescent and light-scattering components which can obscure the green fluorescence of GFP, four different filter sets were used for fluorescence microscopy to determine the optimal set for the visualisation of GFP in sugarcane. GFP expression in the vascular bundles was visualised using a standard fluorescein isothiocyanate (FITC) filter set with maximum excitation at 485 nm (460 - 510 nm) and 540 nm (515 - 565 nm)(Figure 4.8 A), a standard FITC filter set with barrier filter at 420 nm (Figure 4.8 B), a filter with excitation at 450 - 490 nm, and barrier filter at 520 nm (Figure 4.8 C), and filter set 487909 with excitation filter BP450 - 490 nm, chromatic beam splitter FT510 and barrier filter LP520 (Figure 4.8 D). Different microscopes were used for these analyses. Filter sets (A) and (B): Olympus BH2 (PM-20), (C): Zeiss MC 63 and (D):

Zeiss Axiophot. The light source of the Zeiss MC 63 microscope illuminates the sample from below. With the other two microscopes that were used, the samples were illuminated from above. Optimal visualisation of GFP in sugarcane cells was obtained using the Zeiss Axiophot microscope and filter set 487909.

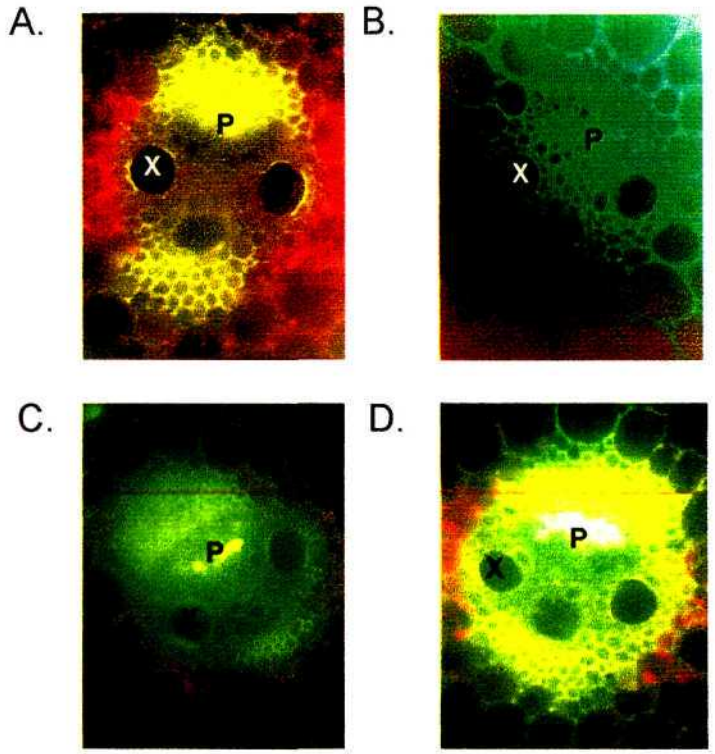

Figure 4.8 Visualisation of GFP expression in phloem cells using different filter sets and microscopes. A.) Standard FITC filter set with maximum excitation at 485 nm (460 - 510 nm) and 540 nm (515 - 565 nm). B.) Standard FITC filter set with barrier filter at 420 nm. C.)Excitation filter at 450 - 490 nm, chromatic beam splitter at 510 nm and barrier filter at 520 nm. D.) Filter set 487909 with excitation filter BP450 - 490 nm, chromatic beam splitter FT510 and barrier filter LP520. In A, B and D the samples were illuminated from above and in C illumination was from below. X: xylem; P: phloem.

In 3 of the 4 GFP transformants GFP expression was observed in the phloem cells of vascular bundles of the mature culm as shown in Figure 4.9 (D, F). A lower level of fluorescence was observed in younger tissues and in vascular bundles situated on the periphery of the culm sections. The control plant sections did not show any fluorescence in the phloem, as shown in Figure 4.9 (C,E).

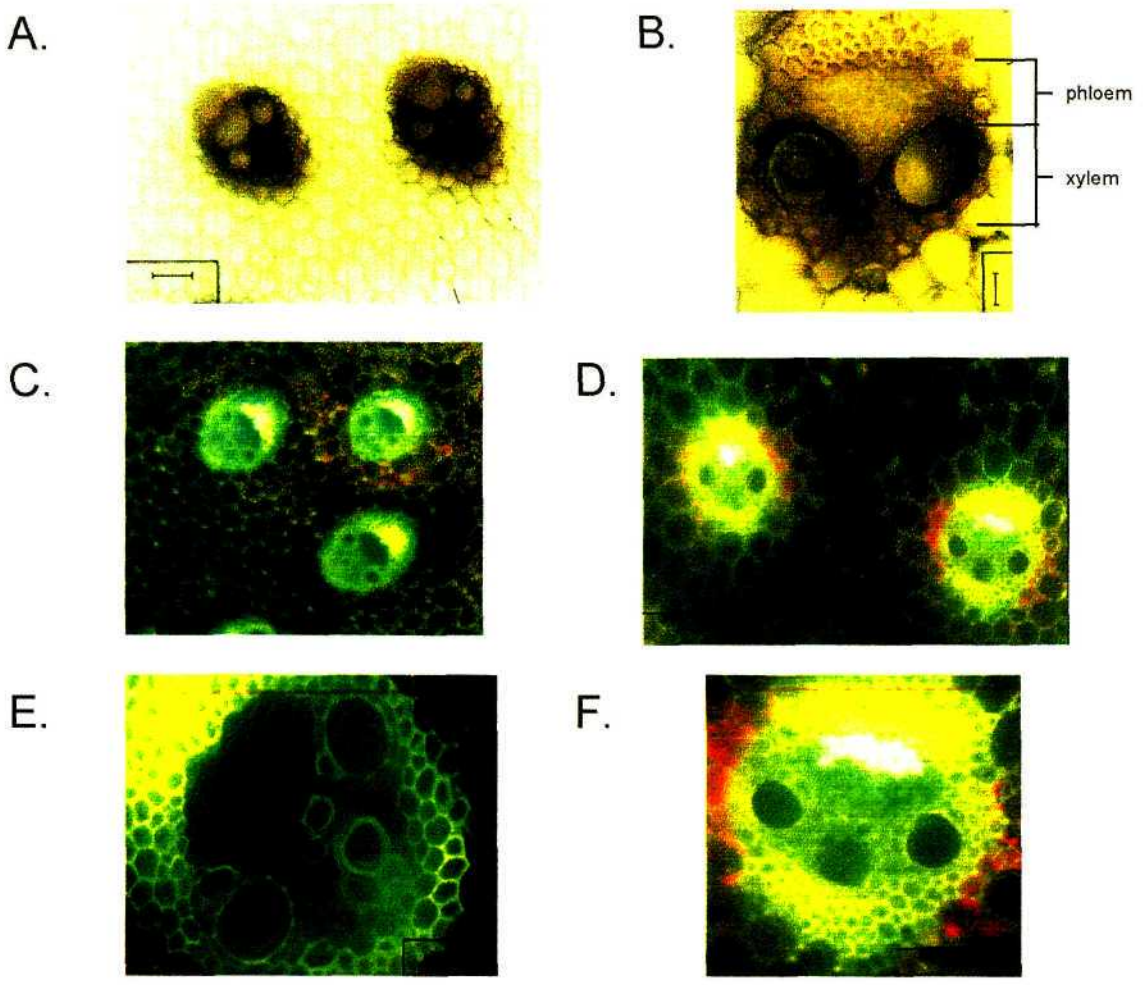

Figure 4.9 Visualisation of GFP expression in sugarcane cells. A.) Vascular bundles in the core of mature sugarcane culm visualised with brightfield illumination. Bar = 100 µm. B.) A single vascular bundle in the core of mature sugarcane culm visualised with brightfield illumination. Bar = 25 µm. C.) Vascular bundles in the culm of mature untransformed NCo310 visualised with blue light (450 - 490 nm). Bar = 100 µm. D.) Vascular bundles in the culm of mature transformant GFP 1.1E visualised with blue light (450 - 490 nm). Bar = 100 µm. E.) A single vascular bundle in mature untransformed NCo310 visualised with blue light (450 - 490 nm). Bar = 25 µm. F.) A single vascular bundle in mature transformant GFP 1.1E visualised with blue light (450 - 490 nm). Bar = 25 µm. The position of the phloem cells is indicated in B.

4.5 DISCUSSION

It is evident from the results that the *rolC* promoter from *Agrobacterium rhizogenes* was successfully engineered into sugarcane through microprojectile bombardment. This was done with two different types of genetic constructs where either GUS or GFP was used as a reporter gene. The only other monocotyledonous plant in which *rolC* has been evaluated before is rice (Matsuki *et al.*, 1989).

DNA analyses confirmed that the foreign DNA was incorporated into the sugarcane genome. The expected size products were amplified from the transformants indicating that no rearrangement of the genes had taken place in the plant cells. Southern blot results revealed that all the GUS transformants and all the GFP transformants resulted from single transformation events and that, in each case, different plants were regenerated from a single piece of transformed callus. The fact that all the plants transformed with GUS and GFP constructs respectively contained the *rolC* promoter and reporter gene inserted into the same locus in the sugarcane genome, makes it impossible to determine whether the insertion of the DNA into different regions of the genome can lead to variation in reporter gene expression levels. For the determination of tissue-specificity of the *rolC* promoter in sugarcane, however, different transformants were not essential, and consequently the above-mentioned plants were used in all subsequent analyses.

Fragments of the expected sizes for the reporter gene mRNAs were obtained from RT-PCR analysis which confirmed that the intact messengers were transcribed in the sugarcane cells. The RT-PCR technique that was used to determine the presence of the transcripts is not a quantitative method and northern blots were not performed. No conclusion can therefore be made about the level of reporter gene mRNA in the plants. Expression of GUS and GFP was detected in the sugarcane transformants by histochemical analysis, allowing the tissue-specificity of the promoter to be determined.

GUS staining patterns in the culm tissues revealed a tissue-specific expression of the β-glucuronidase in the phloem. This result is consistent with expression studies in other transgenic plants using the *rolC* promoter, including tobacco (Scmülling *et al.*, 1989), rice (Matsuki *et al.*, 1989) and hybrid aspen (Nilsson *et al.*, 1996). Although "phloem" can include companion cells, sieve tubes, phloem parenchyma and fibre cells, which are very different cell types (Graham *et al.*, 1997), the specific cell type where *rolC* is active in the sugarcane phloem tissues was not determined. It does, however, warrant further investigation, and the analysis of reporter gene expression in the leaf tissue where the cell types in the vascular bundles are better defined (Graham *et al.*, 1997) can be done. Strong staining was also seen in the horizontal vascular bundles in the nodal tissue (Fahn, 1990). The different cell types in these bundles were not distinguished and it is therefore difficult to explain the strong signal in the nodes. Clearing the tissues in CLP after incubation in the GUS assay solution (Beeckman and Engler, 1994) increased the intensity of the blue color in sugarcane cells where the protein is being expressed.

GFP expression was also restricted to the phloem cells in the sugarcane culm tissue. A very specific filter set and microscope had to be used for the visualisation of GFP, because plant tissues contain various autofluorescent and light-scattering components which can obscure the green fluorescence, especially when expression levels are low. Clear results could only be obtained with one of the four filter sets (Figure 4.8 D) that were used. The same filter set was used to obtain different results with two different microscopes (Figure 4.8 C and D). It was evident that the best results were obtained when the samples were illuminated from above. Laser-scanning confocal microscopy is often used for the visualisation of GFP in plant tissue (Haseloff and Amos, 1995; Kain *et al.*, 1995), but was not available for the present study.

It has been reported that plants expressing high levels of GFP are difficult to regenerate (Haseloff *et al.*, 1997). The protein may be a source of fluorescence-related free radicals and it has been advised that the protein should be targeted to

a more localised compartment within the cell (Haseloff *et al.*, 1997). In jellyfish photocytes, where naturally high levels of GFP are well tolerated, the protein is found sequestered in cytoplasmic granules. In contrast, the mature protein is found throughout the cytoplasm and nucleoplasm of transformed *Arabidopsis* cells (Haseloff *et al.*, 1997). GFP expression was limited to the vascular bundles in the current investigation, and these same problems were not encountered. The results discussed here were obtained by using the modified version of the GFP gene (mGFP4), and the possibility that different results will be obtained when using the wild-type gene was not investigated.

The organ-related activation of reporter gene expression (roots>stems>leaves) that has been reported for transgenic tobacco (Sugaya *et al.*, 1989) was not investigated in sugarcane tissues although higher GUS expression was observed in the mature culm tissue than in the younger parts of the culm. GFP expression in the mature culm was stronger in the vascular bundles situated in the core and decreased toward the peripheral regions. This may, at least in part, be attributed to the fact that peripheral vascular bundles in sugarcane contain particularly small or no phloem cells (Jacobsen *et al.*, 1992).

The pattern of reporter gene expression in the sugarcane culm can be directly compared to the sucrose gradient in the plant. Sucrose levels increase from young to mature tissue (from top to bottom) and from the peripheral region to the core in the internodes (Whittaker and Botha, 1997). These results suggest that sucrose influences *rolC* activity in sugarcane. The *rolC* promoter has previously been shown to be activated by sucrose in the phloem cells of transgenic tobacco (Yokoyama *et al.*,1994). Results obtained in the present study were not compared to previous reports, since the sucrose levels were not quantified. Such analyses can be included in future investigations.

Activity of the *rolC* promoter was determined in the planted crop only and not in subsequent ratoon crops. This indicates that the promoter is stable at least under

glass house conditions. In potatoes the expression of GUS under control of the *rolC* promoter has been shown to be stable when the potatoes were stored at 10°C for extended periods (Graham *et al.*, 1997). Because unstable transgene expression poses a major difficulty for biotechnology, the stability of the expression driven by the *rolC* promoter in sugarcane ratoon crops and under field conditions still has to be investigated.

4.6 CONCLUSIONS

Both GUS and GFP are expressed in sugarcane plants under the control of the *rolC* promoter from *Agrobacterium rhizogenes*, confirming that the *rolC* promoter is active in sugarcane. Expression of these proteins is restricted to the phloem, and expression levels are influenced by the sucrose content in the mature sugarcane plant.

CHAPTER 5

ISOLATION OF THE PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE β-SUBUNIT (PFP-β) PROMOTER

5.1 ABSTRACT

Activity of pyrophosphate-dependent phosphofructokinase (PFP) in sugarcane increases with culm maturity and is negatively correlated with sucrose levels in the culm. The enzyme is also apparently preferentially expressed in the peripheral areas of the mature culm. These properties suggest that the PFP promoter may be used to drive transgene expression in the mature sugarcane culm. Enrichment of transgene expression products in this area of the plant will be of great benefit for the manipulation of sucrose metabolism.

Sequence information of the PFP-β gene of castor bean and potato was used to design degenerate primers for the PCR screening of sugarcane cDNA libraries. Two fragments were isolated and their sequences showed high homology to the castor bean and potato genes. A mature culm cDNA library was screened using one of the amplified fragments as a probe and three positive clones were identified. These clones all contained the same length incomplete cDNA fragment and sequence analysis revealed that the clones were identical. The incomplete cDNA fragment was used to screen a sugarcane genomic library and four positive clones were isolated. Three of these contained the 5' end and the other one the 3' end of the PFP-β gene. A Deletion Factory™ system was used to analyse the sequence of one of the clones that contained the 5' end of the gene.

A part of the coding region containing the first five exons as well as the 5' flanking region of the gene was identified. Sequence-specific primers were designed for the amplification of two fragments in the promoter area. These fragments were of different lengths, one containing 1 072 bp of the promoter region and the other containing the same fragment plus the first intron of the gene. Expression vectors

containing these promoter fragments together with the GUS reporter gene were constructed. PFP promoter activity was determined by the quantification of transient reporter gene expression in sugarcane callus. Transient activity was compared to that of promoters that have previously been tested in sugarcane.

Results indicated that the region of the PFP-β promoter that was included in the constructs is capable of driving foreign gene expression in sugarcane callus. Although expression levels were low in comparison to the other promoters that were tested, further analysis is needed to ensure that all sequences that are crucial for optimal promoter activity are included.

5.2 INTRODUCTION

Pyrophosphate dependent phosphofructokinase (PFP) catalyses the reversible conversion of fructose 6-phosphate (Fru-6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fru-1,6-P_2) and inorganic phosphate (Pi). The activity of the enzyme is high in plant cells, especially those that have a high biosynthetic activity (Dennis and Greyson, 1987).

PFP is, in most plants, composed of two immunologically unrelated subunits, α and β (Kruger and Dennis, 1987). The α-subunit of 65 - 68 kD may be involved in the regulation of the enzyme by binding the allosteric activator fructose 2,6-bisphosphate (Cheng and Tao, 1990), while the smaller β-subunit of 60 - 64 kD is the catalytic subunit. It contains putative binding sites for Fru-6-P and PPi. The PFP enzyme from the intestinal anaerobe *Giardia lamblia* has been shown to have only one subunit which is similar to the β- rather than the α-subunit of plant PFP (Rozario *et al.*, 1995). Both subunits are expressed in most tissues (Carlisle *et al.*, 1990), but differential expression of the genes has been reported (Botha and Botha, 1991; Botha and Botha, 1993a; Botha and Botha, 1993b; Blakeley *et al.*, 1992). This differential expression of the subunits, both of which are required to produce an enzyme with significant activity, is difficult to explain (Dennis and Blakeley, 1995).

Although the kinetic properties of PFP have been studied in detail, the physiological role of the enzyme is still being investigated. Possible roles for the enzyme in both the regulation of gluconeogenesis and biosynthetic activity in plant cells have been suggested (Dennis and Greyson, 1987; Huber and Akazawa, 1986; Nakamura *et al.*, 1992; Botha and Botha, 1993a; Botha and Botha, 1993b).

In sugarcane, sucrose accumulation is suggested to be primarily regulated within the translocation system and at the level of the sink (Moore, 1995). There is likely to be substantial competition for incoming sucrose for storage and biosynthetic activity, including structural fiber in sugarcane sink tissue (Whittaker and Botha, 1996). PFP activity was shown to increase from internode 3, reaching optimal activity at internode 6 and 7, after which the enzyme declines to internode 10. An inverse correlation between peak PFP activity/expression levels and the prevailing sucrose content of internodal tissue, and a positive relationship between PFP activity and the insoluble matter content was observed (Whittaker and Botha, 1996). Sucrose content in the sugarcane culm was shown to increase from internodes 3 to 9 (Whittaker and Botha, 1997).

The distribution of sucrose, fiber and PFP activity within an internode was investigated, and PFP activity correlated positively with the increase in fiber deposition from the core to the periphery. There was an inverse trend in sucrose accumulation, which correlated negatively with PFP activity (Whittaker and Botha, 1996).

It is hypothesised that PFP activity in sugarcane sink tissues is likely to be associated with metabolic processes involving sucrose degradation and utilisation, rather than increased storage (Whittaker and Botha, 1996). PFP and sucrose synthase have also been suggested to play a role in controlling sink strength (Black *et al.*, 1995).

Molecular technology, through the manipulation of key enzymes, can be used to increase sucrose yield as well as facilitate the study of sucrose metabolism in

sugarcane. In future studies where enzymes will be down regulated or over expressed, the use of specific promoter elements that are active in the mature culm where the changes are required will be of great importance. The properties of PFP in sugarcane have made it an ideal candidate to use in these studies. Since the β-subunit has been shown to be the catalytic subunit of the enzyme, it was hypothesised that the promoter of PFP-β may confer differential expression in the sugarcane culm.

5.3 MATERIALS AND METHODS

5.3.1 Degenerate and specific primer design

Degenerate primers were designed after the PFP-β gene sequences from castor bean (accession number Z32850) and potato (accession number JO5629) were compared and an area of high homology identified. Initially, two primers were designed for the amplification of sugarcane PFP from cDNA libraries (5.3.2), namely PFP-B1, a forward primer, and PFP-B2, a reverse primer. Four primers were then designed for the sequencing and amplification of the 5' flanking region of the PFP-β gene (5.3.12 and 5.3.13). The details of all the primers that were designed for this study are summarised in Table 5.1.

Table 5.1 Oligonucleotide primers designed for the amplification of PFP-β cDNA and 5' flanking region.

Primer	Sequence (5' to 3')	Length (bp)	Tm (°C)	Degenerate/ Specific
PFP-B1	ATI-GAT-TTC-ATI-CCI-GAG-GT	20	51.2	D
PFP-B2	TCA-TCI-ACA-ACA-TCA-TGI-GCC	21	55.9	D
PFP-PROM1	GGA-AGA-ACG-TCG-TCT-GAT-TGC	21	59.8	S
PROM-F	TCA-CTG-TGG-ATC-CCT-AGT-CAA-AGC-GAG	27	66.5	S
PROM-R	CGC-CGC-CGG-ATC-CTG-CCT-TCG-CCT-TGG	27	75.6	S
PFP-E2	CAA-ACA-AGT-TAG-GAT-CCA-GCT-TCG-C	25	63	S

*Bam*H I sites in sequences of primers PROM-F, PROM-R and PFP-E2 are underlined.

5.3.2 Amplification of PFP fragments from cDNA libraries

Two cDNA libraries from sugarcane (leafroll and mature culm) were screened by PCR using each of the degenerate primers, PFP-B1 and PFP-B2, in combination with

one of the library vector-specific primers, T7 and pUC18/M13 reverse primer (R). The PCR reaction mixtures contained 1.5 µl of cDNA library template (~3 x 10^9 pfu ml^{-1}), 1x *Taq* Polymerase buffer (10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; 1 mg ml^{-1} gelatin; pH 8.3, 20°C), 0.5 µM of each primer, 0.2 mM of each dNTP and 1 unit of *Taq* DNA Polymerase (Boehringer Mannheim) in a volume of 20 µl. The samples were overlaid with 30 µl mineral oil and subjected to 42 cycles of PCR using the following cycling parameters: 1 cycle of 94°C for 1 min; 10 cycles of 94°C for 45 s, 50°C for 30 s and 72°C for 2 min; 30 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 1 min. The last cycle was followed by a 2 min extension at 72°C. PCR amplification products were separated in 1.5% (w/v) agarose gels. The fragments were extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN).

5.3.3 Cloning of cDNA fragments

The fragments were cloned using the pCR-Script (SK+) cloning kit (Promega). The manufacturer's instructions were followed, except for the use of *E.coli* HB101 cells instead of *Epicurian coli* supercompetent cells for transformation. Clones were identified using blue/white selection on X-Gal-containing LB medium (3.3.7), and insert sizes were determined by PCR amplification of the inserted fragments using the vector-specific T7 promoter and pUC18/M13 reverse primers.

5.3.4 Sequence determination of cDNA fragments

Sequence determination of amplified cDNA fragments was done using the Dye Terminator Cycle Sequencing Ready Reaction with Ampli*Taq* DNA Polymerase FS (Perkin Elmer Applied Biosystems), and sequences were analysed using a Perkin Elmer ABI Prism 310 Genetic analyser. An average of 500 nucleotides were sequenced per reaction.

5.3.5 Screening cDNA and genomic DNA libraries

Apart from the host cell strains and the fragments used as probes, the same method was used to screen the cDNA and genomic libraries. The amplified mature culm (internode 7) cDNA library in Lambda Zap II (Stratagene) had a titre of 3x10^9 pfu ml^{-1}

and the amplified genomic library in Lambda Fix II (Stratagene), $1.7x10^9$ pfu ml^{-1}. The libraries were screened using MagnaGraph nylon membranes (MSI). In the first round, 250 000 plaque forming units (pfu) were screened using 10 plates.

Host cells of the cDNA library were XL1-Blue MRF' and of the genomic library, XL1-Blue MRA (P2). The cells were grown to an OD_{600} of 1.0 in Luria broth (1% (w/v) NaCl; 1% (w/v) tryptone; 0.5% (w/v) yeast extract) supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$. Cells were centrifuged and suspended in 10 mM $MgSO_4$. Before use, the cells were diluted to an OD_{600} of 0.5 with 10 mM $MgSO_4$. For the first round, 200 µl of diluted cells were added to the calculated volume of the library and incubated at 37°C for 15 min. Top agarose (1% (w/v) NZ-amine (casein hydrolysate); 0.5% (w/v) NaCl; 0.5% (w/v) bacto yeast extract; 0.2% (w/v) $MgSO_4.7H_2O$; 0.7% (w/v) agarose) was melted, dispensed into 4 ml aliquots and left to cool to 48°C. The top agarose was added to the cell-phage suspension and poured onto prewarmed, dry NZCYM plates (1% (w/v) NZ-amine (casein hydrolysate); 0.5% (w/v) NaCl; 0.5% (w/v) bacto yeast extract; 0.2% (w/v) $MgSO_4.7H_2O$; 1.5% (w/v) agar). After an incubation of 16 h at 37°C plaques were visible on the agar surface. The plates were stored at 4°C for at least 2 h before the phages were transferred to nylon membranes. Dry nylon membranes were placed on top of the agar and were left for 20 min. The phage DNA was denatured by soaking the membrane in denaturing solution (1.5 M NaCl; 0.5 N NaOH) for 2 min, neutralised by soaking in neutralisation solution (1.5 M NaCl; 0.5 M Tris, pH 8.0) for 5 min and rinsed in 2x SSC; 0.2 M Tris, pH 7.5 for 30 s. The membrane was then blotted dry on filter paper and the DNA was linked to the nylon membrane by exposing it to UV light for 2.5 min at 120 mJ cm^{-1}.

Prehybridisation was done in RapidHyb buffer (Amersham) for 4 h at 50°C. Random primer labelling of probes was done using the Prime It II kit (Stratagene) and hybridisation was done for 16 h at 50°C. The membranes were washed in 2x SSC; 0.1% (w/v) SDS for 20 min at 42°C and again with 0.5x SSC; 0.1% (w/v) SDS for 20 min at 50°C. The membranes were exposed to X-ray film for 24 h. Based on the

hybridisation results, positive plaques were punched out of the agar plates with the back of a pasteur pipette. These agar plugs were placed into 500 µl SM-buffer (0.58% (w/v) NaCl; 0.2% (w/v) $MgSO_4.7H_2O$; 50 mM Tris (pH 7.5); 0.01% (v/v) gelatin) with 20 µl chloroform. Second and third round screening was carried out in exactly the same manner, except that fewer pfu's were plated on each plate to facilitate single plaque isolation.

In the case of positive cDNA library clones, a phagemid rescue protocol (Stratagene) was followed to isolate the pBluescript (SK+) plasmid containing the positive insert from the phage by use of a helper phage.

5.3.6 Southern blot analysis

DNA from the following plants were used for a Southern blot analysis of PFP-β: 1.) *Saccharum officinarum* (Black Cheribon), 2.) *Saccharum spontaneum* (Coimbatore), 3.) N21 (commercial sugarcane variety), 4.) NCo310 (commercial sugarcane variety), 5.) *Solanum tuberosum* (potato), 6.) *Ricinus communis* (castor bean) and 7.) *Zea mays* (maize). Twenty micrograms of genomic DNA from each plant was treated with the restriction endonuclease *Eco*R I. The rest of the procedure was done as described (3.3.9), with the incomplete PFP cDNA fragment isolated from sugarcane clone C3 (5.4.2) being used as a probe.

5.3.7 Northern blot analysis

The size of the sugarcane PFP-β mRNA was determined using northern blot analysis. Twenty micrograms of total RNA from leafroll tissue (variety NCo310) was separated in a 1.2% (w/v) agarose gel. The rest of the procedure was done as described (3.3.10). The incomplete PFP-β cDNA fragment in clone C3 (5.4.2) was used as the probe.

5.3.8 Isolation of Lambda-DNA

Lambda DNA was isolated from positive genomic library clones using the QIAGEN Lambda mini kit (QIAGEN). Plate lysates were prepared according to the method

described in Sambrook *et al.* (1989), and these lysates were used directly for the isolation of Lambda DNA. The DNA was treated with 0.2 mg ml^{-1} RnaseA (Boehringer Mannheim) for 1 h at 37°C, after which a chloroform:isoamylalcohol (24:1) extraction was done. The purified Lambda DNA was quantified spectrophotometrically and by visual comparison to a known concentration of Lambda DNA (Boehringer Mannheim) in an ethidium bromide-stained (0.01 mg ml^{-1}) 0.8% (w/v) agarose gel.

5.3.9 Characterisation of positive genomic library clones

The insert sizes of the positive genomic library clones were determined by treating 1 µg of the Lambda DNA from each clone with the restriction enzyme *Not* I for 2 h at 37°C. The *Not* I restriction sites are located on both sides of the inserted plant genomic DNA in Lambda Fix II (Stratagene). After restriction the DNA fragments were separated in an ethidium bromide-containing (0.01 mg ml^{-1}) 1% (w/v) agarose gel. The DNA was then transferred to a nylon membrane as described in 3.3.6.

Two separate probes were prepared from the incomplete PFP-β cDNA fragment (5.4.2) for the characterisation of the positive genomic clones. One probe, 5'EIB340, was 340 bp in length and was prepared by an *Eco*R I-*Bgl* II double digestion of the cDNA. This fragment was located on the 5' end of the PFP-β cDNA. The second fragment, 3'EVEI650, was 650 bp in length and was located on the 3' end of the cDNA fragment. The fragment was prepared by treating the cDNA with *Eco*R V and *Eco*R I. These probes were prepared to determine whether the 5' end or the 3' end of the PFP-β gene was contained in the genomic clones.

Twenty five nanograms of each fragment was radioactively labelled using the Prime-It II random primer labelling kit (Stratagene) in the presence of [α-^{32}P]dCTP (Amersham; 3000 Ci $mmol^{-1}$). After a 4 h prehybridisation in RapidHyb buffer (Amersham) at 48°C, the probe was denatured for 5 min in boiling water and added to the prehybridisation buffer. Hybridisation was done for 16 h at 48°C after which the membrane was washed once at 45°C for 20 min in a solution containing 2x SSC and 0.1% (w/v) SDS. The membrane was sealed in a plastic bag and exposed to X-ray

film for 16 h. After the film was developed, the radioactive probe was stripped from the membrane by pouring boiling 0.1% (w/v) SDS onto it and leaving it to cool to room temperature. The membrane was rinsed in 0.1x SSC before it was prehybridised again and probed with the second fragment.

5.3.10 Construction of a Deletion Factory™

The analysis of the positive genomic clones was done using the Deletion Factory System Version 2.0 (GibcoBRL). This system is based on the generation of nested deletions across large DNA inserts by intramolecular transposition using the engineered transposon, γδ (Tn *1000)*. After transposition, one transposon end always abuts a deletion endpoint, serving as the sequencing primer binding site. Deletion derivatives are recovered by plating on microbiological media, and selecting for loss of a contra-selectable marker. This selection, in conjunction with screening for the presence of a downstream selectable marker, results in nested deletions that extend for varying distances from one transposon end, through the contra-selectable marker, and into, but not beyond, the cloned insert DNA. Deletion endpoints can be mapped by determining the size of the subclone using agarose gel electrophoresis.

Four steps are involved in the Deletion Factory System, namely cloning of the insert DNA into the cosmid pDELTA 2, transforming competent cells, screening for deletions and isolating plasmid DNA for sequencing.

- *Cloning of the genomic DNA insert into pDELTA 2*

The Lambda DNA from the positive genomic library clone G2, was treated with the restriction enzyme *Not* I to remove the insert from the phage arms. Two insert fragments were formed by this digestion and they were both included in a ligation reaction with pDELTA 2, which was prepared by a *Not* I digestion and an alkaline phosphatase treatment using standard protocols (Ausubel *et al.*, 1997). The phage arms had only one cohesive end each and could therefore not be cloned during this reaction. The ligation reaction mixture contained 1x Ligase buffer (66 mM Tris-HCl; 5 mM $MgCl_2$; 1 mM DTT; 1 mM ATP; pH 7.5, 20°C), 5 units of T4 DNA Ligase

(Boehringer Mannheim), 2.5 µg Lambda DNA from clone G2 and 250 ng of prepared pDELTA 2 cosmid, and was incubated at 22°C for 5 h. Transformation of DH10B cells (GibcoBRL) was done following the protocol supplied with the Deletion Factory System, and positive clones were selected on Luria plates (3.3.7) containing: 0.2 mg ml^{-1} ampicillin, 0.05 mg ml^{-1} kanamycin and 0.05 mg ml^{-1} X-Gal.

Plasmid DNA was prepared from selected clones using an alkaline lysis method (modified method of Ish-Horowicz and Burke, 1981). Because two clonable fragments were formed after *Not* I digestion of clone G2 Lambda DNA, the pDELTA 2 clones containing the 5' end region of PFP-β had to be identified. The DNA was treated with restriction enzymes *Eco*R I and *Hind* III, and after separation of the fragments in a 0.8% (w/v) agarose gel, they were transferred to a nylon membrane. This Southern blot was probed with 5'EIB340 (5.3.9).

The insert size of the positively identified clone, pΔ2-PFPC, was determined by removing it from pDELTA 2 by *Not* I digestion.

- *Generating nested deletions*

pΔ2-PFPC plasmid DNA was used to transform *E.coli* DF1 cells (GibcoBRL) where deletions were generated by the action of γδ transposase (*tnpA*), which specifically recognises the transposon end sequence. DF1 provides *tnpA* on a compatible chloramphenicol resistance plasmid. Transformants were grown in Luria broth (1% (w/v) NaCl; 1% (w/v) tryptone; 0.5% (w/v) yeast extract) containing 0.2 mg ml^{-1} ampicillin, 0.05 mg ml^{-1} kanamycin and 0.015 mg ml^{-1} chloramphenicol. Clockwise deletion transformants were subsequently grown in Luria broth containing 0.2 mg ml^{-1} ampicillin and 0.015 mg ml^{-1} chloramphenicol, and counterclockwise deletion transformants in Luria broth containing 0.05 mg ml^{-1} kanamycin and 0.015 mg ml^{-1} chloramphenicol. Plasmid DNA was isolated from these transformants and used to transform DH10B cells. The cells were plated out on two different sets of Luria plates, the one set containing 0.2 mg ml^{-1} ampicillin and 5% (w/v) sucrose, and the other containing 0.05 mg ml^{-1} kanamycin and 0.1 mg ml^{-1} streptomycin. Plasmid DNA was

purified from these deletion subclones and the insert sizes determined by comparing the mobility of undigested and digested DNA in a 1% (w/v) agarose gel.

5.3.11 Sequence determination of cloned inserts

Inserts of deletion subclones were sequenced as described in 5.3.4. The Contig Manager function of DNASIS for Windows Version 2.0 (Hitachi Software) was used to consecutively arrange the sequence of each clone with those of its neighbors by locating regions at the ends of clones that overlapped with those of neighboring clones. Open reading frames were identified and a partial gene sequence of PFP-β, including the 5' untranslated region, was obtained. The sequences were compared to the non-redundant (NR) nucleotide sequence database by BLASTN, and to the deduced amino acid database by BLASTX (Altschul *et al.*, 1990). A restriction map of the sequence was obtained and was used in the construction of the expression vectors.

Comparative sequence analysis of the obtained sequences and that of castor bean and potato PFP-β was done using the multiple alignment (Higgins-Sharp) function of DNASIS for Windows Version 2.0.

5.3.12 Isolation of the 5' flanking region of sugarcane PFP-β

Analysis of the obtained sequence upstream of the translation initiation codon, ATG, was used to design three primers, namely PROM-F, PROM-R and PFP-E2 (Table 5.1), for the specific amplification of two fragments in this region. PROM-F was used in combination with PROM-R to amplify a 1 088 bp fragment containing the 5' flanking region excluding the ATG codon, and with PFP-E2 to amplify a 1 380 bp fragment containing this 1 088 bp region as well as the first intron, the first exon and 17 bp of the second exon of PFP-β. The primers contained *Bam*H I restriction sites for the cloning of these fragments.

The PCR mixtures were as described in 5.3.2, except for the use of 100 ng cosmid DNA from subclone K7 as template. Cycling parameters were as follows: 1 cycle of

94°C for 3 min, 55°C for 1 min and 72°C for 2 min; 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. The last cycle was followed by a 7 min extension at 72°C.

5.3.13 Construction of expression vectors with the GUS reporter gene

A promoterless vector, pGUS000, was used in the construction of expression vectors. For the determination of PFP-β promoter activity, transient reporter gene expression in sugarcane callus had to be quantified. Two expression vectors were constructed for this analysis:

a). pGUS1072 was constructed by the cloning of the amplified, *Bam*H I treated 1 072 bp fragment (5.3.12) into the *Bam*H I cloning site of pGUS000.

b.) pGUS1355 was constructed by cloning the *Bam*H I treated 1 355 bp fragment containing the promoter area, first exon and first intron of PFP-β (5.3.12) into the *Bam*H I cloning site of pGUS000.

Standard cloning techniques were used in the construction of these vectors (Sambrook *et al.*, 1989), and characterisation of the final constructs was done by restriction analysis.

5.3.14 Transient GUS expression in sugarcane callus

The following seven constructs were used for the transient GUS expression analysis of different promoters in sugarcane callus:

a.) pGUS520: Maize *Ubi-1* promoter + GUS, b.) pAHC27: Maize *Ubi-1* promoter + intron + GUS (Christensen *et al.*, 1992), c.) pBI221: CaMV *35S* promoter + GUS (Jefferson *et al.*, 1987), d.) pMON19468: CaMV *35S* promoter + intron + GUS, e.) pGUS 1072: PFP-β promoter + GUS, f.) pGUS1355: PFP-β promoter + intron + GUS and g.) pGUS000: Promoterless vector + GUS.

Particle bombardment of sugarcane callus was done as described in 4.3.3. Three bombardments were performed with each construct. Histochemical GUS assays were performed 48 h after bombardment as described in 4.3.6. Blue foci in the callus were counted after staining for 36 h.

5.4 RESULTS

5.4.1 Amplification of PFP-β from cDNA libraries

Amplification products were formed with 3 of the 4 primer combinations that were used in each of the cDNA libraries, as shown in Figure 5.1. No products were amplified using the primer combination B2/R.

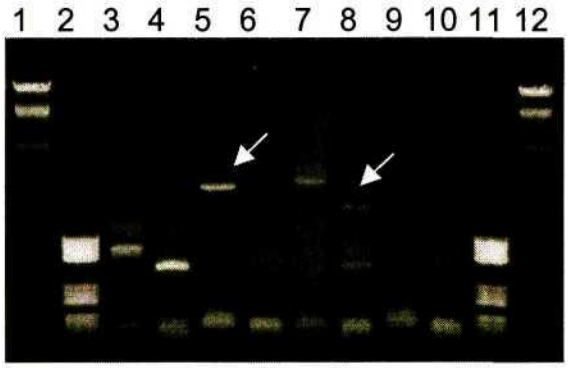

Figure 5.1 Amplification of PFP-β cDNA from leafroll and mature culm cDNA libraries. Amplification was done using each of the degenerate primers, PFP-B1(B1) and PFP-B2 (B2), in combination with one of the library vector-specific primers, T7 and pUC18/M13 reverse (R) primer. Lanes 3 to 6: amplification products from the leafroll cDNA library, lanes 7 to 10: amplification products from the mature culm library, lanes 3 and 7: B1/T7, lanes 4 and 8: B2/T7, lanes 5 and 9: B1/R, lanes 6 and 10: B2/R. The sizes of amplified fragments were determined by comparing them to marker 3 (lanes 1 and 12) and marker 5 (lanes 2 and 11). Amplified fragments that were isolated for further analysis, B1/R from the leafroll library and B2/T7 from the mature culm library, are indicated with arrows.

The sizes of the amplified fragments varied from 250 bp to 1 100 bp and the result confirmed that the PFP-β cDNA was present in these libraries in both orientations. The two largest fragments that could be reamplified, namely the 1 100 bp B1/R fragment from the leafroll library (Figure 5.1 lane 5) and the 800 bp B2/T7 fragment from the mature culm library (Figure 5.1 lane 8), were isolated for further characterisation.

The sequences of these fragments were compared to the international database. The only homologies that were reported were to the PFP-β genes of castor bean (75%), rice (84%) and potato (73%).

5.4.2 Screening the sugarcane mature culm cDNA library

Because PFP activity was shown to reach a peak in internode 7 of the sugarcane culm (Whittaker and Botha, 1996), the library from this tissue was screened for the PFP-β cDNA fragment. The amplified fragment, B2/T7 (5.4.1), which contained the most 5' region of the cDNA, was used as the probe.

A number of 250 000 pfu's of the library were screened. Three rounds of screening were completed and 3 positive clones, namely C3, C4 and C6, were finally isolated after the third round. After phagemid rescue, sequence analysis revealed that the 3 final clones all contained an incomplete PFP-β cDNA fragment of 1 800 bp. No initiation codon was present in the fragments and the reported size of PFP-β cDNA in castor bean is closer to 2000 bp (Carlisle *et al.*, 1990). The inserts of clones C3 and C4 were cloned in the same orientation in pBluescript, while that of C6 was inserted in the opposite orientation. Clone C6 contained a second insert of 1100 bp. Sequence analysis was done and this insert was partially homologous to mitochondrial malate dehydrogenase. No further analysis of this fragment was done.

5.4.3 Southern and northern blot analysis

Different hybridisation patterns were observed for different plants after the Southern blot as shown in Figure 5.2. The sugarcane cDNA probe hybridised to the DNA of all the plants that were analysed.

All four sugarcane varieties (lanes 1 to 4) as well as *Zea mays* (lane 7) showed similar results. Two bands of 4 160 and 2 280 bp respectively were seen in these sample lanes. In the *Solanum* lane (lane 5) 2 bands of sizes 3 100 and 2 800 bp were seen, while bands of 2 500 and 1 500 bp respectively were present in the *Ricinus* lane (lane 6). The signals were of approximately the same strength.

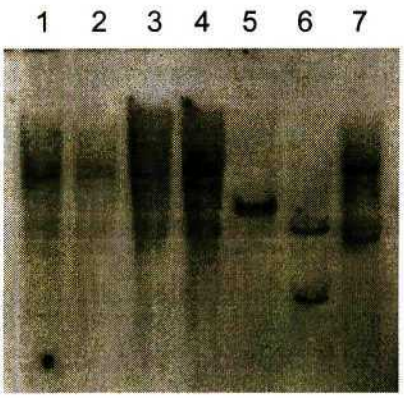

Figure 5.2 Southern blot analysis of PFP-β in sugarcane and other plants. 20 µg of genomic DNA from different plants was digested with *Eco*R I and separated in a 0.8% (w/v) agarose gel. Lane 1: *Saccharum officinarum* (Black Cheribon), lane 2: *Saccharum spontaneum* (Coimbatore), lane 3: N21 (commercial sugarcane variety), lane 4: NCo310 (commercial sugarcane variety), lane 5: *Solanum tuberosum* (potato), lane 6: *Ricinus communis* (castor bean), lane 7: *Zea mays* (maize). The incomplete sugarcane PFP-β cDNA fragment (5.4.2) was used as probe.

The sugarcane PFP-β cDNA fragment hybridised strongly to an mRNA of approximately 2 300 bp that can be seen in the result of the northern blot in Figure 5.3.

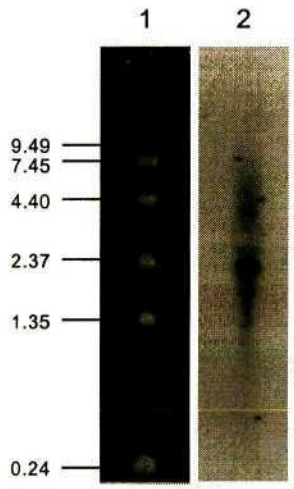

Figure 5.3 Northern blot analysis of PFP-β in sugarcane leafroll. Twenty micrograms of total RNA was separated and the incomplete PFP-β cDNA fragment (5.4.2) was used as a probe. Lane 1: RNA ladder, lane 2: leafroll RNA. The sizes of the fragments are given in kb.

5.4.4 Screening of a sugarcane genomic library and characterisation of positive clones

A total number of 2 x 10^6 pfu's of the genomic library was screened in 3 sets of screening experiments consisting of 3 rounds each. The incomplete cDNA fragment isolated from the cDNA library clone C3 (5.4.2) was used as a probe. Four final positive clones, namely G2, G3, G4 and G9, were isolated.

Insert sizes of the positive clones were determined by *Not* I digestion and two types of clones were identified. Two fragments were formed from inserts in clones G2, G3 and G4, while *Not* I digestion removed a single fragment from the phage arms in clone G9 as shown in Figure 5.4 A.

Because both types of clones were isolated from the genomic library using the same probe, the possibility that the one type of clone contained the 5' end and the other type the 3' end of the PFP-β gene sequence, was investigated. A Southern blot of the 4 clones digested with the restriction enzyme *Not* I, was probed consecutively with 2 probes (5.3.9) located on different ends of the PFP-β cDNA fragment. The results are shown in Figure 5.4 B and C.

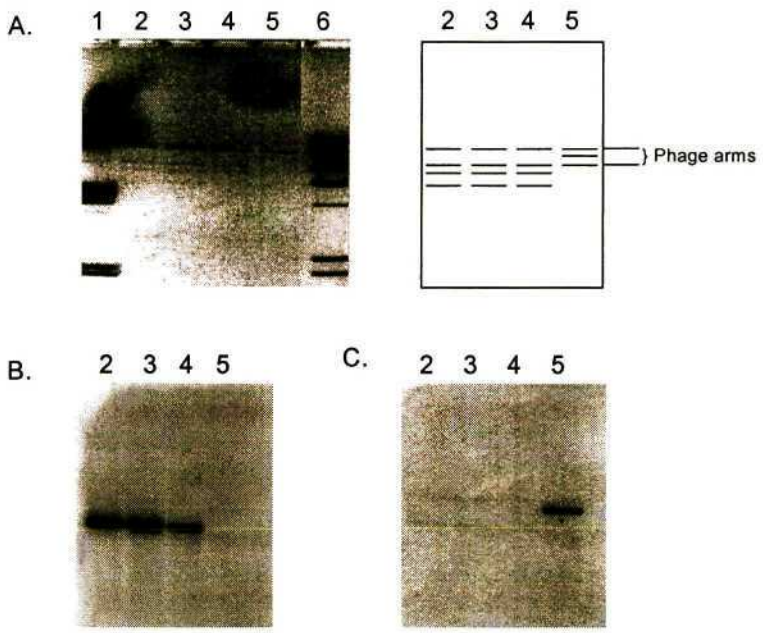

Figure 5.4 Characterisation of positive genomic library clones by restriction and hybridisation analyses. A.) Lambda DNA from genomic library clones G2 (lane 2), G3 (lane 3), G4 (lane 4) and G9 (lane 5) was treated with *Not* I. Sizes of the insert fragments and phage arms were determined by comparing to marker 3 (lane 1) and 2 (lane 6). To clarify, a diagram of the fragments are shown next to the gel photograph. B.) Southern blot analysis of insert fragments obtained in (A) probed with 5'EIB340. C.) Southern blot analysis of insert fragments obtained in (A) probed with 3'EVEI650.

Hybridisation of 5'EIB340 to one of the two insert fragments of G2, G3 and G4, but not to G9 was observed. The other probe, 3'EVEI650, only hybridised to the insert fragment of G9. Clone G2, which contained the 5' end of the gene, was chosen for further analysis and the isolation of the promoter sequence.

5.4.5 Construction of a Deletion Factory™

The insert fragments of G2 were cloned into the cosmid pDELTA2 and the resulting cosmids containing parts of the coding sequence of PFP-β were identified by Southern blot analysis. The 5'EIB340 fragment (5.3.9) was used as a probe. The positively identified cosmid, pΔ2-PFPC, was 15 kb in length with an insert size of approximately 7 kb.

Deletion subclones were obtained after DF1 cells were transformed with pΔ2-PFPC. Clockwise deletion transformants (A) were selected on medium containing ampicillin and sucrose and a total of 9 clones were isolated. A total number of 32 counterclockwise deletion transformants (K) were selected on medium containing kanamycin and streptomycin.

The sequences of all the clones were analysed and overlapping sequences were identified by DNASIS analysis. The overlaps of the A-clone sequences were very large possibly because the clockwise deletion reactions were not efficient. The K-clones, on the other hand, showed sufficient overlapping and large fragments could be analysed.

Initially the K-clones were clustered together in 4 separate groups (CONA to COND) that did not overlap with each other. After the translation initiation codon was identified, the 5' upstream sequence was analysed and a specific primer, PFP-PROM1 (Table 5.1) was designed for the sequencing of an intermediate region (PROM) which linked 2 of the groups (CONC and COND) together. The overlapping fragment clusters as well as the sizes of the consensus sequences are shown in Figure 5.5.

Sequence analysis also revealed that CONB, CONC and COND contained parts of the coding sequence and the 5' flanking region of PFP-β as shown in Figure 5.5.

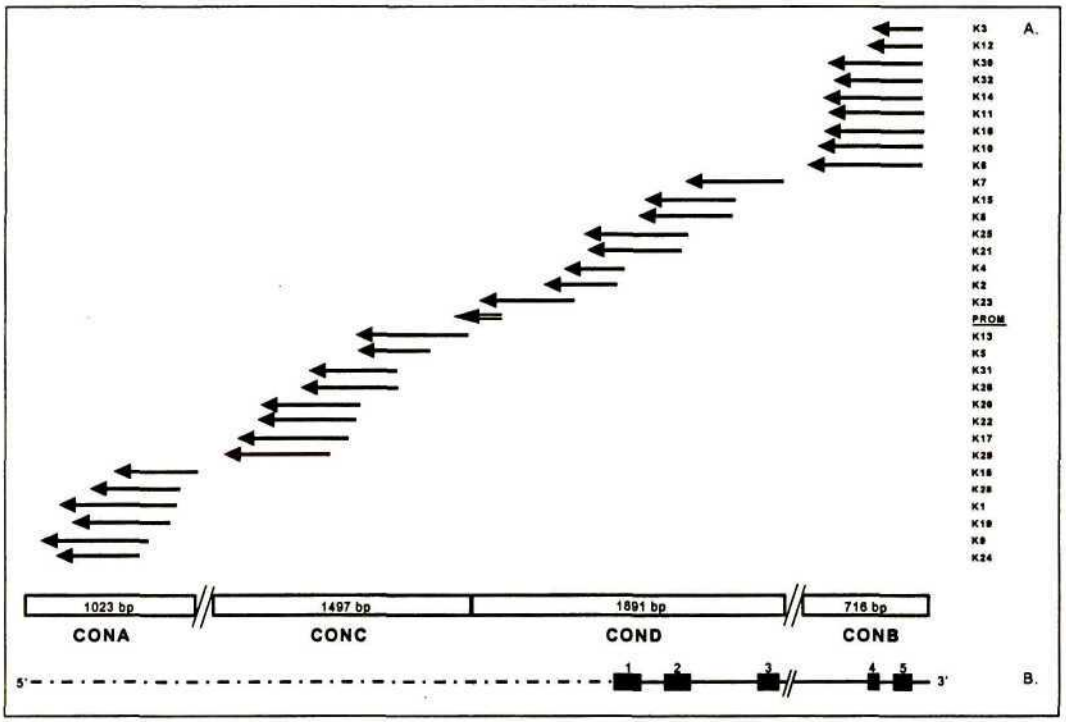

Figure 5.5 Deletion Factory: Counterclockwise deletion subclones (K). A.)The 32 K-clones are clustered together in four groups, CONA to COND, according to deletion size. The specific primer, PFP-PROM1, was used to sequence an intermediate region (PROM, shown by an open arrow) which links CONC and COND together. B.) The exons that are included in the sequence are illustrated by dark boxes and introns by solid lines. The 5' flanking region is shown as a dashed line.

5.4.6 Comparative sequence analysis of the sugarcane, castor bean and potato PFP-β coding sequences

The first 5 exons of the PFP-β gene was identified (Appendix I) and the sequence was compared to that of castor bean and potato. Multiple base substitutions and in-frame insertions or deletions were observed. Most of the mutations that had an effect on the amino acid sequence occurs at the 5' end of the gene. Intron lengths and sequences are not conserved between these plants. For the region that was analysed, a 72.7% homology between sugarcane and castor bean and a 71.9% homology between sugarcane and potato was observed on nucleotide level. Comparison of the same

region in castor bean and potato showed a 75.4% homology. On amino acid level the observed homology in the same region was as follows: 77.9% homology between sugarcane and castor bean, 74.9% homology between sugarcane and potato and 78.7% homology between castor bean and potato.

5.4.7 Sequence analysis of the sugarcane PFP-β promoter

The complete sequence of a 1 747 bp fragment upstream of the ATG codon is shown in Figure 5.6.

5'CAACTGCACCTAGTCGGCACTTGTCCCAAGCCACGCTAGCTAGCCACTCGCTGCGGCCGGGTCTGGCCTCTGGTGATCCTCCAATTACC
TGGACTAAGTTTAAGCCTGGTGGACCCTTCAATTACCTAGACCAAGTTAAGCTGTCATCATTGGCTCACGTTCAACTTAATCATCTTTAGA
GTGAGTTGTTCTAACTCGTATAAGCATGGCTCATGAGTCCGCATCATATACACAACCCAATACATCTTGGAATTGGACTCCGTATCGTGAT
AGATGTGAGTTGTTCTAACTTGCATGAGTGTGGGTTATATAAGTCTGGATGAAAGAAGCTAGGCTATCGGATAGTTAGGGGAAGGTGGCCC
AAAAAAAAGAGGTTGAAATTTTGGTTCTTTAATATACATATAACTAGGTATAGGTAGATCTACAATGTGTAAAAAATTGAAAAAATTAATT
ACATAAAAGTATAGACCTATAGATACAACAAAATCTCTATACTAAAGCATATGATATTATATGTTCACTGTGTAGCCCTAGTCAAAGCGAG
TATAAAAAAATTGATTGGTATTTTTTAATTTTCTGTAATTTTGTATGATTTTAGAATTCTCAACTAGTTTAAAAGAAAAGAAAACAAAAGGG
CTACTGTACAGCTAGGCCATGGGCTTTGGCCTAGACTACTGCGCAGCCAGCCCAGCCTGGGGCCGAATCCAAGATCCACCATAATAGGCCC
AAGCGAAAAAAGAAAAAAAGAAGTTTTGCATTAAGAACACTTTTGTTTCTAAAACAGTTTAAACCCTTCTACTAATTCTTTGTCTGTAGTA
TATTTACAGCCGGAGCCCTGCTATTGTTTCTAATTCCTCCTCAATCCTTCTCGTCTTGGTGCATCAGACGACGTCTTCCGAGCGGAGGGAG
GAGGCGATGACGAGACCGAGGCGGATTAGTAGGTTAGACGAAAAAATTTTAGTGGCAGAAATTTTGCTTCTTTAGTATTTCGTAGCATGAT
TGTATGATTTTTAGAATTCTCAACTAGTTTAAAAGAAAAAGAAAAAAAAGAAAGAAAAAGGGCTAATGTGCGGCTAGGCCATGGGCTTTGG
CCCAGACCACTGCACAGCCAGCCTAGAATGTGGCCGAATCCTAGACCCGTCACAACAGGCCCAAGTGAAAAAAAGAAAAAAAAAGAAAAAA
GAAGTTTTGTATTAGAAACCCTAAATTTGTTTCTAAAACTGTTTAAACTCTTCTACTATTTCTTTGTCTCTGGTATCTTTGCATCTGGAAC
TCGTTATTATTAAAGAGACAAAATTTCTGCCACCAAATTTTTATCATCCAACCTACTAATTATTATTATTATTATTATTATTATTATT
ATTATTATTATTATTATTATTATTATTATTATAGATTAGATTTTTTATTAATTATAATTTTATAGTATGTGTGTTTCATGTTATAAAT
CTTTGTAATTCTTTTTATAATGTTGGTCAAGATGCTTTGTCGACAAGAGTGAAAGGCGGTTTTCATTGGGATTATGATCTGCTGCCATGCC
TCCATCTGCGCGCGGGCGTTGCTAACAAACGAAAAAAAAAAAGAATACTCCAAGAAGGAAAGAGAACCACATATGACCATGCATGGAAATTG
GACCGAGCCTTAGTCGTTTCTAATGTTAGCAACGCACGCCCTCGGATATGAGTCCCCGCCCACCCCTCCTCTCGCATCGTCGTGTTCTNCC
AATCCAAGGCGAAGGCAGGCA 3'

Figure 5.6 Sequence of 1 747 bp of the 5' flanking region of sugarcane PFP-β.

Useful restriction sites and known promoter motifs with their positions in the sequence are shown in Table 5.2.

Table 5.2 Restriction sites (A) and promoter motifs (B) in the promoter region of PFP-β.

A.

Restriction endonuclease	Sequence	Cutting position
***Acc* I**	GT/MKAC	1495
***Dra* I**	TTT/AAA	614 787 1030 1223
***EcoR* I**	G/AATTC	598 1014
***Fok* I**	GGATGNNNNNNNN/	332
***Fok* I**	/NNNNNNNNNNNNNCATCC	1304
***Hind* II**	GTY/RAC	1496
***Nco* I**	C/CATGG	653 1079
***Nde* I**	CA/TATG	503 1616
***Nhe* I**	G/CTAGC	36 40
***Nru* I**	TCG/CGA	2026
***Rsa* I**	GT/AC	643
***Sal* I**	G/TCGAC	1494
***Sma* I**	CCC/GGG	1909
***Spe* I**	A/CTAGT	607 1023
***Sph* I**	GCATG/C	1929
***Taq* I**	T/CGA	1495 1876

B.

Motif	Pattern	Position
CAP site	C-A-N-Y-Y-Y	859 - 864
HSP-70.5	G-A-T-T-G-G	556 - 561
INF.1	A-A-G-T-G-A	1153 - 1158
MSP-CS	A-G-A-T-C-T	418 - 423
napB	C-A-T-G-C-A	1622 - 1627
NF-E1.5	T-A-T-C-T-T	1255 - 1260
OCT-B2-SV40	C-T-T-G-C-A-T	290 - 296
SEF4	R-T-T-T-T-T-R	1006 - 1012, 1309 - 1315
TATA Box	T-A-T-A-W-A-W	307 - 313, 545 - 551, 1448 - 1454
UAS2UP1	T-G-A-T-T-G-G-T	555 - 562

5.4.8 Transient GUS expression analysis in sugarcane

Although sequence analysis of the 5' flanking region of PFP-β revealed the presence of promoter motifs (Table 5.2), the ability of this sequence to act as a promoter and drive foreign gene expression had to be determined. Due to the fact that the 5' flanking sequence was extremely AT-rich, an area had to be identified that would allow the design of a primer with a GC-content of approximately 50%. Such an area was located approximately 1 090 bp upstream from the ATG codon. The primers, PROM-F and PROM-R, were thus designed for the amplification of this portion of the 5' flanking region. Since the inclusion of intron sequences in promoter constructs has resulted in higher foreign gene expression levels in monocotyledonous plants (Mascarenhas *et al.*, 1990; Maas *et al.*, 1991), the first intron of PFP-β was included in one of the promoter constructs. The two expression vectors containing the promoter (pGUS1072) and the promoter plus the first intron (pGUS1355) of sugarcane PFP-β, respectively, are shown in Figure 5.7.

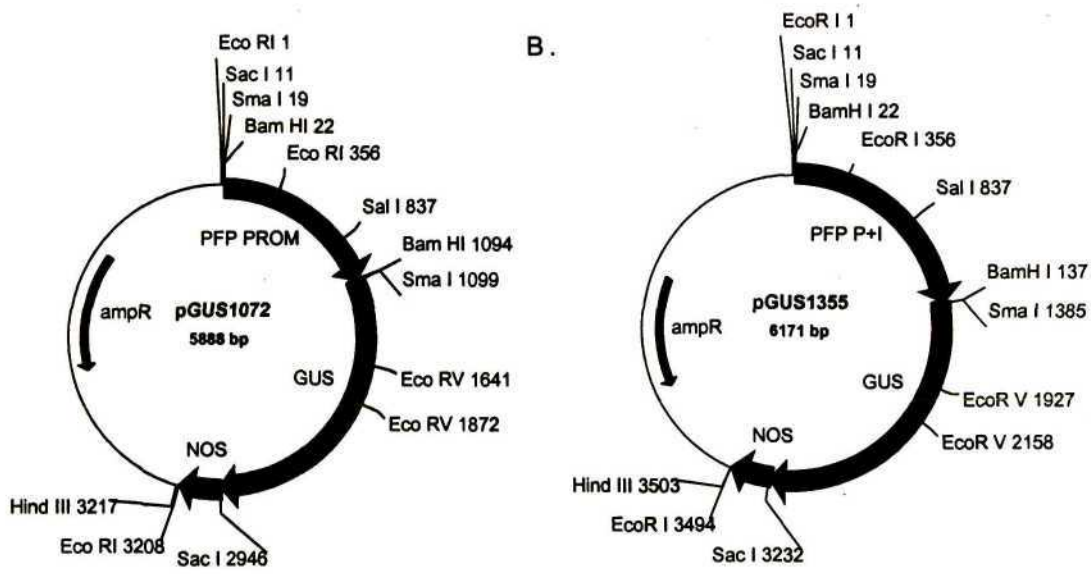

Figure 5.7 Schematic representation of the expression vectors, pGUS1072 and pGUS1355. A.) pGUS1072. The amplified and *Bam*H I treated 1 072 bp fragment containing the putative PFP-β promoter (PFP PROM) was cloned into the *Bam*H I cloning site of the promoterless vector, pGUS000. B.) pGUS1355. The 1 355 bp fragment containing the putative promoter as well as the first exon and first intron of PFP-β (PFP P + I), was treated with *Bam*H I and cloned into pGUS000.

Blue foci were observed with all constructs tested, with the exception of the promoterless control, pGUS000. A variety of intensities of blue coloration ranging from extremely dark to pale blue was observed. Furthermore, blue foci produced from transient expression driven by both PFP promoter constructs were mostly located in the non-embryogenic callus (Taylor *et al.*, 1992). With all the other constructs tested, blue foci were predominantly seen in the white embryogenic callus (Taylor *et al.*, 1992). The results obtained in the transient expression assay were normalised against the levels of expression with pAHC27 (expressed as a percentage), and are shown in Figure 5.8. pAHC27 was used as reference since *Ubi-1* has previously been shown to be the most efficient promoter for driving GUS expression in sugarcane (Gallo-Meagher and Irvine, 1993).

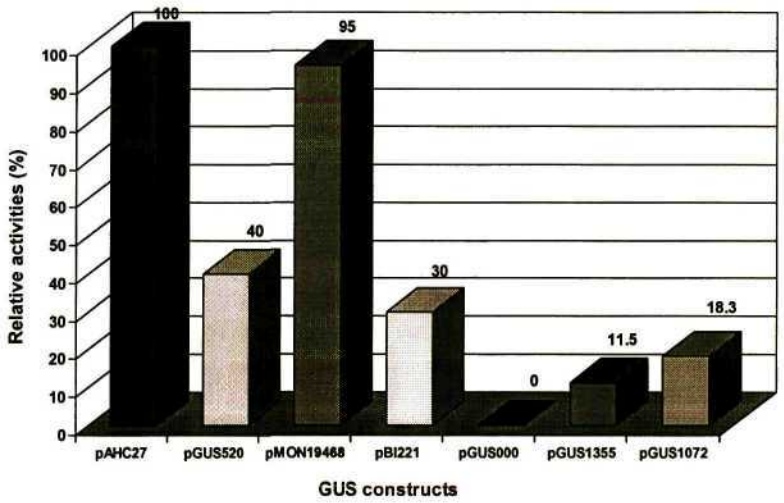

Figure 5.8 Transient GUS expression in sugarcane callus using different promoter constructs. Blue foci were counted after transformation of sugarcane callus. The result of pAHC27 was set to an arbitrary value of 100%, and the other results were calculated in comparison to this value. The constructs are described in 5.3.14.

Blue foci were observed with all constructs tested, with the exception of the promoterless control, pGUS000. A variety of intensities of blue coloration ranging from extremely dark to pale blue was observed. Furthermore, blue foci produced from transient expression driven by both PFP promoter constructs were mostly located in the non-embryogenic callus (Taylor *et al.*, 1992). With all the other constructs tested, blue foci were predominantly seen in the white embryogenic callus (Taylor *et al.*, 1992). The results obtained in the transient expression assay were normalised against the levels of expression with pAHC27 (expressed as a percentage), and are shown in Figure 5.8. pAHC27 was used as reference since *Ubi-1* has previously been shown to be the most efficient promoter for driving GUS expression in sugarcane (Gallo-Meagher and Irvine, 1993).

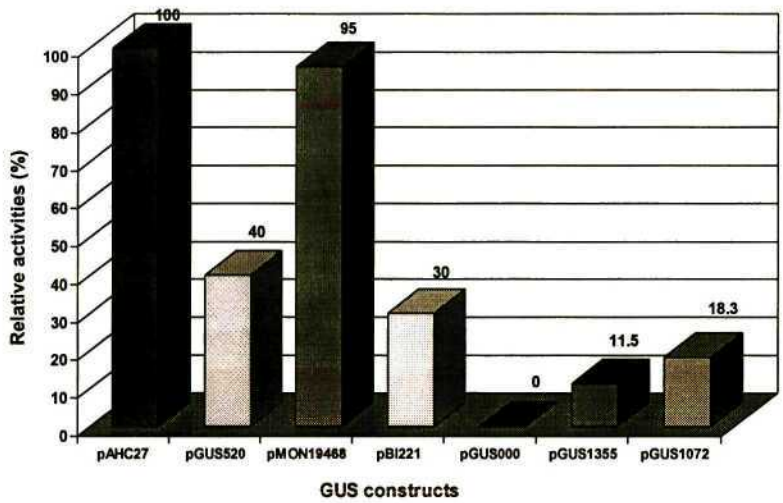

Figure 5.8 Transient GUS expression in sugarcane callus using different promoter constructs. Blue foci were counted after transformation of sugarcane callus. The result of pAHC27 was set to an arbitrary value of 100%, and the other results were calculated in comparison to this value. The constructs are described in 5.3.14.

5.5 DISCUSSION

Two tissue-specific cDNA libraries from sugarcane were screened for PFP-β by PCR using degenerate primers. Amplification products were formed with most primer combinations indicating that the PFP-β cDNA is present in both the leafroll and the mature culm library in both orientations. The sizes of the amplified fragments also indicate that cDNAs of different sizes are present in the libraries, probably due to incomplete cDNA synthesis in some cases. Because PFP activity reaches a peak in internode 7 in the mature sugarcane plant (Whittaker and Botha, 1996), the mature culm cDNA library was screened for the full-length cDNA fragment. However, all the clones that were isolated from this library contained a 1 800 bp incomplete cDNA fragment. The absence of an initiation codon in the open reading frame and the fact that the reported size of PFP-β cDNA is close to 2 000 bp (Carlisle *et al.*, 1990), revealed that the fragments were not complete. Although the full-length cDNA could not be obtained from this library, the isolated cDNA fragment was successfully used as probe in Southern and northern hybridisation analyses and for the screening of the genomic library.

Southern blot analysis of sugarcane genomic DNA revealed that PFP-β may be encoded by a single gene. Similar results have been reported for castor bean (Blakeley *et al.*, 1992). This result is, however, surprising for sugarcane because of the complex polyploid nature of the plant. The monocotyledonous plants that were analysed, namely sugarcane and maize, showed the same hybridisation pattern when probed with PFP-β cDNA. Potato and castor bean showed different hybridisation patterns, and in all plants two bands were observed which may be an indication of the low copy number of the gene in these plants. Identical hybridisation patterns were observed for all sugarcane varieties that were analysed.

The presence of a single mRNA of approximately 2 300 bp was revealed by northern blot analysis. This result is consistent with northern blots from castor bean (Blakeley *et al.*, 1992) and potato (Carlisle *et al.*, 1990). The signal was strong and indicates a high expression level of the gene in leafroll. Expression levels in different

sugarcane tissues still have to be determined to confirm the results that were obtained in metabolic studies of PFP (Whittaker and Botha, 1996).

Two different types of clones were isolated from the genomic library and these contain the 5' and the 3' end of the PFP-β gene respectively. The present study is focussed on the isolation of the promoter area and therefore the clones containing the 5' end of the gene were analysed first. The complete sequence of the gene will, however, be determined in the future since the sequence of sugarcane PFP-β is not yet available and will be of value for further studies.

Sequences of deletion subclones were analysed and the first five exons and the 5' untranslatable region of PFP-β were identified. The gaps that exist in the regions between CONB and COND, and between CONA and CONC (Figure 5.4) can be filled by analysing more deletion subclones. For the purpose of the present study, however, the available sequences were adequate. When the sequence of the coding region that was contained in these clones was compared to the same region in castor bean and potato genes, a homology of approximately 72% was observed.

A portion of 1 747 bp of the 5' flanking region of PFP-β has so far been sequenced and the presence of promoter motifs has been indicated. In the absence of data on the binding of nuclear factors in relation to PFP expression, the significance of these motifs cannot be determined before deletion analyses of the 5' flanking region have been carried out. For the present study, however, the question as to whether the 5' flanking region of the isolated PFP-β gene was a functional promoter or not had to be answered. The possibility existed that the 5' flanking region that was analysed would not drive high levels of reporter gene expression. This was taken into account by including the first intron of PFP-β with the putative promoter region in pGUS1355 to possibly enhance reporter gene expression. Introns of some genes have been postulated to stimulate gene expression, for example the addition of an intron to the 5' untranslated region of the maize *Sh1* gene increased reporter gene expression approximately 100-fold (Maas *et al.*, 1991).

Results obtained from transient GUS expression analysis indicated that the 5' flanking region of PFP-β that was analysed was capable of driving foreign gene expression in sugarcane callus. The expression levels obtained were very low in comparison to those obtained with the *Ubi* (pAHC27) and *35S* (pMON19468) promoters, which included introns in the constructs. However, the difference in expression levels was not as pronounced when compared to the constructs containing the *Ubi* and *35S* promoters without the introns, pGUS520 and pBI221, respectively. Expression levels obtained with pAHC27 and pBI221 in callus can be compared to a previous report where the same constructs were used for transient GUS expression in expanding sugarcane leaves (Gallo-Meagher and Irvine, 1993).

Expression levels obtained with the construct containing the first exon and intron (pGUS1355) were slightly lower than with the construct containing only the promoter (pGUS1072). This result may be explained by the possible presence of *cis*-active regulatory elements in the first exon of PFP-β that could influence gene expression. However, in a study where the effects of promoter, intron and enhancer elements on transient expression in sugarcane protoplasts were investigated, the potential influence of precise sequence context around regulatory elements was emphasised. It was reported that the inclusion of the *Adh1* intron in constructs containing either the *nos* or the *35S* promoter caused a 30 - 70% reduction in GUS expression in sugarcane (Rathus *et al.*, 1993), which was in contrast to the trend from cereals (Mascarenhas *et al.*, 1990). Since the source, size and spatial position of the intron, as well as the combination of the intron enhancer with a surrounding exon region have been shown to strongly influence gene expression (Maas *et al.*, 1991), further analyses with constructs containing the first PFP-β intron should be carried out. It would appear from the results that the expression of the PFP-β promoter was predominant in non-embryogenic callus (Taylor *et al.*, 1992). The implications of this observation can be determined by testing expression in other sugarcane tissues.

Now that it has been established that the 5' flanking region of PFP-β is a functional promoter, further characterisation can be done to determine the sequences required

for optimal activity and possible tissue-specificity. The stability of gene expression driven by this promoter must also be determined in transgenic sugarcane.

5.6 CONCLUSIONS

The gene of PFP-β was isolated from sugarcane, and the sequences of the first five exons and 1 747 bp of 5' flanking region were determined. Transient expression analysis of a reporter gene in sugarcane callus indicated that the 5' flanking region of the isolated gene is a functional promoter that can be used to drive foreign gene expression.

CHAPTER 6
CONCLUSIONS

Two different approaches were followed in the present study to isolate tissue-specific promoter sequences from sugarcane. Due to the limited knowledge of the sugarcane genome, and the fact that very few sugarcane genes have been characterised, a non-directed approach was initially followed to obtain differentially expressed genes.

The original RNA differential display method (Liang and Pardee, 1992) was modified to facilitate the rapid screening of cDNA populations with a large number of primers. Although all four putative culm-preferential fragments that were isolated from the sugarcane culm were shown to be false positives, several important suggestions regarding the differential display technique can be made from the results obtained and from reports in the literature.

Smears resulting from non-specific amplification reactions using an oligo(dT) primer can be eliminated by removing the oligo(dT) primer after reverse transcription, and using random primers for PCR. Alternatively, reverse transcription primed with a simple oligo(dT) primer, and subsequent PCR using an anchored dT primer together with random decamers, can increase the specificity of the amplification reactions. The cDNA template concentration for RT-PCR was shown to be very important for the generation of reproducible amplification profiles. Two identical samples from each tissue should be amplified to verify the reproducibility of the banding pattern. It should also be noted that differential display is not a quantitative method under the low stringency PCR conditions that are used. Longer primers and higher annealing temperatures could increase the sensitivity of the technique.

In future, other techniques could be evaluated for the isolation of differentially expressed genes from sugarcane. Genes that are differentially expressed during fruit ripening in strawberries were identified recently by using a procedure that combines a differential screening technique with a Southern blot screening of a subtractive

library by means of PCR (MAST-PCR-SBDS). The cDNA library was generated by using a modified magnet-assisted subtraction technique (MAST) (Medina-Escobar *et al.*, 1997). The authors describe the technique as being a sensitive, easy, low-cost and reliable means of obtaining differentially expressed genes. In contrast to the small fraction (10 to 20%) of fragments identified by the differential display technique that turn out to be true differentially expressed genes, 31 of the 45 clones (69%) that were characterised by MAST-PCR-SBDS were positive (Medina-Escobar *et al.*, 1997). The results described in the report, and the fact that it was developed in a plant system, make this technique attractive for the identification of differentially expressed genes in sugarcane.

The *Agrobacterium rhizogenes rolC* promoter was shown to drive phloem-specific reporter gene expression in sugarcane culm tissue. The specific cell type in the phloem where expression occurs, and the stability of gene expression under control of the *rolC* promoter in ratoon crops and under field conditions still have to be investigated. The reporter gene expression pattern observed in this study could be directly compared to the sucrose gradient, which suggested that sucrose influences *rolC* promoter activity in sugarcane. The advantage of a promoter that is activated by high sucrose levels is that it will only drive expression in plant tissues where the sucrose levels are high enough for its activation. The properties of the *rolC* promoter that have been observed in the present study have shown that it could be a very useful promoter in future pest and disease resistance programmes, as well as in metabolic studies in sugarcane.

The GUS and mGFP4 reporter genes were used with success in mature sugarcane tissue. Although a staining period of 48 h was required for the visualisation of GUS expression driven by the *rolC* promoter, clear results were nevertheless obtained. The increased staining time may be a reflection of the promoter strength. Clearing of pigmented tissue with CLP was shown to increase the coloration in tissues where GUS was expressed. GFP expression could only be visualised when an appropriate filter set and microscope were used for fluorescence microscopy because of the

presence of autofluorescent and light scattering components in the plant tissues. Several other modified versions of GFP are available and should be evaluated for use as reporter genes in sugarcane.

A directed approach was followed to isolate the gene and promoter of PFP-β from sugarcane. Screening of cDNA libraries using PCR revealed that PFP is present in the leafroll and in internode 7 of the sugarcane culm. Since no other sugarcane cDNA libraries were available, the presence of the transcript in other tissues was not investigated. Northern hybridisation analysis or RT-PCR of different sugarcane tissues can be done to determine whether PFP is expressed in all tissues, and the levels of expression can be compared to results obtained from metabolic studies. Southern blot analysis revealed that PFP is a low copy number gene in sugarcane, castor bean, potato and maize. PFP-β mRNA in sugarcane was shown to be 2.3 kb in length, which is also the reported length of the transcripts in castor bean and potato.

Two types of clones containing different portions of the PFP-β gene were isolated from the sugarcane genomic library. The complete sequence of the PFP-β gene from sugarcane has not been available, but can now be determined from the genomic library clones. Apart from the first five exons of the gene, 1747 bp of the 5' flanking area of the PFP-β gene was sequenced, and a number of promoter motifs were identified. Preliminary activity analysis revealed that the 5' flanking region of the isolated PFP-β gene is a functional promoter. Further analyses can now be done to characterise the promoter, and identify *cis*-acting sequences that are essential for optimal promoter activity. The tissue-specificity and stability of the promoter will be investigated by stable expression studies in different sugarcane tissues. Since the enzyme has been postulated to play a key role in sucrose breakdown in sugarcane, the effect of sucrose on promoter activity can also be investigated.

The two most significant contributions of the present study are the following:

- A functional promoter has been isolated from sugarcane.

- The *Agrobacterium rolC* promoter can drive phloem-specific expression in the sugarcane culm.

These elements may be used in future investigations into the genetic manipulation of sugarcane.

REFERENCES

Aasheim, H-C., Deggerdal, A., Smeland, E.B. and Homes, E. 1994 A simple subtraction method for the isolation of cell-specific genes using magnetic monodisperse polymer particles. *BioTechniques*, **16**: 716 - 722.

Albrecht, H., van de Rhee, M.D. and Bol, J.F. 1992 Analysis of *cis*-regulatory elements involved in induction of a tobacco PR-5 gene by virus infection. *Plant Molecular Biology*, **18**: 155 - 158.

Altamura, M.M., Archiletti, T., Capone, I. and Costantino, P. 1991 Histological analysis of *Agrobacterium rhizogenes rolB*-GUS gene fusions in transgenic tobacco. *New Phytology*, **118**: 69 - 78.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990 Basic local alignment search tool. *Journal of Molecular Biology*, **215**: 403 - 410.

An, G. and Kim, Y. 1993 Techniques for isolating and characterising plant transcription promoters, enhancers, and terminators. In *Methods in plant molecular biology and biotechnology*. Glick, B.R. and Thompson, J.E. (Eds.), ISBN 0-8493-5164-2, pp.155 - 166. C.R.C. Press, London.

An, G., Luo, G., Veltri, R.W. and O'Hara, S.M. 1996 Sensitive, non-radioactive differential display method using chemiluminescent detection. *BioTechniques*, **20**: 342 - 346.

Anderberg, R.J. and Walker-Simmons, M.K. 1992 Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases. *National Academy of Science. Proceedings*, **89**: 10183 - 10187.

ap Rees, T., Green, J.H. and Wilson, P.M. 1985 Pyrophosphate:fructose 6-phosphate 1-phosphotransferase and glycolysis in non-photosynthetic tissues of higher plants. *Chemical Journal*, **227**: 229 - 304.

ap Rees, T. 1995 Prospects of manipulating plant metabolism. *Trends in Biotechnology*, **13**: 375 - 378.

Apel, K. and Kloppstech, K. 1978 The plastid membranes of barley: light-induced appearance of mRNA coding for the apoprotein of the light-harvesting chlorophyll a/b-protein. *European Journal of Biochemistry*, **85**: 581 - 588.

Arias, J.A., Dixon, R.A. and Lamb, C.J. 1993 Dissection of the functional architecture of a plant defence gene promoter using a homologous *in vitro* transcription initiation system. *The Plant Cell*, **5**: 485 - 496.

Ausubel, F.A., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds.) 1997 *Current Protocols in Molecular Biology*. John Wiley and sons, New York.

Barendse, G.W.M. and Peeters, T.J.M. 1995 Multiple hormonal control in plants. *Acta Botanica Neerlandica*, **44**: 3 - 17.

Bartels, D., Hanke, C., Schneider, K., Michel, D. and Salamini, F. 1992 A dessication-related Elip-like gene from the resurrection plant *Craterostigma plantagineum* is regulated by light and ABA. *EMBO Journal*, **8**: 2771 - 2778.

Bauer, D., Müller, H., Reich, J., Riedel, H., Ahrenkiel, V., Warthoe, P. and Strauss, M. 1993 Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Research*, **21**: 4272 - 4280.

Baulcombe, D.C., Chapman, S. and Santa Cruz, S. 1995 Jellyfish green fluorescent protein as a reporter for virus infections. *The Plant Journal*, **7**: 1045 - 1053.

Beaudoin, N. and Rothstein, S.J. 1997 Developmental regulation of two tomato lipoxygenase promoters in transgenic tobacco and tomato. *Plant Molecular Biology*, **33**: 835 - 846.

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

Benfey, P.N. and Chua, N-H. 1990 The cauliflower mosaic virus *35S* promoter: Combinatorial regulation of transcription in plants. *Science*, **250**: 959 - 966.

Bertioli, D.J., Schlichter, U.H.A., Adams, M.J., Burrows, P.R., Steinbiß, H-H. and Antoniw, J.F. 1995 An analysis of differential display shows a strong bias towards high copy number mRNAs. *Nucleic Acids Research*, **23**: 4520 - 4523.

Bidney, D., Scelonge, C., Martich, J., Burrus, M., Sims, L. and Huffman, G. 1992 Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. *Plant Molecular Biology*, **18**: 301 - 303.

Birch, R.G. and Franks, T. 1991 Development and optimisation of microprojectile systems for plant genetic transformation. *Australian Journal of Plant Physiology*, **18**: 453 - 469.

Birch, R.G., Bower, R., Elliot, A., Potier, B., Franks, T. and Coredeiro, G. 1996 Expression of foreign genes in sugarcane. *The XXII Congress of the International Society of Sugarcane Technologists. Proceedings*, p. 368.

Birch, R.G. 1997 Plant transformation: problems and strategies for practical application. *Annual Review of Plant Physiology and Plant Molecular Biology*, **48**: 297 - 326.

Black, C.C., Loboda, T., Chen, J-Q. and Sung, S-J.S. 1995 Can sucrose cleavage enzymes serve as markers for sink strength and is sucrose a signal molecule during plant sink development? In *Sucrose metabolism, Biochemistry, Physiology and Molecular Biology*, Vol. 14. Pontis, H.G., Salerno, G.L. and Echeverria, E.J. (Eds.), ISBN 0-943088-31-3, pp. 49 - 64. American Society of Plant Physiologists, USA.

Blakeley, S.D., Crews, L., Todd, J.F. and Dennis, D.T. 1992 Expression of the genes for the α- and β-subunits of pyrophosphate-dependent phosphofructokinase in germinating and developing seeds from *Ricinus communis*. *Plant Physiology*, **99**: 1245 - 1250.

Bokman, S.H. and Ward, W.W. 1981 Renaturation of *Aequorea* green fluorescent protein. *Biochemical and Biophysical Research Communication*, **101**: 1372 - 1380.

Botha, F.C., de Vries, C. and Small, J.G.C. 1988 Immunological characterisation of the pyrophosphate-dependent fructose-6-phosphate phosphotransferase. *Plant Cell Physiology*, **29**: 223 - 229.

Botha, A-M. and Botha, F.C. 1991 Pyrophosphate-dependent phosphofructokinase of *Citrillus lanatus:* molecular forms and expression of subunits. *Plant Physiology*, **96**: 1185 - 1192.

Botha, A-M. and Botha, F.C. 1993a Induction of pyrophosphate-dependent phosphofructokinase in watermelon (*Citrillus lanatus*) cotyledons coincides with insufficient cytosolic D-fructose 1,6-bisphosphate 1-phosphohydrolase to sustain gluconeogenesis. *Plant Physiology*, **101**: 1385 - 1390.

Botha, A-M. and Botha, F.C. 1993b Effect of the radicle, and hormones on the subunit composition and molecular form of pyrophosphate-dependent phosphofructokinase in the cotyledons of *Citrillus lanatus*. *Australian Journal of Plant Physiology*, **20**: 265 - 273.

Botha, F.C., Whittaker, A., Vorster, D.J. and Black, K.G. 1996 Sucrose accumulation rate, carbon partitioning and expression of key enzyme activities in sugarcane stem tissue. In *Sugarcane: Research towards efficient and sustainable production*. Wilson, J.R., Hogarth, D.M., Campbell, J.A. and Garside, A.L. (Eds.), ISBN 0-643-05941-5, pp. 98 - 101. CSIRO Division of tropical crops and pastures, Brisbane.

Boutry, M. and Chua, N-H. 1985 A nuclear gene encoding the beta subunit of the mitochondrial ATPase in *N.plumbaginifolia*. *EMBO Journal*, **4**: 2159 - 2165.

Boutry, M., Nagy, F., Poulsen, C., Aoyagi, K. and Chua, N-H. 1987 Expression of a bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. *EMBO Journal*, **6**: 307 - 311.

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

Brand, A. 1995 GFP in *Drosphila*. *Trends in Genetics*, **11**: 324 - 325.

Bray, E.A. 1994 Alterations in gene expression in response to water deficit. In *Stress-induced gene expression in plants*. Basra, A.S. (Ed.), ISBN 3-7186-5466-0, pp. 1 - 25. Harwood Academic Press, Switzerland.

Breathnach, U. and Chambon, P. 1981 Organisation and expression of eukaryotic split genes coding for proteins. *Annual Review of Biochemistry*, **50**: 349 - 384.

Broglie, R., Coruzzi, G., Fraley, R.T., Rogers, S.G. and Horsch, R.B. 1984 Light-regulated expression of a pea ribulose-1,5-bisphosphate carboxylase small subunit gene in transformed plants. *Science*, **224**: 838 - 843.

Brown, D.C.W., Tian, L., Buckley, D.J., 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.

Buckley, D.J., Brown, D.C.W., Lefebvre, M., McGrath, A. and Tian, L. 1995 A particle accelerating device for delivering DNA material into plant cells. *Computers and Electronics in Agriculture*, **13**: 67 - 74.

Butow, R.A. and Fox, T.D. 1990 Organelle transformation: shoot first, ask questions later. *Trends in Biological Sciences*, **15**: 465 - 467.

Buzby, J.S., Yamada, T. and Tobin, E.M. 1990 A light-regulated DNA-binding activity interacts with a conserved region of a *Lemna gibba rbcS* promoter. *The Plant Cell*, **2**: 805 - 814.

Callard, D., Bernard, L. and Mazzolini, L. 1994 A method for the elimination of false positives generated by the mRNA differential display technique. *BioTechniques*, **16**: 1096 - 1103.

Callis, J., Fromm, M. and Walbot, V. 1987 Introns increase gene expression in cultured maize cells. *Genes and Development*, **1**: 1183 - 1200.

Capone, I., Cardarelli, M., Mariotti, D., Pomponi, M., Paolis, A.D. and Costantino, P. 1991 Different promoter regions control level and tissue-specificity of expression of *Agrobacterium rhizogenes rolB* gene in plants. *Plant Molecular Biology*, **16**: 427 - 436.

Cardarelli, M., Mariotti, D., Pomponi, M, Spanò, L., Capone, I. and Costantino, P. 1987 *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. *Molecular and General Genetics*, **209**: 475 - 480.

Carlisle, S.M., Blakeley, S.D., Hemmingsen, S.M., Trevanion, S.J., Hiyoshi, T., Kruger, N.J. and Dennis, D.T. 1990 Pyrophosphate-dependent phosphofructokinase: conservation of protein sequence between the α- and β-subunits and with the ATP-dependent phosphofructokinase. *Journal of Biological Chemistry*, **265**: 18366 - 18371.

Carnal, N.W. and Black, C.C. 1979 Pyrophosphate-dependent phosphofructokinase. A new glycolytic enzyme in pineapple leaves. *Biochemical and Biophysical Research Communications*, **86**: 20 - 26.

Carnal, N.W. and Black, C.C. 1983 Phosphofructokinase activities in photosynthetic organisms. *Plant Physiology*, **71**: 150 - 155.

Casas, A.M., Nelson, D.E., Raghothama, K.G., D'Urzo, M.P., Singh, N.K. and Bressan, R.A. 1992 Expression of osmotin-like genes in the halophyte *Atriplex nummularia L.*. *Plant Physiology*, **99**: 329 - 337.

Castresana, C., De Carvalho, F., Gheysen, G., Habets, M., Inzé, D. and Van Montagu, M. 1990 Tissue-specific and pathogen induced regulation of a *Nicotiana plumbaginifolia* β-1,3-glucanase gene. *The Plant Cell*, **2**: 1131 - 1143.

Cattivelli, L. and Bartels, D. 1990 Molecular cloning and characterisation of cold-regulated genes in barley. *Plant Physiology*, **93**: 1504 - 1510.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. 1994 Green fluorescent protein as a marker for gene expression. *Science*, **263**: 802 - 805.

Chen, W.H., Gartland, K.M.A., Davey, M.R., Sotak, R., Garland, J.S., Mulligan, B.J., Power, J.B. and Cocking, E.C. 1987 Transformation of sugarcane protoplasts by direct uptake of a selectable chimeric gene. *Plant Cell Reports*, **6**: 297 - 301.

Chen, W.H., Davey, M.R., Power, J.B. and Cocking, E.C. 1988 Sugarcane protoplasts: factors affecting division and plant regeneration. *Plant Cell Reports*, **7**: 344 - 347.

Chen, Z., Swisshelm, K. and Sager, R. 1994 A cautionary note on reaction tubes for differential display and cDNA amplification in thermal cycling. *BioTechniques*, **16**: 1002 - 1006.

Cheng, H-F. and Tao, M. 1990 Differential proteolysis of the subunits of pyrophosphate-dependent 6-phosphofructo-1-phosphotransferase. *Journal of Biological Chemistry*, **265**: 2173 - 2177.

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

Chilton, M., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F. and Tempé, J. 1982 *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. *Nature,* **295**: 432 - 434.

Chomczynski, P. and Sacchi, N. 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, **162**: 156 - 159.

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

Christensen, A.H., Sharrock, R.A. and Quail, P.H. 1992 Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology*, **18**: 675 - 689.

Christou, P., McCabe, D.E., Martinell, B.J. and Swain, W.F. 1990 Soybean genetic engineering- commercial production of transgenic plants. *Trends in Biotechnology*, **8**: 145 - 151.

Christou, P., Ford, T.L. 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 embryos. *Bio/Technology*, **9**: 957 - 962.

Christou, P. 1992 Genetic transformation of crop plants using microprojectile bombardment. *The Plant Journal*, **2**: 275 - 281.

Christou, P. 1993 Particle gun mediated transformation. *Current Opinion in Biotechnology*, **4**: 135 - 141.

Christou, P. 1996 Transformation technology. *Trends in Plant Science*, **1**: 423 - 431.

Claes, B., Dekeseyer, R., Villaroell, R., Van den Bulcke, R., Bauw, G., Van Montagu, M. 1990 Characterisation of a rice gene showing organ-specific expression in response to salt and drought. *The Plant Cell*, **2**: 19 - 27.

Cody, C.W., Prasher, D.C., Westler, W.M., Pendergast, F.G. and Ward, W.W. 1993 Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. *Biochemistry*, **32**: 1212 - 1218.

Cohen, A. and Bray, E.A. 1990 Characterisation of three mRNAs that accumulate in wilted tomato leaves in response to elevated levels of endogenous abscisic acid. *Planta*, **182**: 27 - 33.

Cohen, A. and Bray, E.A. 1992 Nucleotide sequence of an ABA-induced tomato gene that is expressed in wilted vegetative organs and developing seeds. *Plant Molecular Biology*, **18**: 411 - 413.

Crameri, A., Whitehorn, E.A., Tate, E. and Stemmer, W.P.C. 1996 Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnology*, **14**: 315 - 319.

Czarnecka, E., Key, J.L. and Gurley, W.B. 1989 Regulatory domains of the Gmhsp17.5-E heat shock promoter of soybean. *Molecular and Cellular Biology*, **9**: 3457 - 3463.

Czarnecka-Verner, E., Dulce-Barros, M. and Gurley, W.B. 1994 Regulation of heat shock gene expression. In *Stress-induced gene expression in plants*. Basra, A.S. (Ed.), ISBN 3-7186-5466-0, pp. 131 - 162. Harwood Academic Publishers, Switzerland.

Dancer, J.E. and ap Rees, T. 1989 Relationship between pyrophosphate:fructose-6-phosphate 1-phosphotransferase, sucrose breakdown and respiration. *Journal of Plant Physiology*, **135**: 197 - 206.

Delauney, A.J. and Verma, D.P.S. 1990 A soybean gene encoding Δ^1-pyrroline-5-carboxylate reductase was isolated by functional complementation in *E.coli* and is found to be osmoregulated. *Molecular and General Genetics*, **221**: 299 - 305.

Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983 A plant DNA mini preparation: Version II. *Plant Molecular Biology Reporter*, **1**: 19 - 21.

Dennis, D.T. and Greyson, M.F. 1987 Fructose 6-phosphate metabolism in plants. *Plant Physiology*, **69**: 394 - 404.

Dennis, D.T. and Blakeley, S.D. 1995 The regulation of carbon partitioning in plants. In Carbon partitioning and source-sink interactions in plants. Madore, M.A. and Lucas, W.J. (Eds.), *Current topics in Plant Physiology*, **13**: 258 - 267.

Depater, B.S. and Schilperoort, R.A. 1992 Structure and expression of a root-specific rice gene. *Plant Molecular Biology*, **18**: 161- 164.

Dixon, R.A. and Lamb, C.J. 1990 Molecular communication in interactions between plants and microbial pathogens. *Annual Review of Plant Physiology and Plant Molecular Biology*, **41**: 339 - 367.

Doss, R.P. 1996 Differential display without radioactivity - a modified procedure. *BioTechniques*, **21**: 408 - 410.

Draper, J., Scott, R., Armitage, P. and Walden, R. (Eds.), 1988 *Plant genetic transformation and gene expression: A laboratory manual.* ISBN 0-632-02172-1, pp. 324 -326. Blackwell Scientific Publications.

Duff, S., Moorhead, G.B.G., Lefebvre, D.D. and Plaxton, W.C. 1989 Phosphate starvation inducible bypasses of adenylate and phosphate dependent glycolytic enzymes in *Brassica nigra* suspension cells. *Plant Physiology*, **90**: 1275 - 1278.

Eisermann, A., Tiller, K. and Link, G. 1990 *In vitro* transcription and DNA binding characteristics of chloroplast and etioplast extracts from mustard *(Sinapis alba)* indicate differential usage of the *psbA* promoter. *EMBO Journal*, **9**: 3981 - 3987.

Etzold, T., Fritz, C.C., Schell, J. and Schreier, P.H. 1987 A point mutation in the chloroplast 16S ribosomal RNA gene of a streptomycin resistant *Nicotiana tabacum*. *FEBS Letters*, **219**: 343 - 346.

Fahn, A. (Ed.), 1990 Vascular system of monocotyledons. In *Plant anatomy*. 4th edition, ISBN 0-08-037491-3, pp. 204 -209. Pergamon Press, Oxford, England.

Faktor, O., Kooter, J.M., Dixon, R.A. and Lamb, C.J. 1996 Functional dissection of a bean chalcone synthase gene promoter in transgenic tobacco plants reveals motifs essential for floral expression. *Plant Molecular Biology*, **32**: 849 - 859.

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

Finer, J.J. and McMullen, M.D. 1990 Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. *Plant Cell Reports*, **8**: 586 - 589.

Finer, J.J., Vain, P., Jones, M.W. and McMullen, M.D. 1992 Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Reports*, **11**: 323 - 328.

Flach, J., Bossie, M., Vogel, J., Corbett, A.H., Jinks, T., Willins, D.A. and Silver, P.A. 1994 A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. *Molecular and Cellular Biology*, **14**: 8399 - 8407.

Fladung, M. and Ahuju, M.R. 1997 Excision of the maize transposable element *Ac* in periclinal chimeric leaves of *35S-Ac-rolC* transgenic aspen-*Populus*. *Plant Molecular Biology*, **33**: 1097 - 1103.

Fluhr, R. and Chua, N-H. 1986 Developmental regulation of two genes encoding ribulose-bisphosphate carboxylase small subunit in pea and transgenic petunia plants. *National Academy of Science. Proceedings*, **83**: 2358 - 2362.

Forde, B.G. 1994 AT-rich elements (ATREs) in the promoter regions of nodulin and other higher plant genes: a novel class of *cis*-acting regulatory element? In *Plant promoters and transcription factors*. Nover, L. (Ed.), ISBN 3-540-57288-0, pp. 87 - 105. Springer-Verlag, Germany.

Fraley, R. 1992 Sustaining the food supply. *Bio/Technology*, **10**: 40 - 43.

Fraley, R. and Schell, J. 1992 Plant biotechnology: editorial review. *Current opinion in Biotechnology*, **3**: 139 - 140.

Franks, T. and Birch, R. 1991 Gene transfer into intact sugarcane cells using microprojectile bombardment. *Australian Journal of Plant Physiology*, **18**: 471 - 480.

Fujii, N. and Uchimiya, H. 1991 Conditions favorable for the somatic embryogenesis in carrot cell culture enhance expression of the *rolC* promoter-GUS fusion gene. *Plant Physiology*, **95**: 238 - 241.

Fukuda, Y., Ohme, M. and Shinshi, H. 1991 Gene structure and expression of a tobacco endochitinase gene in suspension-cultured tobacco cells. *Plant Molecular Biology*, **16**: 1 - 10.

Fukuda, Y. and Shinshi, H. 1994 Characterisation of a novel *cis*-acting element that is responsive to a fungal elicitor in the promoter of a tobacco class I chitinase gene. *Plant Molecular Biology*, **24**: 485 - 493.

Galas, D. and Schmitz, A. 1978 DNase footprinting: A simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Research*, **5**: 3157 - 3170.

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

Gambley, R.L., Ford, R. and Smith, G.R. 1993 Microprojectile transformation of sugarcane meristems and regeneration of shoots expressing β-glucuronidase. *Plant Cell Reports*, **12**: 343 - 346.

Garamszegi, N., Garamszegi, Z.P., Rogers, M.S., DeMarco, S.J. and Strehler, E.E. 1997 Application of a chimeric green fluorescent protein to study protein-protein interactions. *BioTechniques*, **23**: 864 - 872.

Gasser, C.S. and Fraley, R.T. 1989 Genetically engineered plants for crop improvement. *Science*, **244**: 1293 - 1299.

Gatehouse, A.M.R., Hilder, V.A. and Boulter, D. (Eds.) 1992 *Plant genetic manipulation for crop protection*. ISBN 0-85198-707-9, C.A.B. International, U.K.

Gatz, C. 1997 Chemical control of gene expression. *Annual review of Plant Physiology and Plant Molecular Biology*, **48**: 89 - 108.

Gery, S. and Lavi, S. 1997 Purification and cloning of differential display products. *BioTechniques*, **23**: 198 - 202.

Gibson, S. and Somerville,C. 1993 Isolating plant genes. *Trends in Biotechnology*, **11**:306 - 313.

Gilmartin, P.M., Sarokin, L., Memelink, J. and Chua, N-H. 1990 Molecular light switches for plant genes. *The Plant Cell*, **2**: 369 - 378.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P-C., Bouvier-Durand, M. and Vartanian, N. 1994 Current advances in abscisic acid action and signalling. *Plant Molecular Biology*, **26**: 1557 - 1577.

Glick, B.R. and Thompson, J.E. (Eds.) 1993 *Methods in plant molecular biology and biotechnology*. ISBN 0-8493-5164-2. CRC Press, London.

Goddijn, O.J.M. and Pen, J. 1995 Plants as bioreactors. Trends in *Biotechnology*, **13**: 379 - 387.

Gómez, J., Sanchez-Martinez, D., Stiefel, V., Rigau, J., Puigdomènech, P. and Pagès, M. 1988 A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. *Nature*, **334**: 262 - 264.

Graham, M.W., Craig, S. and Waterhouse, P.M. 1997 Expression patterns of vascular-specific promoters *rolC* and *Sh* in transgenic potatoes and their use in engineering PLRV-resistant plants. *Plant Molecular Biology*, **33**: 729 - 735.

Green, A.E. and Alison, R.F. 1994 Recombination between viral RNA and transgenic plant transcripts. *Science*, **263**: 1423 - 1425.

Greenberg, B.M. and Glick, B.R. 1993 The use of recombinant DNA technology to produce genetically modified plants. In *Methods in Plant Molecular Biology and Biotechnology*. Glick, B.R. and Thompson, J.E. (Eds.), ISBN 0-8493-5164-2 pp.1 - 10.CRC Press, London.

Grierson, D. and Covey, S. 1984 *Plant molecular biology*. ISBN 0-216-91631-2, pp. 37 - 49. Blackie and Son, Ltd., Glasgow.

Grimmig, B. and Matern, U. 1997 Structure of the parsley caffeoyl-CoA *O*-methyltransferase gene, harbouring a novel elicitor-responsive *cis*-acting element. *Plant Molecular Biology*, **33**: 323 - 341.

Groenewald, J.H., Groenewald, S., Whittaker, A., Huckett, B.I. and Botha, F.C. 1995 Molecular agriculture: prospects for the production of alternative commodities in sugarcane through genetic engineering. *Proceedings of the South African Sugarcane Technologists' Association*: 14 - 20.

Gruber, M.Y. and Crosby, W.L. 1993 Vectors for plant transformation. In *Methods in plant molecular biology and biotechnology*. Glick, B.R. and Thompson, J.E. (Eds.), ISBN 0-8493-5164-2, pp. 89 - 119. CRC Press, London.

Gubler, F. and Jacobsen, J.V. 1992 Gibberellin-responsive elements in the promoter of a barley high pI α-amylase gene. *The Plant Cell*, **4**: 1435 - 1441.

Guerrero, F.D. and Mullet, J.E. 1988 Reduction of turgor induces rapid changes in leaf translatable RNA. *Plant Physiology*, **88**: 401 - 408.

Guilfoyle, T.J. 1997 The structure and function of plant gene promoters. In *Genetic engineering: principles and methods*, Vol. 19. Setlow, J.K. (Ed.), ISBN 0-306-45681-8, pp.15 - 47. Plenum publishing corporation, New York.

Guilley, H., Dudley, R.K., Jonard, G., Balazs, E. and Richards, K.E. 1982 Transcription of cauliflower mosaic virus DNA: Detection of promoter sequences, and characterisation of transcription. *Cell*, **30**: 763 - 773.

Guivarc'h, A., Spena, A., Noin, M., Besnard, C. and Chriqui, D. 1996 The pleiotropic effects induced by the *rolC* gene in transgenic plants are caused by expression restricted to protophloem and companion cells. *Transgene Research*, **5**: 3 - 11.

Guy, C.L. 1990 Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annual review of Plant Physiology and Plant Molecular Biology*, **41**: 187 - 223.

Hadman, M., Adam, B-L., Wright, G.L. Jr. and Bos, T.J. 1995 Modifications to the differential display technique reduce background and increase sensitivity. *Analytical Biochemistry*, **226**: 383 - 386.

Hagen, G., Li, Y., Liu, Z., Ulmasov, T. and Guilfoyle, T. 1994 Promoter analysis of two auxin-responsive genes: *GH3* and *SAUR*. In *Abstracts of the 4th International Congress of Plant Molecular Biology*, Amsterdam, 906.

Hain, R., Stabel, P., Czernilofsky, A.P., Steinbi, H-H., Herrera-Estrella, L. and Schell, J. 1985 Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. *Molecular and General Genetics*, **199**: 161 - 167.

Hajela, R.K., Horvath, D.P., Gilmar, S.J. and Thomashow, M.F. 1990 Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana*. *Plant Physiology*, **93**: 1246 - 1252.

Hara, E., Yamaguchi, T., Tahara, H., Tsuyama, N., Trusrui, H., Ide, T. and Oda, K. 1993 DNA-DNA subtractive cDNA cloning using oligo(dT)$_{30}$-latex and PCR: Identification of cellular genes which are overexpressed in senescent human diploid fibroblasts. *Analytical Biochemistry*, **214**: 58 - 64.

Haring, M.A. and De Block, M. 1990 New roads towards chloroplast transformation in higher plants. *Physiologia Plantarum*, **79**: 218 - 221.

Haseloff, J. and Amos, B. 1995 GFP in plants. *Trends in Genetics*, **11**: 328 - 329.

Haseloff, J., Siemering, K.R., Prasher, D.C. and Hodge, S. 1997 Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *National Academy of Sciences. Proceedings*, **94**: 2122 - 2127.

Hatzopoulos, A.K., Schlokat, U. and Gruss, P. 1988 Enhancers and other cis-regulatory sequences. In *Transcription and splicing*. Hames, B.D. and Glover, D.M. (Eds.), ISBN 1-85221-076-1, pp. 43 - 96. IRL Press, Oxford.

Hauptmann, R.M., Vasil, V., Ozias-Akins, P., Tabaeizadeh, Z., Rogers, S.G., Fraley, R.T., Horsch, R.B. and Vasil, I.K. 1988 Evaluation of selectable markers for obtaining stable transformants in the Graminae. *Plant Physiology*, **86**: 602 - 606.

Hayford, M.B., Medford, J.I., Hoffman, N.L., Rogers, S.G. and Klee, H.J. 1988 Development of a plant transformation selection system based on expression of genes encoding gentamicin acetyltransferase. *Plant Physiology*, **86**: 1216 - 1222.

Heim, R., Prasher, D.C. and Tsien, R.Y. 1994 Wavelength mutations and posttranscriptional autoxidation of green fluorescent protein. *National Academy of Sciences. Proceedings*, **91**: 12501 - 12504.

Herrera-Estrella, A., Van Montagu, M. and Wang, K. 1990 A bacterial peptide acting as a plant nuclear targeting signal: the amino-terminal portion of *Agrobacterium Vir*D2 protein directs a beta-galactosidase fusion protein into tobacco nuclei. *National Academy of Sciences. Proceedings*, **87**: 9534 - 9537.

Hiatt , A. (Ed.)1993 *Transgenic plants: fundamentals and applications*. ISBN 0-8247-8766-8, pp. 283 - 300. Marcel Dekker, New York.

Higgs, S., Traul, D., Davis, B.S., Kamrud, K.I., Wilcox, C.L. and Beaty, B.J. 1996 Green fluorescent protein expressed in living mosquitoes without the requirement of transformation. *BioTechniques*, **21**: 660 - 664.

Ho, L.C., Grange, R.I. and Shaw, A.F. 1989 Source/sink regulation. In *Transport of photoassimilates*. Baker, D.A. and Milburn, J.A. (Eds.), pp. 306 - 343. Longman Scientific and Technical, UK.

Hodgkinson, S. 1995 GFP in *Dictyostelium*. *Trends in Genetics*, **11**: 327 - 328.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. 1983 A binary plant vector strategy based on separation of *vir*- and T-region *of the Agrobacterium tumefaciens* Ti-plasmid. *Nature*, **303**: 179 - 181.

Honeycutt, R.J., Sobral, B.W.S., Keim, P. and Irvine, J.E. 1992 A rapid DNA extraction method for sugarcane and its relatives. *Plant Molecular Biology Reporter*, **10**: 66 - 72.

Hooykaas, P.J.J. and Schilperoort, R.A. 1992 *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology*, **19**: 15 - 38.

Houde, M., Danyluk, J., Laliberté, J-F., Rassart, E., Dhindsa, R.S., Sarhan, F. 1992 Cloning, characterisation and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. *Plant Physiology*, **99**: 1381 - 1387.

Hu, W. and Cheng, C-H. 1995 Expression of *Aequorea* green fluorescent protein in plant cells. *FEBS Letters*, **369**: 331 - 334.

Hu, C.A.A., Delauney, A.J. and Verma, D.P.S. 1992 A bifunctional enzyme catalyses the first two steps in proline biosynthesis in plants. *National Academy of Science, Proceedings*, **89**: 9354 - 9358.

Huber, S.C. and Akazawa, T. 1986 A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. *Plant Physiology*, **81**: 1008 - 1013.

Iida, A., Yamashita, T., Yamada, Y. and Morikawa, H. 1991 Efficiency of particle-bombardment-mediated transformation is influenced by cell cycle stage in synchronised cultured cells of tobacco. *Plant Physiology*, **97**: 1585 - 1587.

Iida, A., Nagasawa, A. and Oeda, K. 1995 Positive and negative *cis*-regulatory regions in the soybean glycinin promoter identified by quantitative transient gene expression. *Plant Cell Reports*, **14**: 539 - 544.

Ish-Horowicz, D. and Burke, J.F. 1981 Rapid and efficient cosmid cloning. *Nucleic Acids Research*, **9**: 2989 - 2992.

Jacobsen, K.R., Fisher, D.G., Maretzki, A. and Moore, P.H. 1992 Developmental changes in the anatomy of the sugarcane stem in relation to phloem unloading and sucrose storage. *Botanica Acta*, **105**: 70 - 80.

Jefferson, R.A. 1987 Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter*, **5**: 387 - 405.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987 GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal*, **6**: 3901 - 3907.

Jorgensen, R.A. 1995 Cosuppression, flower color patterns, and metastable gene expression states. *Science*, **268**: 686 - 691.

Kain, S.R., Adams, M., Kondepudi, A., Yang, T-T., Ward, W.W. and Kitts, P. 1995 Green fluorescent protein as a reporter of gene expression and protein localisation. *BioTechniques*, **19**: 650 - 655.

Kaiser, T. and Batschauer, A. 1995 *Cis*-acting elements of the *CHS1* gene from white mustard controlling promoter activity and spatial patterns of expression. *Plant Molecular Biology*, **28**: 231 - 243.

Kanaya, K., Tabata, T., Iwabuchi, M. and Uchimiya, H. 1990 Specific binding of nuclear protein from tobacco hairy roots cultured *in vitro* to a 5' upstream region of the *rolC* gene of the Ri plasmid. *Plant Cell Physiology*, **31**: 941 - 946.

Katagiri, F., Lam, E. and Chua, N-H. 1989 Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. *Nature*, **340**: 727 - 730.

Kehoe, D.M., Degenhardt, J., Winicov, I., Tobin, E.M. 1994 Two 10-bp regions are critical for phytochrome regulation of a *Lemna gibba Lhcb* gene promoter. *The Plant Cell*, **6**: 1123 - 1134.

Kemper, E.L., Da Silva, M.J. and Arruda, P. 1996 Effect of microprojectile bombardment 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.

Kikkert, J.R. 1993 The Biolistic® PDS-1000/He device. *Plant Cell, Tissue and Organ Culture*, **33**: 221 - 226.

Kim, S-R., Choi, J-L., Costa, M.A. and An, G. 1992 Identification of G-box sequence as an essential element for methyl jasmonate response of potato proteinase inhibitor II promoter. *Plant Physiology*, **99**: 627 - 631.

Kim, Y., Buckley, K., Costa, M.A. and Gynheung, A. 1994 A 20 nucleotide upstream element is essential for the nopaline synthase (*nos*) promoter activity. *Plant Molecular Biology*, **24**: 105 - 117.

Kirch, H-H., Van Berkel, J., Glaczinski, H., Salamini, F. and Gebhardt, C. 1997 Structural organisation, expression and promoter activity of a cold-stress-inducible gene of potato (*Solanum tuberosum L.*). *Plant Molecular Biology*, **33**: 897 - 909.

Klee, H.J. and Rogers, S.G. 1989 Plant gene vectors and genetic transformation: Plant transformation systems based on the use of *Agrobacterium tumefaciens*. In *Cell culture and somatic cell genetics of plants*. Schell, J. and Vasil, I.K. (Eds.), pp.1 - 22. Academic Press, New York.

Klein, T.M., Wolf, E.D., Wu, R. and Sanford, J.C. 1987 High velocity microprojectiles for delivering nucleic acids into living cells. *Nature*, **327**: 70 - 73.

Klein, T.M., Harper, E.C., Svab, Z., Sanford, J.C., Fromm, M.E. and Maliga, P. 1988 Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. *National Academy of Sciences. Proceedings*, **85**: 8502 - 8505.

Köhler, R.H., Zipfel, W.R., Webb, W.W. and Hanson, M.R. 1997 The green fluorescent protein as a marker to visualise plant mitochondria *in vivo*. *The Plant Journal*, **11**: 613 - 621.

Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Z., Körber, H., Redei, G.P. and Schell, J. 1989 High-frequency T-DNA mediated gene tagging in plants. *National Academy of Sciences. Proceedings*, **86**: 8467 - 8471.

Koprek, T., Hänsch, R., Nerlich, A., Mendel, R.R. and Schulze, J. 1996 Fertile transgenic barley of different cultivars obtained by adjustment of bombardment conditions to tissue response. *Plant Science*, **119**: 79 - 91.

Kruger, N.J. and Dennis, D.T. 1987 Molecular properties of pyrophosphate:fructose 6-phosphate phosphotransferase from potato tubers. *Archives of Biochemistry and Biophysics*, **269**: 228 - 279.

Kuhlemeier, C., Green, P. and Chua, N-H. 1987 Regulation of gene expression in higher plants. *Annual Review of Plant Physiology*, **38**: 221 - 257.

Lam, E. 1994 Analysis of tissue-specific elements in the CaMV *35S* promoter. In *Plant promoters and transcription factors*. Nover, L. (Ed.), ISNB 0-387-57288-0, pp. 181 - 195. Springer-Verlag, Germany.

Lanahan, M.B., Ho, T-H.D., Rogers, S.W. and Rogers, J.C. 1992 A gibberellin response complex in cereal α-amylase gene promoters. *The Plant Cell*, **4**: 203 - 211.

Last, D.I., Brettel, R.I.S., Chamberlain, D.A., Chaudhury, A.M., Larkin, P.J., Marsh, E.L., Peacock, W.J. and Dennis, E.S. 1991 pEmu: an improved vector for gene expression in cereal cells. *Theoretical and Applied Genetics*, **81**: 581 - 588.

Lee, S.W., Tomasetto, C. and Sager, R. 1991 Positive selection of candidate tumor-suppressor genes by subtractive hybridisation. *National Academy of Science. Proceedings*, **88**: 2825 - 2829.

Lefebvre, D.D. and Gellatly, K.S. 1997 Fundamentals of gene structure and control. In *Plant metabolism* (2nd. Ed.) Dennis, D.T., Turpin, D.H., Lefebvre, D.D. and Layzell, D.B. (Eds.), ISBN 0-582-25906-1, pp. 3 - 16. Longman Singapore Publishers, Singapore.

Leffel, S.M., Mabon, S.A. and Stewart, C.N. 1997 Applications of green fluorescent protein in plants. *BioTechniques*, **23**: 912 - 918.

Lerchl, J., Geigenberger, P., Stitt, M. and Sonnewald, U. 1995 Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. *The Plant Cell*, **7**: 259 - 270.

Li, L., Qu, R., De Kochko, A., Fauquet, C. and Beachy, R.N. 1993 An improved rice transformation system using the biolistic method. *Plant Cell Reports*, **12**: 250 - 255.

Liang P. and Pardee A.B. 1992 Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, **257**: 967 - 971.

Liang, P., Averboukh, L. and Pardee, B. 1993 Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimisation. *Nucleic Acids Research*, **21**: 3269 - 3275.

Link, G. 1994 Plastid differentiation: organelle promoters and transcription factors. In *Plant promoters and transcription factors*. Nover, L. (Ed.), ISBN 0-387-57288-0, pp. 65 - 81. Springer-Verlag, Germany.

Linskens, M.H.K., Feng, J., Andrews, W.H., Enlow, B.E., Saati, S.M., Tonkin, L.A., Funk, W.D. and Villeponteau, B. 1995 Cataloging altered gene expression in young and senescent cells using enhanced differential display. *Nucleic Acids Research*, **23**: 3244 - 3251.

Liu, L-J. and Shearn, A. 1995 Rapid PCR for RNA differential display in a conventional heat block thermal cycler. *BioTechniques*, **19**: 44 - 46.

Lohmann, J., Schickle, H. and Bosch, T.C.G. 1995 REN display, a rapid and efficient method for non-radioactive differential display and mRNA isolation. *BioTechniques*, **18**: 200 - 202.

Lonsdale, D., Önde, 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.

Ludwig, S.R., Bowen, B., Beach, L. and Wessler, S.R. 1990 A regulatory gene as a novel visible marker for maize transformation. *Science*, **247**: 449 - 450.

Luo, G., An, G. and Wu, R. 1994 A PCR differential screening method for rapid isolation of clones from a cDNA library. *BioTechniques*, **16**: 672 - 675.

Maas, C., Laufs, J., Grant, S., Korfhage, C. and Werr, W. 1991 The combination of a novel stimulatory element in the first exon of the maize *Shrunken-1* gene with the following intron enhances reporter gene expression up to 1000-fold. *Plant Molecular Biology*, **16**: 199 - 207.

Macdonald, F.D. and Preiss, J. 1986 The subcellular location and characteristics of pyrophosphate:fructose-6-phosphate 1-phosphotransferase from suspension cultured cells of soybean. *Planta*, **167**: 240 - 245.

Martin, T., Schmidt, R., Altmann, T. and Frommer, W.B. 1992 Non-destructive assay system for detection of β-glucuronidase activity in higher plants. *Plant Molecular Biology Reporter*, **10**: 37 - 46.

Mascarenhas, D., Mettler, I.J., Pierce, D.A. and Lowe, H.W. 1990 Intron-mediated enhancement of heterologous gene expression in maize. *Plant Molecular Biology*, **15**: 913 - 920.

Matsuki, R., Onodera, H., Yamauchi, T. and Uchimiya, H. 1989 Tissue-specific expression of the *rolC* promoter of the Ri plasmid in transgenic rice plants. *Molecular and general Genetics,* **220**: 12 - 16.

Maurel, C., Brevet, J., Barbier-Brygoo, H., Guern, J. and Tempé, J. 1990 Auxin regulates the promoter of the root-inducing *rolB* gene of *Agrobacterium rhizogenes* in transgenic tobacco. *Molecular and General Genetics*, **223**: 58 - 64.

Mauro, M.L., Trovato, M., Paolis, A.D., Gallelli, A., Costantino, P. and Altamura, M.M. 1996 The plant oncogene *rolD* stimulated flowering in transgenic tobacco plants. *Developmental Biology*, **180**: 693 - 700.

McCabe, D.E., Swain, W.F., Martinell, B.J. and Christou, P. 1988 Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology*, **6**: 923 - 926.

McCabe, D. and Christou, P. 1993 Direct DNA transfer using electric discharge particle acceleration (ACCELL™ technology). *Plant Cell, Tissue and Organ Culture*, **33**: 227 - 236.

McClelland, M., Mathieu-Daude, F. and Welsh, J. 1995 RNA fingerprinting and differential display using arbitrarily primed PCR. *Trends in Genetics*, **11**: 242 - 246.

McDonnell, R., Clark, R., Smith, W. and Hinchee, M. 1987 A simplified method for the detection of neomycin phosphotransferase activity in transformed plant tissues. *Plant Molecular Biology Reporter*, **5**: 380 - 386.

McElroy, D., Zhang, W., Cao, J. and Wu, R. 1990 Isolation of an efficient actin promoter for use in rice transformation. *The Plant Cell*, **2**: 163 - 171.

Medina-Escobar, N., Cárdenas, J., Valpuesta, V., Muñoz-Blanco, J. and Caballero, J.L. 1997 Cloning and characterisation of cDNAs from genes differentially expressed during the strawberry ripening process by a MAST-PCR-SBDS method. *Analytical Biochemistry*, **248**: 288 - 296.

Meier, I., Hahlbrock, K. and Somssich, I.E. 1991 Elicitor-inducible and constitutive *in vivo* DNA footprints indicate novel *cis*-acting elements in the promoter of a parsley gene encoding pathogenesis-related protein 1. *The Plant Cell*, **3**: 309 - 315.

Meins, F.J., Neuhaus, J-M., Sperisen, C. and Ryals, J. 1992 The primary structure of plant pathogenesis-related glucanohydrolases and their genes. In *genes involved in plant defense*. Boller, T. and Meins, F. (Eds.), pp. 245 - 282. Springer Verlag, New York.

Mendel, R.R., Müller, 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.

Michel, D., Salamini, F., Bartels, D., Dale, P., Baga, M. and Szalay, A. 1993 Analysis of a dessication and ABA-responsive promoter isolated from the resurrected plant *Craterostigma plantagineum*. *The Plant Journal*, **4**: 29 - 40.

Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., Gotoh, Y., Katayose, Y., Nakamura, S., Honkura, R., Nishimiya, S., Ueno, K., Mochizuki, A., Tanimoto, H., Tsugawa, H., Otsuki, Y. and Ohashi, Y. 1996 Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiology*, **37**: 49 - 59.

Miyoshi, H., Usami, T. and Tanaka, I. 1995 High level of GUS gene expression driven by pollen-specific promoters in electroporated lily pollen protoplasts. *Sexual Plant Reproduction*, **8**: 205 - 209.

Mol, J.N.M., Holton, T.A. and Koes, R.E. 1995 Floriculture: genetic engineering of commercial traits. *Trends in Biotechnology*, **13**: 350 - 355.

Moore, L., Warner, G. and Strobel, G. 1979 Involvement of plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. *Plasmid*, **2**: 617 - 626.

Moore, P.H. 1995 Temporal and spatial regulation of sucrose metabolism in the sugarcane stem. *Australian Journal of Plant Physiology*, **22**: 661 - 679.

Moore, P.H. 1996 Progress in sugarcane molecular biology. *The XXII Congress of the International Society of Sugarcane Technologists. Proceedings*, p. 353.

Morrish, F., Songstad, D.D., Armstrong, C.L. 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*. Hiatt, A. (Ed.), ISBN 0-8247-8766-8, pp. 133 - 171. Marcel Dekker, New York.

Moss, J.B. and Rosenthal, N. 1994 Analysis of gene expression patterns in the embryonic mouse myotome with the green fluorescent protein, a new vital marker. *Journal of Cell Biochemistry*, **S18D**: 489 - 494.

Mukumoto, F., Hirose, S., Imaseki, H. and Yamazaki, K. 1993 DNA sequence requirement of a TATA element-binding protein from *Arabidopsis* for transcription *in vitro*. *Plant Molecular Biology*, **23**: 995 - 1003.

Mundy, J. and Chua, N-H. 1988 Abscisic acid and water-stress induce the expression of a novel rice gene. *EMBO Journal*, **7**: 2279 - 2286.

Murashige, T. and Skoog, F. 1962 A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, **15**: 473 - 479.

Nagata, N., Kosono, S., Sekine, M., Shinmyō, A. and Syōno, K. 1995 The regulatory functions of the *rolB* and *rolC* genes of *Agrobacterium rhizogenes* are conserved in the homologous genes (*Ngrol*) of *Nicotiana glauca* in tobacco genetic tumors. *Plant Cell Physiology*, **36**: 1003 - 1012.

Nagatani, N., Takumi, S., Tomiyama, M., Shimada, T. and Tamiya, E. 1997 Semi-real imaging of the gene expression of a maize polyubiquitin promoter-GFP gene in transgenic rice. *Plant Science*, **124**: 49 - 56.

Nakamura, T., Handa, T., Oono, Y., Kanaya, K., Michikawa, M. and Uchimiya, H. 1988 Organ-specific mRNA in transgenic tobacco plants possessing T-DNA of Ri plasmids. *Plant Science*, **56**: 213 - 218.

Nakamura, N., Suzuki, Y. and Suzuki, H. 1992 Pyrophosphate-dependent phosphofructokinase from pollen: properties and possible roles in sucrose metabolism. *Plant Physiology*, **86**: 616 - 622.

Nelson, D.E., Raghothama, K.G., Singh, N.K., Hasegawa, P.M. and Bressan, R.A. 1992 Analysis of structure and transcriptional activation of an osmotin gene. *Plant Molecular Biology*, **19**: 577 - 588.

Neven, L.G., Haskell, D.W., Hofig, A., Li, Q-B. and Guy, C.L. 1993 Characterisation of a spinach gene responsive to low temperatures and water stress. *Plant Molecular Biology*, **21**: 291 - 305.

Niedz, R.P., Sussman, M.R. and Satterlee, J.S. 1995 Green fluorescent protein: an *in vivo* reporter of plant gene expression. *Plant Cell Reports*, **14**: 403 - 406.

Nilsson, O., Moritz, T., Imbault, N., Sandberg, G. and Olsson, O. 1993 Hormonal characterisation of transgenic tobacco plants expressing the *rolC* gene of *Agrobacterium rhizogenes* TL-DNA. *Plant Physiology*, **102**: 363 - 371.

Nilsson, O., Little, C.H.A., Sandberg, G. and Olsson, O. 1996 Expression of two heterologous promoters, *Agrobacterium rhizogenes rolC* and cauliflower mosaic virus *35S*, in the stem of transgenic hybrid aspen plants during the annual cycle of growth and dormancy. *Plant Molecular Biology*, **31**: 887 - 895.

Nilsson, O. and Olsson, O. 1997 Getting to the root: The role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. *Physiologia Plantarum*, **100**: 463 - 473.

Nilsson, O., Tuominen, H., Sandberg, G. and Olsson, O. 1997 The *Agrobacterium rhizogenes rolB* and *rolC* promoters are expressed in pericycle cells competent to serve as root initials in transgenic hybrid aspen. *Plant Physiology*, **100**: 456 - 462.

Nishiuchi, T., Nakamura, T., Abe, T., Kodama, H., Nishimura, M. and Iba, K. 1995 Tissue-specific and light-responsive regulation of the promoter region of the *Arabidopsis thaliana* chloroplast ω-3 fatty acid desaturase gene *(FAD7)*. *Plant Molecular Biology*, **29**: 599 - 609.

Nunberg, A.N., Li, Z., Chung, H-J., Reddy, A.S. and Thomas, T.L. 1995 Proximal promoter sequences of sunflower helianthinin genes confer regionalised seed-specific expression. *Journal of Plant Physiology*, **145**: 600 - 605.

Oard, J.H., Paige, D.F., Simmonds, J.A. and Gradziel, T.M. 1990 Transient gene expression in maize, rice, and wheat cells using an airgun apparatus. *Plant Physiology*, **92**: 334 - 339.

Oard, J. 1993 Development of an airgun device for particle bombardment. *Plant Cell, Tissue and Organ Culture*, **33**: 247 - 250.

Oeller, P.W., Min-Wong, L., Taylor, L.P., Pike, D.A. and Theologis, A. 1991 Reversible inhibition of tomato fruit senescence by antisense RNA. *Science*, **254**: 437 - 439.

Oono, Y., Handa, T., Kanaya, K. and Uchimiya, H. 1987 the TL-DNA gene of Ri plasmid responsible for dwarfness of tobacco plants. *Japanese Journal of Genetics*, **62**: 501 - 505.

O'Brien, W.E., Bowein, S. and Wood, H.G. 1975 Isolation and characterisation of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii. Journal of Biological Chemistry*, **250**: 8690 - 8695.

Palm, C.J., Costa, M.A., An, G. and Ryan, C.A. 1990 Wound-inducible nuclear protein binds DNA fragments that regulate proteinase inhibitor II gene from potato. *National Academy of Science. Proceedings*, **87**: 603 - 607.

Palva, E.P. 1994 Gene expression under low temperature stress. In *Stress-induced gene expression in plants*. Basra, A.S. (Ed.), ISBN 3-7186-5466-0, pp. 103 - 130. Harwood Academic Publishers, Switzerland.

Pang, S-Z., DeBoer, D.L., Wan, Y., Ye, G., Layton, J.G., Neher, M.K., Armstrong, C.L., Fry, J.E., Hinchee, M.A.W. and Fromm, M.E. 1996 An improved green fluorescent protein as a vital marker in plants. *Plant Physiology*, **112**: 893 - 900.

Peferoen, M. 1992 Engineering of insect-resistant plants with *Bacillus thuringiensis* crystal protein genes. In *Plant genetic manipulation for crop protection*. Gatehouse, A.M.R., Hilder, V.A. and Boulter, D. (Eds.), ISBN 0851987079, pp. 135 - 154. C.A.B. International, U.K.

Pen, J., Sijmons, P.C., Van Ooijen, A.J.J. and Hoekema, A. 1993 Protein purification in transgenic crops: analysis of plant molecular farming. In *Transgenic plants: fundamentals and applications*. A.Hiatt (Ed.), ISBN 0-8247-8766-8, pp. 239 - 254. Marcel Dekker, Inc., New York.

Pines, J. 1995 GFP in mammalian cells. *Trends in Genetics*, **11**: 326 - 327.

Pla, M., Gómez, J., Goday, A. and Pagès, M. 1991 Regulation of the abscisic acid-responsive gene *rab28* in maize *viviparous* mutants. *Molecular and General Genetics*, **230**: 394 - 400.

Plaxton, W.C. 1996 The organisation and regulation of plant glycolysis. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**: 185 - 214.

Potier, B.A.M. and Birch, R.G. 1997a Differentially expressed genes from a sugarcane stem cDNA library. In *Abstracts of the pathology and molecular biology workshop of the International Society of Sugarcane Technologists,* South Africa.

Potier, B.A.M. and Birch, R.G. 1997b Differentially expressed genes from a sugarcane stem cDNA library: Expression pattern of the transcripts and promoter recovery. In *Abstracts of the 5th international congress of Plant Molecular Biology*, Singapore.

Quatrano, R.S., Marcotte, W.R and Guiltinan, M. 1993 Regulation of gene expression by abscisic acid. In *Control of plant gene expression*. Verma, D.P.S. (Ed.), ISBN 0-8493-8866-X/93, pp. 69 - 90. CRC Press, Florida.

Rasmussen, J.L., Kikkert, J.R., Roy, M.K. and Sanford, J.C. 1994 Biolistic transformation of tobacco and maize suspension cells using bacterial cells as microprojectiles. *Plant Cell Reports*, **13**: 212 - 217.

Rathus, C., Bower, R. and Birch, R.G. 1993 Effects of promoter, intron and enhancer elements on transient gene expression in sugarcane and carrot protoplasts. *Plant Molecular Biology*, **23**: 613 - 618.

Reeves, R.E., Serrano, R. and South, D.J. 1976 6-Phosphofructokinase (pyrophosphate). Properties of the enzyme from *Entamoeba histolytica* and its reaction mechanism. *Journal of Biological Chemistry*, **251**: 2958 - 2962.

Reeves, S.A., Rubio, M-P. and Louis, D.N. 1995 General method for PCR amplification and direct sequencing of mRNA differential display products. *BioTechniques*, **18**: 18 - 20.

Reichel, C., Mathur, J., Eckes, P., Langenkemper, K., Koncz, C., Schell, J., Reiss, B. and Maas, C. 1996 Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *National Academy of Science. Proceedings*, **93**: 5888 - 5893.

Reimann-Phillip, U. and Beachy, R. 1993 Coat-protein mediated resistance in transgenic tobacco expressing the tobacco mosaic virus coat protein from tissue-specific promoters. *Molecular Plant-microbe Interactions*, **6**: 323 - 330.

Reznikoff, W., Siegele, D.A., Cowing, D.W. and Gross, C.A. 1985 The regulation of transcription initiation in bacteria. *Annual Review of Genetics*, **19**: 355 - 387.

Rhode, W., Becker, D. and Randles, J.W. 1995 The promoter of coconut foliar decay-associated circular single-stranded DNA directs phloem-specific reporter gene expression in transgenic tobacco. *Plant Molecular Biology*, **27**: 623 - 628.

Riggs, C.D. and Chrispeels, M.J. 1987 Luciferase reporter gene cassettes for plant gene expression studies. *Nucleic Acids Research*, **15**: 8115 - 8121.

Roby, D., Broglie, K., Cressman, R., Biddle, P., Chet, I. and Broglie, R. 1990 Activation of a bean chitinase promoter in transgenic tobacco plants by phytopathogenic fungi. *The Plant Cell,* **2**: 999 - 1007.

Roby, D., Broglie, K., Gaynor, J and Broglie, R. 1991 Regulation of a chitinase gene promoter by ethylene and elicitors in bean protoplasts. *Plant Physiology*, **97**: 433 - 439.

Rompf, R. and Kahl, G. 1997 mRNA Differential display in agarose gels. *BioTechniques*, **23**: 28 - 32.

Rosenthal, N. 1987 Identification of regulatory elements of cloned genes and functional assays. *Methods in Enzymology*, **152**:704 - 720.

Ross, A.F. 1966 Systemic effects of local lesion formation. In *Viruses of plants*. Beemster, A.B.R. and Dijkstra, J. (Eds.), pp.127 - 150. North Holland Publishers, Amsterdam.

Rouwendal, G.J.A., Mendes, O., Wolbert, E.J.H. and de Boer, A.D. 1997 Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. *Plant Molecular Biology*, **33**: 989 - 999.

Rozario, C., Smith, M.W. and Muller, M. 1995 Primary sequence of a putative inorganic pyrophosphate linked phosphofructokinase of *Giardia lamblia*. *Biochimica et Biophysica Acta*, **1260**: 218 - 222.

Sabularse, D.C. and Anderson, R.L. 1981 D-Fructose 2,6-bisphosphate: A naturally occurring activator for inorganic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase in plants. *Biochemical and Biophysical Research Communication*, **103**: 848 - 855.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989 *Molecular cloning: A laboratory manual*, 2nd edition. Cold Spring Harbor, New York.

Sargent, T.D. 1987 Isolation of differentially expressed genes. *Methods in Enzymology*, **152**: 423 - 432.

Sato, S., Newell, C., Kolacz, K., Tredo, L., Finer, J. and Hinchee, M. 1993 Stable transformation via particle bombardment in two different soybean regeneration systems. *Plant Cell Reports*, **12**: 408 - 413.

Sautter, C., Waldner, H., Neuhaus-Url, G., Galli, A., Neuhaus, G. and Potrykus, I. 1991 Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. *Bio/Technology*, **9**: 1080 - 1085.

Schaffner, M.A. and Fischer, R.L. 1990 Transcriptional activation by heat and cold of a thiol protease gene in tomato. *Plant Physiology*, **93**: 1486 - 1491.

Schäffner, A.R. and Sheen, J. 1991 Maize *rbcS* promoter activity depends on sequence elements not found in dicot *rbcS* promoters. *The Plant Cell*, **3**: 997 - 1012.

Scharf, K-D., Materna, T., Treuter, E. and Nover, L. 1994 Heat stress promoters and transcription factors. In *Plant promoters and transcription factors*. Nover, L. (Ed.), ISBN 0-387-57288-0, pp.125 - 162. Springer-Verlag, Switzerland.

Schmülling, T., Schell, J. and Spena, A. 1988 Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO Journal*, **7**: 2621 - 2629.

Schmülling, T., Schell, J. and Spena, A. 1989 Promoters of the *rolA, B* and *C genes of Agrobacterium rhizogenes* are differentially regulated in transgenic plants. *The Plant Cell*, **1**: 665 - 670.

Schraml, P., Shipman, R., Stulz, P. and Ludwig, C.U. 1993 cDNA subtraction library construction using a magnet-assisted subtraction technique (MAST). *Trends in Genetics*, **3**: 70 - 71.

Sessa, G., Meller, Y. and Fluhr, R. 1995 A GCC element and a G-box motif participate in ethylene-induced expression of the *PRB-1b* gene. *Plant Molecular Biology*, **28**: 145 - 153.

Setlow, J.K. (Ed.) 1997 *Genetic engineering: principles and methods*, Vol. 19. ISBN 0-306-45681-8, pp.15 - 47. Plenum publishing corporation, New York.

Shah, D.M., Rommens, C.M.T. and Beachy, R.N. 1995 Resistance to diseases and insects in transgenic plants: progress and application to agriculture. *Trends in Biotechnology*, **13**: 362 - 368.

Sharma, P., Lönneborg, A. and Stougaard, P. 1993 PCR-based construction of subtractive cDNA library using magnetic beads. *BioTechniques*, **15**: 610 - 611.

Shaw, C.H., Leemans, J., Van Montagu, M. and Schell, J. 1983 A general method for the transfer of cloned genes into plant cells. *Gene*, **23**: 315 - 330.

Shen, Q. and Ho, T-H.D. 1995 Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel *cis*-acting element. *The Plant Cell*, **7**: 295 - 307.

Shinshi, H., Usami, S. and Ohme-Takagi, M. 1995 Identification of an ethylene-responsive region in the promoter of a tobacco class I chitinase gene. *Plant Molecular Biology*, **27** : 923 - 932.

Singh, K., Dennis, E.S., Ellis, J.G., Llewellyn, D.J., Tokuhisa, J.G., Wahleithner, J.A. and Peacock, W.J. 1990 OCSBF-1, a maize *ocs* enhancer binding factor :isolation and expression during development. *The Plant Cell*, **2**: 891 - 903.

Sinkar, P.S., Pythoud, F., White, F.F., Nester, E.W. and Gordon, M.P. 1988 *rolA* locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants. *Genes and Development*, **2**: 688 - 697.

Smith, C.J.S., Hamilton, A.J. and Grierson, D. 1993 The regulation of tomato ripening by antisense genes. In *Control of plant gene expression*. D.P.S.Verma (Ed.), ISBN0-8493-8866-X, pp. 535 - 546. C.R.C. Press, Florida.

Smith, N.R., Aldersley, M., Li, A., High, A.S., Moynihan, T.P., Markham, A.F. and Robinson, P.A. 1997 Automated differential display using a fluorescently labelled universal primer. *BioTechniques*, **23**: 274 - 279.

Snyman, S.J., Meyer, G.M., Carson, D.L. and Botha, F.C. 1996 Establishment of embryogenic callus and transient gene expression gene expression in selected sugarcane varieties. *South African Journal of Botany*, **62**: 151 - 154.

Sokolov, B. and Prockop, D.J. 1994 A rapid and simple PCR-based method for isolation of cDNAs from differentially expressed genes. *Nucleic Acids Research*, **22**: 4009 - 4015.

Sompayrac, L., Jane, S., Burn, T.C., Tenen, D.G. and Danna, K.J. 1995 Overcoming limitations of the mRNA differential display technique. *Nucleic Acids Research*, **23** : 4738 - 4739.

Somssich, I.E. 1994 Regulatory elements governing pathogenesis-related (PR) gene expression. In *Plant promoters and transcription factors*. Nover, L. (Ed.), ISBN 0-387-57288-0, pp. 163 - 179. Springer-Verlag, Switzerland.

Straub, P.F., Shen, Q. and Ho, T-H.D. 1994 Structure and promoter analysis of an ABA- and stress-regulated barley gene, *HVA1*. *Plant Molecular Biology*, **26**: 617 - 630.

Sturtevant, A.P. and Beachy, R.N. 1993 Virus resistance in transgenic plants: coat protein mediated resistance. In *Transgenic plants: fundamentals and applications*. A.Hiatt (Ed.), ISBN0-8247-8766-8, pp. 93 -114. Marcel Dekker, New York.

Sugaya, S., Hayakawa, K., Handa, T. and Uchimiya, H. 1989 Cell-specific expression of the *rolC* gene of the TL-DNA of Ri plasmid in transgenic tobacco plants. *Plant Cell Physiology*, **30**: 649 - 653.

Sugaya, S. and Uchimiya, H. 1992 Deletion analysis of the 5' upstream region of the *Agrobacterium rhizogenes* Ri plasmid *rolC* gene required for tissue-specific expression. *Plant Physiology*, **99**: 464 - 467.

Sullivan, M.L. and Green, P.J. 1993 Post-transcriptional regulation of nuclear-encoded genes in higher plants: the roles of mRNA stability and translation. *Plant Molecular Biology*, **23**: 1091-1104.

Sun, S.S.M., Maretzki, A., Nagai, C., Houtchens, K. and Bidney, D. 1993 Transformation of *Saccharum spontaneum* by particle bombardment. *Sugar Cane*, **5**: 1 - 7.

Svab, Z., Hajdukiewicz, P. and Maliga, P. 1990 Stable transformation of plastids of higher plants. *National Academy of Science. Proceedings,* **87**: 8526 - 8530.

Taylor, P., Ko, H., Adkins, S., Rathus, C. and Birch, R. 1992 Establishment of embryogenic callus and high protoplast yielding suspension cultures of sugarcane (*Saccharum* spp. hybrids). *Plant Cell, Tissue and Organ Culture*, **28**: 69 - 78.

Terzaghi, W.B. and Cashmore, A.R. 1995 Light-regulated transcription. *Annual review of Plant Physiology and Plant Molecular Biology*, **46**: 445 - 474.

Thangue, N.B.L. and Rigby, P.W.J. 1988 Trans-acting protein factors and the regulation of eukaryotic transcription. In *Transcription and splicing*. Hames, B.D. and Glover, D.M. (Eds.), ISBN1-85221-076-1, pp. 1 - 30. IRL Press, Oxford.

Theodorou, M.E. and Plaxton, W.C. 1992 Metabolic adaptations of plant respiration to nutritional phosphate deprivation. *Plant Physiology*, **101**: 334 - 339.

Tian, L., Séguin, A. and Charest, P.J. 1997 Expression of the green fluorescent protein in conifer tissues. *Plant Cell Reports*, **16**: 267 - 271.

Tobin, E.M. and Silverthorne, J. 1985 Light regulation of gene expression in higher plants. *Annual Review of Plant Physiology*, **36**: 569 - 593.

Todd, J., Blakeley, S.D. and Dennis, D.T. 1995 Structure of the genes encoding the alpha- and beta-subunits of castor pyrophosphate-dependent phosphofructokinase. *Gene*, **152**: 181 - 186.

Tomes, D.T., Weissinger, A.K., Ross, M.R., Higgins, R., Drummond, B.J., Schaaf, S., Malone-Schoneberg, J., Staebell, M., Flynn, P., Anderson, J. and Howard, J. 1990 Transgenic tobacco plants and their progeny derived by microprojectile bombardment of tobacco leaves. *Plant Molecular Biology*, **14**: 261 - 268.

Vain, P., McMullen, M.D. and Finer, J.J. 1993a Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports*, **12**: 84 - 88.

Vain, P., Keen, N., Murillo, J., Rathus, C., Nemes, C. and Finer, J.J. 1993b Development of the particle inflow gun. *Plant Cell, Tissue and Organ Culture*, **33**: 237 - 246.

Van de Rhee, M.D., Lemmers, R. and Bol, J.F. 1993 Analysis of regulatory elements involved in stress-induced and organ-specific expression of tobacco acidic and basic β-1,3-glucanase genes. *Plant Molecular Biology*, **21**: 451 - 461.

Van de Rhee, M.D., Lindhorst, H.J.M. and Bol, J.F. 1994 Pathogen-induced gene expression. In *Stress-induced gene expression in plants*. Basra, A.S. (Ed.), ISBN 3-7186-5466-0, pp. 249 - 284. Harwood Academic Publishers, Switzerland.

Van de Löcht, U., Meier, I., Hahlbrock, K. and Somssich, I.E. 1990 A 125 bp promoter fragment is sufficient for strong elicitor-mediated gene activation in parsley. *EMBO Journal*, **9**: 2945 - 2950.

Van der Geest, A.H.M. and Hall, T.C. 1996 A 68 bp element of the β-phaseolin promoter functions as a seed-specific enhancer. *Plant Molecular Biology*, **32**: 579 - 588.

Van Dillewijn, C. 1952 Growth: general, grand period, growth formulae. In *Botany of sugarcane*. Vol. 1. Van Dillewijn, C. (Ed.), pp. 97 - 162. Veenen and Zonen, The Netherlands.

Van Haute, E., Joos, H., Maes, M., Warren, G., van Montagu, M. and Schell, J. 1983 Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. *EMBO Journal*, **2**: 411 - 415.

Van Loon, L.C. 1990 The nomenclature of pathogenesis-related proteins. *Physiological and Molecular Plant Pathology*, **37**: 229 - 230.

Vasil, V., Brown, S.M., Re, D., Fromm, M.E. and Vasil, I.K. 1991 Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. *Biotechnology*, **9**: 743 - 747.

Velten, J. and Schell, J. 1985 Selection-expression plasmid vectors for use in genetic transformation of higher plants. *Nucleic Acids Research*, **13**: 6981 - 6987.

Vierling, E. and Kimpel, J.A. 1992 Plant responses to environmental stress. *Current Opinion in Biotechnology*, **3**: 164 - 170.

Vilaine, F., Charbonnier, C. and Casse-Delbart, F. 1987 Further insight concerning the TL region of the Ri plasmid of *Agrobacterium rhizogenes* strain A4: Transfer of a 1.9 kb fragment is sufficient to induce transformed roots on tobacco leaf fragments. *Molecular and General Genetics*, **210**: 111 - 115.

Vilardell , J., Goday, A., Freire, M.A., Torrent, M., Martinez, M.C., Torné, J.M. and Pagès, M. 1990 Gene sequence, developmental expression and protein phosphorylation of *RAB-17* in maize. *Plant Molecular Biology*, **14**: 423 - 432.

Vögeli-Lange, R., Fründt, C., Hart, C.M., Nagy, F. and Meins, F. 1994 Developmental, hormonal, and pathogenesis-related regulation of the tobacco class I β-1,3-glucanase B promoter. *Plant Molecular Biology*, **25**: 299 - 311.

Walden, R., Koncz, C. and Schell, J. 1990 The use of gene vectors in plant molecular biology. *Methods in Molecular and Cellular Biology*, **1**: 175 - 182.

Walden, R. and Schell, J. 1990 Techniques in plant molecular biology - progress and problems. *European Journal of Biochemistry*, **192**: 563 - 576.

Walden, R. and Wingender, R. 1995 Gene transfer and plant-regeneration techniques. *Trends in Biotechnology*, **13**: 324 - 331.

Waldron, C., Murphy, E.B., Roberts, J.L., Gustafson, G.D., Armour, S.L. and Malcolm, S.K. 1985 Resistance to hygromycin B - a new marker for plant transformation studies. *Plant Molecular Biology*, **5**: 103 - 108.

Wang, X. and Feuerstein, G.Z. 1995 Direct sequencing of DNA isolated from mRNA differential display. *BioTechniques*, **18**: 448 - 453.

Wang, S. and Hazelrigg, T. 1994 Implications for *bcd* mRNA localisation from spatial distribution of *exu* protein in *Drosophila* oogenesis. *Nature*, **369**: 400 - 402.

Wang, Y-C., Klein, T.M., Fromm, M., Cao, J., Sanford, J.C. and Wu, R. 1988 Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. *Plant Molecular Biology*, **11**: 433 - 439.

Warner, S.A.J., Scott, R. and Draper, J. 1993 Isolation of an asparagus intracellular PR gene (AoPR1) wound-responsive promoter by the inverse PCR and its characterisation in transgenic tobacco. *The Plant Journal*, **3**: 191 - 201.

Watson, J.B. and Margulies, J.E. 1993 Differential cDNA screening strategies to identify novel stage-specific proteins in the developing mammalian brain. *Developments in Neuroscience*, **15**: 77 - 86.

Webb, K.J. and Morris, P. 1992 Methodologies of plant transformation. In *Plant genetic manipulation for crop protection*. Gatehouse, A.M.R., Hilder, V.A. and Boulter, D. (Eds.), ISBN0-85198-707-9, pp. 7 - 44. C.A.B. International, Inc., U.K.

Welsh, J.,Chada, K., Dalal, S.S., Cheng, R., Ralph, D. and McClelland, M. 1992 Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Research*, **20**: 4965 - 4970.

White, F.F. and Nester, E.W. 1980 Hairy root: Plasmid encodes virulence traits in *Agrobacterium rhizogenes*. *Journal of Bacteriology*, **141**: 1134 - 1141.

Whitelaw, C.A., Le Huquet, J.A., Thurman, D.A. and Tomsett, A.B. 1997 The isolation and characterisation of type II metallothionein-like genes from tomato (*Lycopersicon esculatum L.*). *Plant Molecular Biology*, **33**: 503 - 511.

Whittaker, A. and Botha, F.C. 1996 Relationship of PFP expression in sugarcane to fiber and sucrose accumulation. *The XXII Congress of the International Society of Sugarcane Technologists. Proceedings,* pp. 403 - 405.

Williamson, J.D. and Quatrano, R.S. 1988 ABA-regulation of two classes of embryo-specific sequences in mature wheat embryos. *Plant Physiology*, **86**: 208 -215.

Wingender, R., Röhrig, H., Höricke, C. and Schell, J. 1990 *cis*-Regulatory elements involved in ultraviolet light regulation and plant defense. *The Plant Cell*, **2**: 1019 - 1026.

Wolfraim, L.A., Langis, R., Tyson, H. and Dhindsa, R.S. 1993 cDNA sequence, expression and transcript stability of a cold acclimation-specific gene, *cas 18*, of Alfalfa (*Medicago falcata*) cells. *Plant Physiology*, **101**: 1275 - 1282.

Wong, J.H., Kang, T. and Buchanan, B.B. 1988 A novel pyrophosphate-6-P 1-phosphotransferase from carrot roots. Relation to PFK from the same source. *FEBS Letters*, **238**: 405 - 410.

Wong, J-H., Kiss, F., Wu, M-X. and Buchanan, B.B. 1990 Pyrophosphate fructose 6-phosphotransferase from tomato fruit. Evidence for change during ripening. *Plant Physiology*, **94**: 499 - 506.

Yamaguchi-Shinozaki, K., Mundy, J. and Chua, N-H. 1989 Four tightly linked *rab* genes are differentially expressed in rice. *Plant Molecular Biology*, **14**: 29 - 39.

Yamaguchi-Shinozaki, K., Koizumi, M., Urrao, S. and Shinozaki, K. 1992 Molecular cloning and characterization of 9 cDNAs for gene that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. *Plant and Cell Physiology*, **33**: 217 - 224.

Yan, T-F.J. and Tao, M. 1984 Multiple forms of pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase from wheat seedlings. Regulation by fructose 2,6-bisphosphate. *Journal of Biological Chemistry*, **259**: 5087 - 5092.

Yang, N-S. and Russell, D. 1990 Maize sucrose synthase-1 promoter directs phloem cell-specific expression of GUS gene in transgenic tobacco plants. *National Academy of Science. Proceedings*, **87**: 4144 - 4148.

Yang, F., Moss, L.G. and Phillips, G.N. 1996 The molecular structure of green fluorescent protein. *Nature Biotechnology*, **14**: 1246 - 1251.

Yokoyama, R., Hirose, T., Fujii, N., Aspuria, E.T., Kato, A. and Uchimiya, H. 1994 The *rolC* promoter of *Agrobacterium rhizogenes* Ri plasmid is activated by sucrose in transgenic tobacco plants. *Molecular and General Genetics*, **244**: 15 - 22.

Yoshida, H. and Kato, A. 1994 Cold-induced accumulation of RNAs and cloning of cDNAs related to chilling injury in rice. *Breeding Science*, **44**: 361 - 365.

Youvan, D.C. and Michel-Beyerle, M.E. 1996 Structure and fluorescence mechanism of GFP. *Nature Biotechnology*, **14**: 1219 - 1220.

Zebrowski, R., Thorman, J., Norred, T. and Hurst, R.E. 1994 Isolation and identification of rare and differentially expressed genes using subtractive hybridisation. *Analytical Biochemistry*, **222**: 285 - 287.

Zhang, W., McElroy, D. and Wu, R. 1991 Analysis of rice *Act1* 5' region activity in transgenic rice plants. *The Plant Cell*, **3**: 1155 - 1165.

Zhao, S., Ooi, S.L. and Pardee, A.B. 1995 New primer strategy improves precision of differential display. *BioTechniques*, **18**: 842 - 850.

Zimmermann, J. and Schultz, R.M. 1994 Analysis of gene expression in preimplantation mouse embryo: use of mRNA differential display. *National Academy of Science. Proceedings*, **91**: 5456 - 5460.

Zupan, J.R. and Zambryski, P. 1995 Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiology*, **107**: 1041 - 1047.

CURRICULUM VITAE

Sarita Groenewald (neé Greeff) was born on 19 November 1968 in Johannesburg.

1986 - Matriculated at Hoërskool Riebeeck, Randfontein.

1989 - Obtained a Bachelor of Science degree with distinction, at Potchefstroom University for Christian Higher Education (PU for CHE), Potchefstroom, with majors in Biochemistry and Microbiology.

1990 - Obtained a Bachelor of Science (Honours) degree in Biochemistry, with distinction, at PU for CHE.

1993 - Obtained a Master of Science degree in Biochemistry with specialisation in Biotechnology, with distinction, at PU for CHE.
Title of thesis: Cloning and expression of the human genes for GM-CSF and G-CSF.

1994 - Enrolled for a PhD in Plant Molecular Biology at the University of Natal, Pietermaritzburg. Experimental work was carried out in the Biotechnology Department at the South African Sugar Association Experiment Station, Mount Edgecombe.

1994 - Became the first recipient of the FRD Aaron Klug PhD scholarship for molecular biology.

1995 - Married Hennie Groenewald.

APPENDIX I

Nucleotide sequence of the first five exons of the PFP-β gene from sugarcane. The exons are highlighted and the introns are given in lower case. In introns 3 and 4 positions where sequences are still lacking are indicated with //.

```
ATGGCGGCGCCGAGCGGACCATCACCTGGGACTGGGAGGTTGGCGTCGGTTTACAGCGAGGTGCA
GACGAGCCGCCTCCATCACGCGTTCCGGCTCCCCTCCGTCCTCTGCTCCCAATTCTCCCTCGTCG
ATGGACCTCCCAGCTCAGCCACGGGGAACCCGGgtaacgaagacctgcatgcgtcccttggcctt
gatcttgtctcatttccttggtttcttcgtccaggttctttttggccttacggttggttttgatc
tgatccccgccagATGAGATCGCGAAGCTGTTTCCGCTAACTTGTTTGGCAGCCGTCGGCGACA
TTGGTGCCGGCCAAAGAGGCGGTGGAGGGCAAGGCGCTGAAGGTCGGGGTGGTGCTCTCTGGTGG
ACAAGCACCCGGTGGGCACAATGTGATCTGCGGTATCTTCGgtgaggtctctttgatccatttat
agaatcaatgttttcagaattgcaagcaacagtggcagtatggtgtcaccatttccaggtgtcga
tttaaaagtggttggataatataggattgttcaattgcatgtttcccgtttattaattaacatat
ttatctggagatttctacctttttgtcacagacatagtagtacatctcgtggagtaatctgggca
gctggactggggatcgaatttggtgacaacatttagatttggaactaatattggctaataagca
atgagctgctaccattgattagtggagttatttaaaagtagcttattaagatgaagtgcacagct
atttagtatctctcgctaatgcatctgccattttagATTTCTTGCAGAAACACGCAAACGGCAA
CACAATGTATGGATTCAAAGCAGCCCCAGCAGGGCTGATGAAGTGCAAGTACGTGAAACTGAAT
CCGATTTCGTCTATCCCTACAGAAACCAG//
gtgacctccaattggagagacnaatttcttcctcggttgggaagtgtaataatttgaaatgagcc
caagtagaaacccttctctttatccccataGGTGGATTGATACATCGTAGTGGAAGGGATAAGAT
TGAAACACCAGAGCAGgtacgtccctttatctcccaagtttagcctacctaagcgtgttatatac
atcttattccacagacgtaagcatcctatcaactatggctagaatacttccccttgtggggtcat
ccaggttaagcaagccgaagatacagccaacaaacttgagattggaacggacttgttgttagtt
gtgatggcgacgattccaaatatctcatgcttgcctttattgctgtataccttcaggttggttgc
ctggcagagttgcaaatcaccatttctgctgccatcgaaaatgcttctttgttcacttttgttat
gaaataagctctacttaggcttaaaaaataataagatatggtatttttctaaacaatgccatct
tgaattattactgtgataataatgtttattcgtttataactaatggaagaagttacatatgcatg
ctatagggacgcacgatcattgctgattttatgcacgtgtgtaatggggattcggttggtttc
tcacaaattaatcttagtaaaaatgtgaccccatgcttaatcaagtggcatttcttgatgccgt
atagagtgtggaagattgacggatttggcggacatgagcgcggagtacgaaaaatcncc //
caataggggattganagatnggaaggnaaganggnacaccagagcaggtagtccccttntctcc
caagtttngctacataagcttgctaaatacatcttattccntatacttaagcacttctcgaccat
ggctaatatacttcccttgcgggtcatccagTTTAAGCAAGCCGAAGATACAGCCAACAAACTTG
AGTTGTTAGTTGTGATGGCGACGATTCAAATATCTCATGCTTGCCTTTATTGCTGA
TACTtcaggttggttgcttgcagantgcaaatcaccattactgctccatcgaaaatgctctttgt
tcactgttatgaaataagctctanttaggnnaaataataagatatggtatttttctaaacaatg
ccatcttgaattattatgtgataataatgttattgtttataacaatggnagaagttacatatgct
agaatcaatcattgctgattctaatggggattctgtgcttcatcacanataaatttagtaaaaat
gtgacccatgcttaatnagaggcatttctttgtagggggaaagaatctgaagactcgtgttattg
gngtcccaagacaattgatggagatcgagtcgacgccctatagtgagtcgccttagagctcgcgg
ccgcagtcctctagatcgaccngnagcagcaacaagcna
```