

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600



Order Number 9230472

**Isolation of a sugarcane gene promoter and its application to
chimeric gene expression in sugarcane protoplasts**

Barry, Kelly, Ph.D.

University of Hawaii, 1992

U·M·I

300 N. Zeeb Rd.
Ann Arbor, MI 48106



**ISOLATION OF A SUGARCANE GENE
PROMOTER AND ITS APPLICATION TO CHIMERIC GENE
EXPRESSION IN SUGARCANE PROTOPLASTS**

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BOTANICAL SCIENCES (PLANT PHYSIOLOGY)

MAY 1992

By

Kelly Barry

Dissertation Committee:

John Stiles, Chairperson
Michael Harrington
Richard Manshardt
Andrew Maretzki
Paul Moore

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. John Stiles for helpful advice and guidance during the course of developing this thesis.

I am extremely grateful to Dr. Don Heinz and the Hawaiian Sugar Planters' Association for financial support of this thesis.

I would like to thank Dr. Paul Moore of the United States Department of Agriculture for his encouragement and the generous use of research facilities

I am grateful to Dr. William Peacock for allowing me to use the pEmu plasmid in these experiments.

I would also like to thank my husband, William Barry, for his constant support, understanding attitude, and patience.

ABSTRACT

The promoter region of a sugarcane gene has been isolated and characterized from suspension culture cells of *Saccharum ssp.* hybrid H50-7209. The transcription initiation site was determined by primer extension and physical characteristics such as a 5' transcript splice site, a TATA element, and several upstream repetitive sequences were inferred by sequence analysis.

The sugarcane promoter element was isolated by PCR amplification and fused to the GUS reporter gene coding sequence. This chimeric gene construct was transferred to sugarcane protoplasts by electroporation and transient gene expression was monitored by the GUS fluorimetric assay.

The sugarcane promoter was approximately five times more effective than the CaMV 35S promoter at driving heterologous gene expression in sugarcane protoplasts.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	x
CHAPTER 1. LITERATURE REVIEW	1
TRANSFORMATION SYSTEMS	2
Agrobacterium-Mediated Transformation	2
Direct Gene Transfer	5
Electroporation	5
Electroinjection	6
Fusion to Liposomes and Spheroplasts	7
Microinjection	7
Agroinfection	7
Injection into Floral Tillers	8
Microprojectiles	8
DNA viruses	9
Sonication	9
SELECTABLE MARKERS	10
REPORTER GENES	15

PROMOTERS USED IN CHIMERIC GENE CONSTRUCTION ...	19
HYPOTHESIS	31
CHAPTER 2. METHODS	32
TISSUE CULTURE MAINTENANCE	32
ETHANOL PRECIPITATION OF NUCLEIC ACIDS	32
LARGE SCALE PLASMID DNA ISOLATION.....	33
SMALL-SCALE PLASMID DNA ISOLATION	34
SUGARCANE GENOMIC DNA ISOLATION.....	34
BACTERIOPHAGE DNA ISOLATION	35
SUGARCANE RNA ISOLATION	36
GEL ELECTROPHORESIS.....	37
BLUNT-END LIGATION.....	39
PROBE SYNTHESIS.....	39
SUGARCANE GENOMIC LIBRARY CONSTRUCTION	41
SOUTHERN HYBRIDIZATION.....	44
NORTHERN HYBRIDIZATION	45
DNA SEQUENCING	46
RIBONUCLEASE PROTECTION ASSAY.....	47
PRIMER EXTENSION	48
PROTOPLAST FORMATION AND ELECTROPORATION.....	48
GUS FLUORESCENCE ASSAY.....	49
PCR AMPLIFICATION OF PLASMID DNA	50
CHAPTER 3. RESULTS	51
SUSPENSION CELL SENSITIVITY TO HYGROMYCIN AND G418.....	51

ISOLATION OF CLONES FOR ABUNDANT TRANSCRIPTS	51
Plasmid Genomic Library Construction	51
Construction of Genomic Library in EMBL4.....	53
SUBCLONING.....	57
DETERMINATION OF TRANSCRIPTION START SITE.....	61
CONSTRUCTION OF EXPRESSION VECTORS	69
Promoterless GUS Construct.....	69
Sugarcane Promoter Construct.....	70
High Expression Construct pEmuGN.....	71
GUS EXPRESSION ASSAYS	74
CHAPTER 4. DISCUSSION.....	76
ANTIBIOTIC RESISTANCE	76
EXPRESSION	76
ANALYSIS OF SUGARCANE PROMOTER.....	77
ANALYSIS OF PROMOTER FUNCTION	83
POTENTIAL FOR IMPROVEMENT.....	84
REFERENCES	87

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Optimization of Electroporation Conditions.....	74
2. Mean GUS Activities Following Transient Expression in Sugarcane Protoplasts	75
3. <i>Cis</i> -Acting Elements Reported in Plant Promoters	77

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Antibiotic Sensitivity.....	52
2. Northern Probed with λ 1, p1-2, and p1-4.....	54
3. Southern of λ Clones Probed with λ 1.....	55
4. Restriction Map of λ 7 and Subclones.....	56
5. Southern of λ 7 Probed with cDNA.....	58
6. RNA Slot Blot Probed with S5, S1, and p12.....	59
7. Northern Probed with S5.....	60
8. Riboprobes for RNase Protection Assay.....	63
9. RNase Protection Assay with Probes I and D.....	64
10. Primers for Sequencing and PCR.....	65
11. p9 Insert Sequence.....	66
12. RNase Protection Assay with Probes K,L, and M.....	67
13. Primer Extension Analysis.....	68
14. Map of GUS Gene in pBI221.....	69
15. Southern of pSGN and pBI221 Probed with Gel-purified SC.....	72
16. Reporter Gene Constructs Used.....	73
17. Comparison of TATA and Start Sites.....	80
18. Relationship of λ 7, p9 and Promoter Elements.....	82

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
ARE	Anaerobic Response Element
ATP	Adenosine Triphosphate
bis	<i>N,N'</i> -methylenebisacrylamide
bp	base pair
BSA	bovine serum albumin
CaMV	Cauliflower Mosaic Virus
CAT	3 chloramphenicol acetyl transferase
cDNA	complementary DNA
cpm	counts per minute
CsCl	cesium chloride
CTP	Cytidine triphosphate
ddNTP	dideoxynucleotide triphosphate
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DPAM2	4-dichlorophenoxyacetate monooxygenase
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
EPSPs	5-enolpyruvylshikimate-3-phosphate synthase
GS	glutamine synthetase

GTP	Guanosine triphosphate
GUS	β -glucuronidase
kb	kilobase
MOPS	3-[N-Morpholino]propane-sulfonic acid
mRNA	messenger RNA
MUG	2 mM 4-methylumbelliferyl β -D glucuronide
NOS	nopaline synthase
NPTII	aminoglycoside phosphotransferase (3') Type II
nt	nucleotide
OCS	octopine synthase
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
poly(A)	poly-adenylated
PPT	Phosphinothricin
PR1a	pathogenesis related protein 1a
PSII	photosystem II
pvp	polyvinyl-pyrrolidone (MW 40,000)
Q _B	Quinone binding protein
RNA	ribonucleic acid
RNAse	ribonuclease
RPA	RNase Protection Assay
RUBISCO	ribulose bisphosphate carboxylase
SC	sugarcane promoter

SDS	sodium dodecyl sulfate
SV40	Simian virus 40
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
Tris	tris[hydroxymethyl]aminomethane
UAS	upstream activating sequence
UTP	Uridine triphosphate

CHAPTER 1. LITERATURE REVIEW

Sugarcane, *Saccharum ssp.* hybrids, is a major crop throughout the tropical and subtropical world, including Hawaii. Because of its economic importance, means of improving sugarcane productivity are being pursued. Traditional breeding programs are actively producing improved sugarcane cultivars, however there are limitations to this approach. Cultivar development represents an extremely time-consuming and laborious procedure. Additionally, traits introduced by traditional breeding must be available in a plant that can successfully cross with sugarcane and numerous undesired traits will be introduced which must be removed by backcrossing and selection. Direct gene transfer offers a new and promising approach for more efficient introduction of foreign genes into an already desired background.

An essential procedure needed to apply genetic engineering to sugarcane is transformation. Transformation is the process by which DNA, typically a plasmid, is taken up by a cell. Once inside the cell, the DNA either integrates into the host genome giving rise to stable transformation or remains extrachromosomal resulting in transient expression.

With regard to basic research, transformation facilitates the study of gene regulation using *in vitro* methods. This includes the identification of sequences involved in photoregulation (Schindler and Cashmore, 1990; Dehesh et al., 1990; Bruce and Quail, 1990), defense-induced gene expression (Hauffe et al., 1991; Lawton et al., 1991), tissue-specific gene expression (Takaiwa et al., 1991; Bustos et al., 1991; and Aryan et al., 1991), and promoter strength (Maas et al., 1990; Maas et

al., 1991). From a practical point of view, transformation accelerates the enhancement of crop productivity by the introduction of specific genes that impart new and beneficial traits such as improved nutritional qualities (De Clercq et al., 1990; Altenbach et al., 1989; Vandekerckhove et al., 1989; and Hoffman et al., 1987) improved postharvest characteristics (Oeller et al., 1991), herbicide resistance (Chaleff and Ray, 1984; Comai et al., 1985; Shah et al., 1986; De Block et al., 1987;), insect resistance (Vaeck et al., 1987; Fischhoff et al., 1987; Delannay et al., 1989; Vaeck et al., 1989; Perlak et al., 1990), and virus resistance (Powell-Abel et al., 1986; Tumer et al., 1987; Loesch-Fries et al., 1987; Van Dun et al., 1987; Cuzzo et al., 1988; Hemenway et al., 1988; Kaniewski et al., 1990; Van Dun et al., 1988; Van Dun and Bol, 1988; Baulcombe et al., 1986; Harrison et al., 1987; Gerlach et al., 1987).

While significant progress has been made in gene transfer technology, a major limitation has been the lack of promoter diversity for gene regulation, especially in monocots. There is a need for promoters providing constitutive expression, tissue and developmental specificity, and varying degrees of promoter strength.

TRANSFORMATION SYSTEMS

Agrobacterium-mediated Transformation

By far the most widely used and best understood transformation system for plants is *Agrobacterium*-mediated transformation. *Agrobacterium tumefaciens* is a soil bacterial phytopathogen that is a natural system for genetic transfer. Plants infected

with *A. tumefaciens* produce a tumorous growth called a crown gall at the site of infection. Infected plant cells produce opines which are specifically catabolized by the bacteria, and an excess of auxin and cytokinin which causes tumor formation. Transformation requires that the *Agrobacterium* carry an approximately 200 kb extrachromosomal plasmid called *Ti*, for tumor-inducing. A portion of the *Ti* plasmid carries the genes for tumorous growth, opine synthesis, and opine catabolism by the *Agrobacterium* (Van Larebeke et al., 1974; Watson et al., 1975).

Two regions of the *Ti* plasmid are essential for transformation, the T-DNA region and the virulence, or *vir* region. The T-DNA region is approximately 25 kb in size and flanked by 25 bp directly repeated sequences (Yadav et al., 1982; Zambryski et al., 1982) that are the recognition sites for an endonuclease encoded by the *virD* gene of the *vir* region (Yanofsky et al., 1986). Transformation by *Agrobacterium* results in integration of the T-DNA into the host plant's genome. The T-DNA region also contains the genes for phytohormone synthesis (Schroder et al., 1984; Akiyoshi et al., 1984; Barry et al., 1984) and opine synthesis (Otten et al., 1978; Salomon et al., 1984). However, any DNA sequences placed between the T-DNA borders will be transferred to the plant genome and herein lies the basis of plant transformation by means of *Agrobacterium*. The other necessary component for T-DNA transfer is the *vir* region of the *Ti* plasmid. The *vir* region is about 35 kb in size and has at least six genes (Klee et al., 1983). While the *vir* genes are essential for T-DNA transfer, they can act *in trans* (Hoekema et al., 1983).

Plant transformation vectors based on *Agrobacterium* are of two types, cointegrative and binary. With the cointegrative vector system there is homology between the vector and the *Ti* plasmid which allows for integration of the vector within the T-DNA region by recombination (Zambryski et al., 1983; Fraley et al.,

1985; Matzke and Matzke, 1986). The binary vector system does not require recombination. The vector plasmid contains a broad host range origin of replication which allows for autonomous replication of the vector in *Agrobacterium*. The vector contains T-DNA borders flanking the sequence of interest while the *vir* genes are provided *in trans* by a helper *Ti* plasmid (Horsch and Klee, 1986; Koncz and Schell, 1986; Bevan, 1984).

For several dicotyledonous plants such as tobacco and petunia, transformation can occur through the cocultivation of protoplasts with *A. tumefaciens* (Horsch et al., 1984; Marton et al., 1979). However, the lack of an efficient protoplast regeneration protocol renders this transformation system useless for many important plant species. Frequently, in these cases a more applicable procedure is leaf disk transformation, where surface sterilized explants are incubated with *Agrobacterium* (Horsch et al., 1985). This procedure can be used for species that respond to *Agrobacterium* infection and that also produce plants readily from tissue explants.

The major drawback of *Agrobacterium*-mediated transformation is the limited host range of the bacteria. The host range is naturally limited to dicotyledons and reports of *Ti*-mediated transformation of monocotyledons are rare. Tumor formation has been demonstrated for the monocot *Asparagus officinalis* (Hernalsteens et al., 1984). Opine synthesis in infected wound sites was observed in *Chlorophytum capense*, *Narcissus*, and *Zea mays* (Hooykaas-van Slogteren et al., 1984; Graves and Goldman, 1986), however, the presence of transforming DNA in the plant genomes was not demonstrated. The only verified examples of *Agrobacterium*-mediated transformation of monocots are asparagus (Bytebier et al., 1987), yam (*Dioscorea*

bulbifera) (Schafer et al., 1987), rice (Raineri et al., 1990), and maize (Gould et al., 1991).

Monocots, including sugarcane, constitute many of the most important crop plants and thus are desirable targets for plant biotechnology. Consequently, other transformation systems have been investigated as alternatives to *Agrobacterium*-mediated transformation.

Direct Gene Transfer

Direct gene transfer is the process by which plant protoplasts take up naked DNA. This system was first used in plants when petunia protoplasts were transformed by isolated *Ti* plasmid DNA (Davey et al., 1980). The frequency of transformation was improved by the incorporation of PEG and a heat shock treatment (Shillito et al., 1985). PEG-mediated gene transfer has been used to transform several graminaceous monocots, including wheat (Lorz et al., 1985), ryegrass (Potrykus et al., 1985), sugarcane (Chen et al., 1987), rice (Uchimiya et al., 1986), and maize (Armstrong et al., 1990).

PEG-mediated transformation and subsequent plant regeneration have led to the successful recovery of transgenic plants from a number of plant types. These include tobacco (Meyer et al., 1985; Uchimiya et al., 1986; Negrutiu et al., 1987), orchard grass (Horn et al., 1988), moth bean (Koehler et al., 1987a) and rice (Datta et al., 1990; Hayashimoto et al., 1990).

Electroporation

Electroporation is a procedure in which protoplasts are subjected to a high voltage electrical pulse that reversibly permeabilizes the cell membranes and allows

for DNA uptake. The first successful transformation by electroporation was reported by Neumann et al. (1982), who transformed mammalian cells. The electroporation of plant protoplasts was first reported by Fromm et al. (1985), who electroporated carrot, tobacco and maize protoplasts. Since then, transient expression in plant protoplasts has been reported in rice, wheat, sorghum (Ou-Lee et al., 1986; Oard et al., 1989), soybean, petunia, napier grass, *Panicum maximum*, sugarcane (Hauptmann et al., 1987), sugar beet (Lindsey and Jones, 1987), white spruce (Bekkaoui et al., 1988), moth bean (Koehler et al., 1987b), *Alnus incana* (Seguin and Lalonde, 1988), black spruce, jack pine (Tautorus et al., 1989), tomato (Tsukada et al., 1989), and potato (Jones et al., 1989).

Stable transformation of a plant host genome was first demonstrated in tobacco (Shillito et al., 1985). Transgenic callus has been obtained in tobacco (Shillito et al. 1985), maize (Fromm et al., 1986; Huang and Dennis, 1989), soy bean (Christou et al., 1987), sugar beet (Lindsey and Jones, 1989), and moth bean (Koehler et al., 1987b). Transgenic plants have been obtained from tobacco (Paszkowski et al., 1984; Riggs and Bates, 1986), *Brassica napus* (Guerche et al., 1987), orchard grass (Horn et al., 1988), maize (Rhodes et al., 1988), moth bean (Koehler et al., 1987b), lettuce (Chupeau et al., 1989), rice (Toriyama et al., 1988; Shimamoto et al., 1989; Matsuki et al., 1989; Toriyama et al., 1988; Zhang et al., 1988) and *Lycopersicon peruvianum* (Bellini et al., 1989).

Electroinjection

A relatively new approach is electroinjection, whereby nucleic acids are introduced into intact plant cells by means of electric pulses. This technique has been used to introduce tobacco mosaic virus RNA into intact tobacco mesophyll cells

(Morikawa et al., 1986). Lindsey and Jones (1990) used the CAT assay system to demonstrate DNA transfer across untreated sugarbeet cell walls.

Fusion to Liposomes and Spheroplasts

Plasmid DNA may also be introduced into cereal protoplasts by means of liposomes (artificial lipid vesicles), and bacterial spheroplasts containing plasmid DNA. Tobacco protoplasts were transformed by means of liposomes (Deshayes et al., 1985) while rice protoplasts were transformed by *Agrobacterium* spheroplasts (Baba et al., 1986).

Microinjection

Microinjection of nucleic acid into the nuclei of plant protoplasts is a potentially efficient means of gene transfer. Morikawa and Yamada (1985) injected DNA into the nuclei of tobacco while Reich et al (1986) transformed alfalfa protoplasts using fluorescent dyes to visualize the nuclei. Toyoda et al. (1990) observed transient expression of the GUS gene following microinjection of barley coleoptile cells. Although microinjection requires expertise and sophisticated equipment, it is attractive because its potentially high transformation rate makes it possible to screen transformants in the absence of selectable markers. It is also possible to transform intact plant cells via microinjection as demonstrated in tomato (Toyoda et al., 1985) and tobacco (Nims, 1967).

Agroinfection

Agroinfection is the term used to describe transformation with a plant virus that is introduced via the *Ti* plasmid. Tandemly repeated copies of the viral genome

are placed between the T-DNA borders. Upon inoculation with the *Agrobacterium*, a copy of the viral genome excises and spreads systemically throughout the plant. Agroinfection has been used to introduce gemini maize streak virus into maize (Grimsley et al., 1987). Whole maize plants developed symptoms of viral infection, however the virus was not seed transmissible.

Injection into Floral Tillers

De la Pena et al (1987) obtained transgenic rye plants after injecting DNA into floral tillers. They postulate that the DNA is transported to the germ cells which are capable of incorporating the DNA when the cells are in a competent stage of development.

Microprojectiles

Klein et al. (1987) successfully introduced nucleic acids into intact cells of onion by "shooting" tungsten microprojectiles coated with DNA or RNA into the cells. This approach to transformation does not require cell culture or pretreatment of the tissue in any way and only small quantities of nucleic acid are needed. The technique is rapid so many samples can be tested in a short period of time. This approach has been used for the transient transformation of intact plant cells and tissues from onion (Klein et al., 1987), maize (Klein et al., 1988a; 1988b), tobacco (Klein et al., 1988c), and rice, wheat, soybean (Wang et al., 1988; Lonsdale et al., 1990). Microprojectile bombardment has been used for the stable transformation of tobacco (Klein et al., 1988c), soybean (McCabe et al., 1988; Christou et al., 1988) maize (Klein et al., 1989; Spencer et al., 1990) papaya (Fitch et al., 1990), and sugarcane (Maretzki et al., 1990). Fertile transformed plants have been obtained from maize

(Gordon-Kamm et al., 1990; Fromm et al., 1990) and soybean (Christou et al., 1990),.

DNA Viruses

It may be possible to use DNA viruses as transformation vectors. A foreign gene can be transported into and systemically spread within a plant infected with a genetically engineered Cauliflower Mosaic Virus (Brisson et al., 1984). The disadvantages of this system are the narrow host range, no transfer to sexual offspring, and limited space for cloned DNA in the virus.

Sonication

Joersbo and Brunstedt (1990) described the use of mild sonication as a method for transformation of sugarbeet and tobacco protoplasts.

A variety of potential transformation methods are thus available. *Agrobacterium*-mediated transformation may become a reality for grasses and other plants not readily transformed by *Agrobacterium*. Direct gene transfer to protoplasts is routine and efficient. As methods of protoplast regeneration are established, PEG-mediated transformation and electroporation may represent standard methods for the production of transgenic plants. Microprojectile bombardment is a popular and successful method of transformation due to the ability to transform intact plant cells and its relative simplicity. Microinjection is a promising approach to transformation which needs to be further developed.

SELECTABLE MARKERS

Most transformation vectors contain a selectable marker for plant transformation. A selectable marker is a gene whose expression in a transformed cell allows for growth in an environment which is hostile to nontransformed cells. The most widely used markers are antibiotic and herbicide resistance genes. Under ideal conditions all plants that are regenerated in the presence of the selective agent are transformed.

There are several reasons for having a variety of selectable markers. In any transformation system, only a small proportion of plant cells are transformed and it is crucial to have a marker to select transformed cells and suppress growth of wild type cells. Additionally, since not all markers work well in all plant species, when attempting to transform a new plant it is advantageous to evaluate several alternative markers. A third reason for a variety of selectable markers is the ability to introduce multiple genes into a single plant using a different marker for each introduction.

There are several considerations for the development of a useful selectable marker. It is desirable that the compound inhibit cell growth of nontransformed cells rather than kill them. Rapid death of nontransformed cells releases toxic substances into the media that are detrimental to the transformed cells. A desirable trait is to have high-level resistance of transformants with no "escapes" of nontransformed cells. The selectable agent should result in no side effects or interfere with the ability to regenerate plants. Also, a simple and inexpensive assay for rapid verification of expression of the marker in a regenerated plant is a useful feature.

Several antibiotic resistance genes have been used as selectable markers in plants. The most widely used selectable marker is the enzyme aminoglycoside phosphotransferase (3') Type II (NPTII) carried by transposon *Tn5* and originally

isolated by Beck et al. (1982). The enzyme inactivates the aminoglycoside antibiotics of the neomycin family (G418, neomycin, kanamycin) by phosphorylation. Chimeric constructs of NPTII have been incorporated into a large number of plant transformation vectors and have been used successfully to transform many different plant species (An et al., 1985; Klee et al., 1985; Matzke and Matzke, 1986). Vectors containing the kanamycin marker have been used to transform rice (Rainerie et al., 1990) and grapevines (Mullins et al., 1990) by *Agrobacterium* methods. Ishige et al. (1991) transformed several varieties of potato by *Agrobacterium* transformation.

The high natural resistance to kanamycin shown by cultured cells of most grasses has limited the use of this antibiotic for selecting transformed cells (Hauptmann et al., 1988). To avoid false positives, Rainerie et al. (1990) stressed that kanamycin selection must be applied immediately and that the microcalli must be continually dissected to ensure that all parts of the explant tissue remain in contact with selective levels of the antibiotic at all times.

Another useful selectable marker is the bacterial hygromycin phosphotransferase gene which confers resistance to hygromycin (Gritz and Davies, 1983; Van den Elzen et al., 1985; Waldron et al., 1985) by transferring a phosphate from ATP to the antibiotic (Leboul and Davies, 1982). Hauptmann et al. (1988) evaluated hygromycin, kanamycin, and methotrexate as markers for the isolation of stable transformants of graminaceous plants by electroporation. All lines were highly resistant to kanamycin and varied in sensitivity to hygromycin. Hygromycin selection has been used for the successful stable transformation of rice (Datta et al., 1990; Shimamoto et al., 1989) and maize (Huang and Dennis, 1989).

Streptomycin resistance is conferred by the bacterial gene streptomycin phosphotransferase from the transposon *Tn5*. Unlike most other drugs, streptomycin

does not kill plant cells. Sensitive cells respond by bleaching and retarded growth but do not die (Jones et al., 1987).

Bleomycin and phleomycin are glycopeptides which interact with DNA, resulting in single and double stranded breaks. A resistance gene has been isolated from the transposon *Tn5* (Genilloud et al. 1984). Recently a second gene was isolated from the chromosomal DNA of *Streptoalloteichus hindustanus* and referred to as *Sh ble* (Drocourt et al., 1990). A comparison of the two resistance genes in transformed tobacco indicates that phleomycin selection is preferable to that of bleomycin (Perez et al., 1989).

Bromoxynil (3,5-dibromo-4-hydroxybenzoxynitrile) is an inhibitor of PSII and acts by binding a component of the quinone-binding protein complex, inhibiting electron transfer. A resistance gene (*bxn*) was isolated from *Klebsiella ozaenae* and placed under control of a light-regulated tissue-specific promoter, the ribulose biphosphate carboxylase (RUBISCO) small subunit. This chimeric gene was transformed into tobacco and its expression resulted in resistance to high levels of bromoxynil in progeny plants (Stalker et al., 1988).

Gentamicin resistance occurs when the bacterial enzyme aminoglycoside-3-N-acetyltransferase inactivates certain aminoglycoside compounds by acetylation. Gentamicin selection has been used successfully for transformation of petunia, tobacco, tomato, *Arabidopsis* and *Brassica napus* (Hayford et al., 1988).

Sulfonamides are anti-bacterial compounds which act as inhibitors of dihydropteroate synthase (DHPS), an enzyme of the folic acid synthesis pathway. The resistance gene *sulI* encodes a modified DHPS which is insensitive to inhibition by sulfonamides. Guerineau et al. (1990) used a chimeric *sulI* gene regulated by the CaMV 35S promoter to select for transformed tobacco.

Herbicide resistance is generally accomplished by overexpression of the target enzyme or expression of a mutant form of the enzyme which is less sensitive to the herbicide. Several herbicides have been employed for selection and some appear to be more useful than others.

Resistance to the auxin-like hormone 2,4-D was demonstrated by Streber and Willmitzer (1989). They transformed tobacco with a chimeric form of the *tfdA* gene product from *Alcaligenes eutrophus*. The gene codes for 2,4-dichlorophenoxyacetate monooxygenase (DPAM), the first enzyme involved in a 2,4-D degradative pathway. The DPAM gene could not be used as a selectable marker in leaf disc transformation due to a lack of shoot development. The authors suggest that transformed cells cannot develop into shoots because they are overgrown or inhibited by untransformed cells that are rapidly developing in the presence of 2,4-D. Additionally, it was not possible to select transgenic plants during germination on 2,4-D due to a lack of resistance of roots against 2,4-D.

Atrazine is an herbicide which blocks electron transport through association with Q_B of PSII and could potentially be used for transgenic selection. The coding region of the *psbA* gene from atrazine-resistant *Amaranthus hybridus* was fused to the RUBISCO small subunit promoter and transit peptide and used to transform tobacco by *Agrobacterium* methods (Cheung et al., 1988). Actual selection was based on kanamycin, however resistance to atrazine was demonstrated. Because transgenic plants have resistant as well as sensitive PSII reaction centers, photosynthesis is reduced and plants may not survive on continuously high concentrations of atrazine.

Methotrexate is an inhibitor of dihydrofolate reductase (DHFR) and is extremely toxic. It is a folate analogue that binds tightly to the catalytic site of DHFR, blocking the synthesis of glycine, purines, and thymidine. Simonsen and

Levinson (1983) developed a selection scheme using a mutant mouse enzyme that has 260-fold reduced affinity for methotrexate. This altered gene was used as a dominant selectable marker in cultured hamster and mouse cells and it has been used successfully in petunia (Eichholtz et al., 1987), tobacco, *Brassica*, and several grasses (Hauptmann et al., 1988).

Glyphosate interferes with aromatic amino acid biosynthesis by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs). Sensitive cells starve for aromatic amino acids and accumulate shikimate. Tolerance to this compound can be mediated by the presence of an altered enzyme or by overproduction of EPSPs. Comai et al. (1985) expressed a mutant allele of *aroA* from *Salmonella typhimurium* which had a decreased affinity for the enzyme in transgenic tobacco. Shah et al. (1986) obtained glyphosate resistance by overexpression of a chimeric EPSPs gene in transformed petunia and Klee et al. (1987) repeated the work in *Arabidopsis*.

The sulfonylurea herbicides are a relatively new class of chemicals that are effective at very low doses. The target for the herbicide is the enzyme acetolactate synthase (ALS), the first common enzyme in the biosynthesis of branched chain amino acids, valine, leucine, and isoleucine. Mutant forms of ALS have been identified in yeast, bacteria, and plants. Chaleff and Ray (1984) isolated mutant forms from tobacco which were less sensitive to the herbicide. Lee et al. (1988) transferred the mutant tobacco ALS gene to highly sensitive tobacco lines and found that the transgenic plants were resistant to the herbicide application. Haughn et al. (1988) isolated a mutant ALS gene from a resistant line of *Arabidopsis* and used it to transform tobacco. Actual selection was based on kanamycin resistance.

Phosphinothricin (PPT) and Bialaphos are potent herbicides. PPT is an inhibitor of glutamine synthetase (GS) in plants and bacteria. This enzyme plays a

central role in regulation of nitrogen metabolism and it is the only enzyme in plants that can detoxify ammonia. Bialaphos consists of PPT, an analogue of glutamic acid and two alanine residues. The intact tripeptide has little or no inhibitory activity, however, removal of the alanines by intracellular peptidases forms the active PPT. A resistance gene has been characterized and cloned from *Streptomyces hygrosopicus*. The *bar* gene product is phosphinothricin acetyltransferase (PAT), which acetylates the free ammonia of PPT and prevents autotoxicity. De Block et al. (1987; 1989) engineered herbicide resistance in tobacco, tomato, potato, and *Brassica* by expressing a chimeric form of the *bar* gene. Spencer et al. (1990) described the use of a chimeric *bar* gene to produce stable transformation of nonembryogenic Black Mexican Sweet (BMS) maize suspension cells.

REPORTER GENES

A reporter gene has no effect on the growth of transformed versus nontransformed cells, however, its expression imparts an obvious phenotype which allows for identification of transformed cells. Reporter genes have several uses in plant transformation studies. They are useful for establishing and optimizing transformation conditions and they represent an independent means of identifying transformed tissue. However, the primary function of reporter genes has been the analysis of gene promoter function. The *cis*-acting elements of a promoter are joined to a reporter gene that codes for a novel enzymatic activity and the amount of enzymatic activity is used as a measure of the ability of the promoter elements to regulate transcription.

A good reporter gene will have several features. The activity encoded by the reporter gene must be distinguishable from any similar activities present in the cells prior to transformation. There should be no interference from other enzymatic activities in the cells. The enzyme assay should be rapid, sensitive, reproducible, and convenient. Although not essential, a desired feature is the ability of the reporter gene to tolerate amino-terminal fusions.

A reporter gene consisting of the *E. coli lacZ* coding region fused to the nopaline synthase regulatory elements was used in transformation studies of sunflower and tobacco cells (Helmer et al., 1984). The *lacZ* gene encodes β -galactosidase. While the *lacZ* tolerates variations in the length of the amino-terminal segment, endogenous plant galactosidases make assaying for transformation difficult.

The *Ti* plasmid genes for opine synthase have been used as reporter genes. Bevan et al. (1983) demonstrated the expression of nopaline synthase in transformed tobacco while De Greve et al. (1983) assayed for octopine synthase activity. Opine synthesis has been used as evidence of *Ti*-mediated transformation of *Chlorophytum capense*, *Narcissus*, and *Zea mays* (Hooykaas-van Slogteren et al., 1984; Graves and Goldman, 1986). These reporter genes are not often used today due to the difficulty in assay quantitation, the inability to localize enzyme activity, and the high frequency of false positives.

The NPTII gene has been used successfully as a reporter gene in plants. Gene activity was demonstrated by phosphorylation of kanamycin in the presence of [γ -³²P]ATP and tobacco tumor extract (Herrera-Estrella et al., 1983; Bevan and Flavell, 1983) as well as petunia and tobacco cells (Fraley et al., 1983). While this reporter gene is able to accommodate amino-terminal fusions, the enzyme assay is somewhat tedious.

The bacterial gene chloramphenicol acetyl transferase (CAT) was derived from the transposable element *Tn9* and confers resistance to chloramphenicol (Alton & Vapnek, 1979). Fromm et al. (1985; 1986) measured the efficiency of electroporation in plant cells by constructing a chimeric gene consisting of the CaMV 35S promoter fused to the CAT gene and the nopaline synthase (NOS) terminator. The CAT gene has also been used for the analysis of the photoregulation of an oat phytochrome promoter in transgenic rice (Bruce et al., 1989; Bruce and Quail, 1990), deletion analysis of a RUBISCO small subunit promoter (Rolfe and Tobin, 1991), the effects of the first exon and intron of the maize *Shrunken-1* locus on gene expression (Vasil et al., 1989; Maas et al., 1991), and the transient expression of a chloroplast transformation vector in tobacco cells (Daniell et al., 1990).

The luciferase gene from the firefly, *Photinus pyralis*, is a useful reporter gene for eukaryotic gene expression studies. The luciferase gene cDNA was cloned and fused to the CaMV 35S promoter and the NOS termination signals (Ow et al., 1986). This construct was used for the transient expression in electroporated carrot protoplasts and stable transformation of tobacco. Luciferase is a potentially useful reporter due to its commercial availability and sensitive assay. However, the luciferase does not tolerate amino-terminal fusions.

The β -glucuronidase (GUS) reporter gene system was developed by Jefferson et al. (1986) as a gene fusion marker for analysis of gene expression in transformed plants. GUS is encoded by the *uidA* locus of *E. coli* and is a hydrolase which catalyzes the cleavage of many β -glucuronides. This has become a widely used reporter gene due to the enzyme's extreme stability, the tolerance of amino-terminal additions, the availability of several sensitive and quantitative assay procedures, and commercial availability of the reporter constructs.

The GUS reporter gene system has been used extensively in plant studies. GUS has been used as a reporter gene for the analysis of foreign gene expression, as seen by the organ-specific and substrate-induced expression of the rice glutamine synthetase in transformed tobacco (Kozaki et al., 1991). In the area of crop protection, a chalcone synthase promoter-GUS reporter gene fusion was used for screening chemical inducers of plant defenses and natural signal molecules involved in stress responses (Doerner et al., 1990). GUS was used as a marker for *Agrobacterium*-mediated transformation of *Rubus* due to difficulties with regeneration following NPTII selection (Graham et al., 1990). GUS has been used for the identification of efficient promoters for gene expression, as demonstrated by McElroy et al. (1990) who determined that the rice actin promoter is more efficient than the maize *Adh1* and CaMV 35S promoter. The most common use for GUS reporter genes has been the functional analysis of *cis*-elements of gene promoters. Examples of this include the pathogenesis-related 1a promoter of tobacco (Bielmann et al., 1991), the promoter for parsley 4-coumarate:coenzyme A ligase (4CL) (Hauffe et al., 1991), and the French bean β -phaseolin gene promoter (Bustos et al., 1989).

One of the main reasons for the popularity of GUS is the reported lack of detectable glucuronidase activity in higher plants. Jefferson et al. (1987) used the sensitive fluorometric assay to test roots, stems and leaves from wheat, tobacco, tomato, potato, *Brassica napus* and *Arabidopsis thaliana*, potato tubers, and seeds from wheat and tobacco for intrinsic GUS activity. In all these tissues the endogenous GUS activity was below the limits of detection. However, Hu et al. (1990) examined fifty-two plant species and found that intrinsic GUS activity is common among seed plants. The GUS activity is mainly expressed during fruit and seed development and infrequently in the vegetative organs. The intrinsic GUS-like

activities in seeds diminished during germination and the ELISA test was used to detect true GUS activity since the endogenous GUS protein(s) is not antigenically similar to *E. coli* GUS enzyme.

A reporter gene whose expression results in anthocyanin pigmentation has been used in the transformation of maize by microprojectile bombardment (Ludwig et al., 1990). In maize, pigmentation is controlled by members of the *R* gene family. This reporter gene consists of the transcription unit from one of these genes (*Lc*) fused to a constitutive promoter. This reporter has advantages over other reporter genes because visualization of *R* expression does not require a biochemical assay or histochemical staining. Additionally, cell-autonomous expression is observed and may be done so without disturbing the integrity of the plant. A disadvantage of this system is false negatives in tissues which suppress the anthocyanin pathway, as observed for the maize endosperm. Another consideration is the possible interference of anthocyanin pigmentation with cell divisions and regeneration.

PROMOTERS USED IN CHIMERIC GENE CONSTRUCTION

In order for a gene to be expressed in a plant it must have regulatory elements which are recognized by the plant cell. The promoter controls the transcription of a gene and constitutes the first level of gene regulation. The promoter for RNA polymerase II consists of a number of sequence elements required for accurate and efficient initiation of transcription. The TATA box is the basic primary structural unit of the promoter while other less conserved elements such as the CAAT box and the GC box may also be included.

The TATA box was first noticed by Goldberg and Hogness (Goldberg, 1979) when searching for a counterpart to the prokaryotic Pribnow box involved in prokaryotic transcription initiation. In plants, the TATA box is an AT-rich region located 25-40 bp upstream of the transcription initiation site and has the consensus sequence 5'-TCACTATATATAG-3' (Joshi, 1987). The TATA box is required for accurate transcription initiation and functions to fix the location of the start site.

The frequency of initiation is strongly influenced by the sequence elements farther upstream, such as the CAAT box and the GC box. The CAAT box was one of the first promoter elements to be described (Efstratiadis et al., 1980). It is a less strongly conserved element and has the consensus sequence 5'-GGCCAATCT-3'. The CAAT box is located 40 to 110 bp upstream of the start site and appears important in promoting efficient transcription initiation but plays no role in determining its accuracy. The GC-rich sequence has a function similar to the CAAT box. It has a consensus sequence of 5'-CCGCCC-3' or its complement 5'-GGGCGG-3'. This element may occur in one or more copies (McKnight, 1982; Everett et al., 1983) and may be present in addition to a CAAT box as the so-called -100 element (Dierks et al., 1983).

For operational purposes, it is useful to define the promoter as a sequence or sequences of DNA that must be in a relatively fixed location with regard to the start point of transcription. By definition, this includes the TATA box, the CAAT box, and the GC box. However, promoters do not necessarily function alone. The activity of a promoter can be increased by the presence of an enhancer. Enhancers are *cis*-acting DNA elements which are regulatory in function and strongly stimulate the transcription of adjacent genes. Typically, enhancers can produce their effects regardless of their orientation and can stimulate any promoter placed in their vicinity.

Although enhancers preferentially activate proximal promoter elements, they can, with decreased efficiency, act over considerable distances and can even function in a downstream position.

The first enhancer discovered was a 72 bp tandem repeat located more than 100 bp upstream of the early SV40 genes (Benoist and Chambon, 1981; Gruss et al., 1981). Since then, enhancers have been discovered in DNA tumor viruses (Laimins et al., 1984) and a variety of other sources (Boshart et al., 1985; Gillies et al., 1983; Queen and Baltimore, 1983; Morelli et al., 1985).

Response elements are enhancer-like elements consisting of short DNA sequences that have been associated with the tissue-specific expression of genes or with gene expression induced by specific stimuli such as hormones, heat shock, heavy metals, or viral infection. Response elements are recognized by factors that coordinate the transcription of particular groups of genes. They contain short consensus sequences that can be recognized in the appropriate promoters. The elements are not present at fixed distance from the start point, but are usually within 200 bp upstream of the start site. The presence of a single element is usually sufficient to confer the regulatory response, but there may be multiple copies.

Promoter consensus sequences were derived for several types of plant genes by Elliston and Messing (1989) based on available sequence information. These include the storage protein genes, light-induced genes, and stress-induced genes.

The general structure of the monocot storage protein gene promoters is 5'-RRA₂₋₃GAA₂₋₄TG-8-A₂₋₃GA₂₋₅-12-YCTATAAATA-3' (Elliston and Messing, 1989). This is the result of analyzing five zein sequences, two gliadin genes, two glutelin genes, and one hordein gene. The dicot seed storage protein gene promoters conserve a promoter consensus similar to the monocot's consensus. While no discrete

consensus exists, fairly long stretches of poly(A) or poly(T) occur upstream of the TATA consensus, with an interspersed cytosine or guanine. This is based on the analysis of 16 dicot storage protein gene sequences. Another conserved region consisting mainly of C and A residues, the CACA box, has also been recognized in several gliadin and hordein gene promoters (Reeves and Okita, 1987). This CACA box was located at -196 bp in a gliadin gene and was found to enhance the transcriptional regulation of this gene (Aryan et al., 1991). Takaiwa et al. (1991) reported the presence of four direct repeats between position -540 and -192 in a rice glutelin gene. These repeats share homology to the consensus sequence TG(T/A/C)AAA(G/A)(G/T) which was shown to be essential for endosperm-specific expression of prolamine genes in transformed tobacco (Colot et al., 1987; Maier et al., 1987). Shirsat et al. (1989) reported that this consensus sequence may act as an enhancer-like element in determining the level of expression of the pea legumin gene.

Light regulation involving a GT motif described by Kuhlemeier et al. (1987) occurs between -168 and -110 in a pea *rbcS* gene. The consensus sequence 5'-RTGTGGYYA(T/A)TA(T/A)G-3' was derived from pea, soybean, petunia, and tobacco *rbcS* genes (Elliston and Messing, 1989). This sequence is present in *rbcS* sequences, but is not present in other published light-induced gene sequences.

A feature of the light-inducible promoters of dicots is the TATA box and an upstream AG or AGGA-like box. The TATA box takes the form of CATTATATACNRTTT, with the internal TTATATATAC being very highly conserved. The upstream AG box has a rather variable form and is represented by the consensus AGGA₁₋₃TAAGRA₁₋₄NCT₁₋₃. This sequence ends six to ten bp before the beginning of the TATA sequence (Elliston and Messing, 1989). This consensus was derived from the analysis of eight light-induced genes from pea, petunia, and tobacco.

Schaffner and Sheen (1991) determined that the maize *rbcS* genes are regulated by elements distinct from the elements of dicot *rbcS* genes. Mutagenesis of the element GGCCACT at -92 to -97 effected light expression but not dark expression while changes in the element AACGGT at -97 to -104 affected both light and dark expression.

Stress-induced genes such as the heat-shock protein genes and the wound-induced genes share the common feature of induction by some form of physiological stress. The promoter regions of four soybean heat-shock genes show no striking sequence motif, with only a generalized poly(T) region about 30 bp upstream of the TATA box and separated from it by a fairly pyrimidine-rich region (Elliston and Messing, 1989). Three short sequences with the consensus GGTAA(A/T)(A/T)(A/T) were found in the region -326 to -173 of the bean chalcone synthase promoter (Lawton et al., 1991). These sequences function as a transcriptional silencer and may have a key function in determining the organ-specific pattern of promoter activity.

Because most selectable marker and reporter genes are prokaryotic in origin, it has been necessary to fuse the prokaryotic coding region to a promoter derived from a plant gene, plant viral gene, or T-DNA genes. Commonly used regulatory signals are derived from the nopaline synthase (NOS) gene of the *Ti* plasmid (De Block et al., 1984; Herrera-Estrella et al., 1983) and the 35S and 19S promoters from the CaMV (Paszkowski et al., 1984).

The 35S promoter of CaMV is the most frequently used promoter in plant transformation procedures. CaMV, a virus of dicotyledonous plants, has a circular double-stranded DNA genome of about 8 kb. Two distinct promoters have been identified which produce two polyadenylated transcripts (Guilley et al., 1982). The

35S (8.2 kb) transcript is slightly larger than the full-length genome while the second transcript is 19S (1.9 kb) and has the same 3' end as the 35S transcript.

The 35S promoter consists of two domains. Correct initiation of transcription from the 35S promoter is dependent on proximal sequences (0 to -90) that include a TATA element (Odell et al., 1985). The rate of transcription is determined by the upstream region of the 35S promoter (-395 to -86). Deletion of viral sequences upstream of -90 resulted in 1/10 the activity of the full 35S promoter (Odell et al., 1985; Maas et al., 1991). Kay et al. (1987) raised the efficiency of transcription of the 35S promoter by duplication of the transcription-activating sequences (-343 to -90). The upstream sequence caused increased expression when present in both orientations and at a distance, a behavior characteristic of an enhancer.

Benfey et al., (1989) analyzed expression conferred by two domains from the 35S promoter. Expression from domain A (-90 to +8) was strongest in root tissue or tissue destined to become root. Expression from domain B (-343 to -90) was strongest in the cotyledons of seeds and seedlings and in the leaves and stem of mature plants. When both domains were present, expression was detectable in most tissues at all stages of development.

The CaMV 35S promoter is 10- to 40-fold more effective than the NOS promoter in driving the expression of foreign genes introduced into plant cells (Fromm et al., 1985; Hauptmann et al., 1987). Although the CaMV 35S promoter has been widely used as an efficient promoter for high-level constitutive expression of foreign genes in dicotyledonous plants (Kay et al., 1987; Jefferson et al., 1987), this promoter has low activity in monocotyledonous plants. The CaMV 35S promoter has been shown to be 10- to 100-fold less effective in grass cells than in dicotyledonous species (Fromm et al., 1985; Hauptmann et al., 1987). Furthermore, a recent report

suggests that the pattern of 35S promoter activity may not be constitutive (Benfey and Chua, 1989).

In attempts to obtain efficient expression of introduced genes in monocot cells, other promoter elements have been examined. Hauptmann et al. (1987) examined the expression of introduced genes in grass cells, using promoters of the *Alcohol dehydrogenase-1 (Adh1)*, *Alcohol dehydrogenase-2 (Adh2)* and *Shrunken-1 (Sh1)* genes of maize. The *Adh2* and *Sh1* promoters gave no detectable expression in grass protoplasts, while the levels of expression obtained with the *Adh1* promoter was approximately 30% of the CaMV 35S promoter. However, Zhang and Wu (1988) observed that the maize *Adh1* promoter is 10 to 20 times more active than the 35S promoter in transformed rice protoplasts and calli. Disadvantages of the maize *Adh1* promoter is its requirement for induction by anaerobic stress and its lack of constitutive expression in all tissues.

The maize *Adh1* promoter gave very low expression in protoplasts of tobacco. However, the species-specificity of this promoter could be changed by the addition of an octopine synthase (OCS) enhancer from *A. tumefaciens* (Ellis et al., 1987). The first 247 bp upstream of the translation initiation codon of the *Adh1* gene was sufficient to impose anaerobic regulation and accurate initiation of transcription in tobacco. When the same construct was introduced into maize protoplasts, expression was high regardless of whether or not the protoplasts were anaerobically induced (Peacock et al., 1987).

It has been shown in mammalian systems (Gasser et al., 1982; Hamer and Leder, 1979) and in SV40 (Gruss and Houry, 1980) that gene expression may be increased by the presence of an intron. The importance of introns in plant gene expression was first demonstrated by Callis et al. (1987). Expression of the CAT

coding region was increased tenfold by the inclusion of the maize *Adh1* intron 1 in the *Adh1* promoter. Intron 1 stimulated activity only when present in the transcription unit. Additionally, the stimulatory effect was not specific to the *Adh1* promoter. The intron 1 fragment stimulated expression in 35S and NOS promoter genes. CAT activity was stimulated by the maize *Bronze-1 (Bz1)* intron, however it was much less effective than the *Adh1* intron.

Vasil et al. (1989) used transient assays of various grass protoplasts to show that the first intron of the maize *Sh1* greatly increased expression of a reporter gene when transcription was driven by the CaMV 35S promoter. There was up to 91-fold increase in expression when this intron was incorporated in the promoter. The increase was 10-fold greater than that caused by the first intron of the *Adh1* gene of maize.

Maas et al. (1991) described two regulatory elements in the untranslated leader sequence of the maize *Sh1* gene which, in combination, mediated up to a 1000-fold increase in CAT expression in maize and rice protoplasts. The *Sh1* exon 1 and intron 1 sequences were individually or in combination inserted between the 35S transcription start site and the CAT coding sequence. Workers observed a tenfold increase with the insertion of the *Sh1* exon 1 and a 100-fold increase when intron 1 was inserted at the same position. However, if both elements were in front of CAT the activity was up to 1000-fold higher, indicating that the positive effects of each element may be multiplicative rather than additive.

McElroy et al. (1990) isolated the promoter from the rice *Act1* gene which encodes a relatively abundant transcript in all rice tissues and at all developmental stages. The 5' region was fused to GUS and used in transient expression experiments on rice protoplasts. The regulatory elements necessary for maximal *Act1* promoter

activity in transformed rice protoplasts were located within a region 1.3 kb upstream of the *Act1* translation initiation codon. This consisted of 0.83 kb of 5'-flanking sequence, the noncoding exon, and the 5' intron. The 5' intron was essential for efficient gene expression from this promoter. The rice *Act1* promoter was approximately 5 to 10 times more active than the maize *Adh1* promoter in transformed rice cells.

It can be concluded from these experiments that the expression of a foreign gene can be affected by the presence of an intact intron sequence. However, there are genes that do not contain introns but are capable of producing gene products in the absence of splicing. Included in these are a soybean seed storage protein (Vodkin et al., 1983) and the zein storage proteins of maize endosperm (Pederson et al., 1982). Additionally, the expression of some intron-containing genes are not affected by the removal of introns (Chee et al., 1986).

Based on the knowledge available regarding plant promoters, Last et al. (1991) set out to construct a promoter giving high level expression in monocots protoplasts. A series of promoters was constructed based on either a truncated maize *Adh1* promoter (Δ ADH) (region -100 to +106) or a truncated 35S promoter (Δ 35S) (region -90 to +3).

The rationale for construction of the highest expressing promoter was as follows. It has been demonstrated that replacing the single Anaerobic Response Element (ARE), an enhancer of the maize *Adh1* promoter, with additional copies of the enhancer gave an increase in expression under anaerobic conditions (Olive et al., 1990). The 6ARE element consists of one ARE in the natural orientation preceded by five AREs in the reverse orientation. It was previously shown that the addition of the OCS enhancer to the maize *Adh1* promoter caused strong anaerobically inducible

expression in tobacco and in maize protoplasts the expression was high regardless of whether the protoplasts were anaerobically induced (Ellis et al., 1987). Since the 6ARE element is a stronger element than the single ARE, they included the 4OCS element, assuming it might be required to confer high level aerobic expression. Because the maize *Adh1* intron 1 has been shown to increase expression, a fragment containing this intron was included between the promoter and the GUS coding sequence.

In monocot suspension cell protoplasts the most highly expressing construct was based on the truncated *Adh1* promoter. The *Adh1* constructs gave little or no expression in tobacco, however a construct consisting of the truncated 35S promoter and the 4OCS element increased expression in tobacco.

High level gene expression should be useful in cases in which a potentially selectable gene is normally expressed at suboptimal levels. Of equal importance is the construction of promoters that can confer specific gene expression in the transformants, such as constitutive, inducible, stage-specific, or tissue-specific expression. Through techniques such as promoter deletion analysis, several promoter regulatory elements have been determined.

Spena et al. (1985) constructed a chimeric gene of the NPTII coding region under control of the *hsp70* promoter from *Drosophila*. A 457 bp fragment from the *hsp70* gene containing the promoter and 199 nt of untranslated leader sequence was fused to the coding region of NPTII. The DNA sequence responsible for heat induction was mapped to a region of DNA extending 97 bp upstream of the initiation of transcription (Dudler and Travers, 1984). Transformation into tobacco resulted in expression in a heat-regulated fashion.

A stress-induced promoter was isolated and expressed by Enomoto et al. (1990). They fused the 5' flanking 2.4 kb fragment from the tobacco PR1a gene to the GUS reporter gene and introduced this chimeric gene into lettuce by *Agrobacterium*-mediated gene transfer. The chimeric gene was induced by salicylic acid, a normal inducer of the PR1a gene.

Promoter deletion analysis of two tomato genes that are coordinately expressed during pollen maturation revealed the minimal region required for developmentally regulated gene expression in pollen (Twell et al., 1991). The 200 bp proximal promoter contained two upstream activator regions and a 19 bp segment from one of those regions enhanced expression of the 35S promoter in pollen.

Correct light-regulated and leaf-specific expression was observed in transgenic tobacco and petunia (Nagy et al., 1985) following transformation with a 2.4 kb genomic fragment of the gene for pea *rbcS*-E9. This gene codes for the small subunit of ribulose-1,5-biphosphate carboxylase. This expression pattern was preserved in a 5' deletion mutant which had only 352 bp sequence upstream from the transcription start site. Light-regulated and organ-specific expression in transgenic plants has also been obtained with a wheat nuclear gene encoding the chlorophyll a/b-binding protein (Lamppa et al., 1985).

A great deal of research has been aimed at determining regulatory constraints on the expression of plant seed storage proteins. Seed storage proteins are synthesized during seed maturation and are stored in dry seeds as the nitrogen and carbon source for germinating seedlings. Their expression is under temporal and tissue-specific control.

Bustos et al. (1989) showed that the bean storage protein phaseolin 5'-flanking sequence (-795 to +20) was sufficient to confer correct spatial and temporal

regulation upon the GUS gene in transgenic tobacco plants. Similarly-regulated expression of reporter genes was demonstrated for upstream sequences from soybean β -conglycinin (Chen et al., 1988), corn zein (Schernthaner et al., 1988), barley β -hordein (Marris et al., 1988), and Faba bean legumin (Baumlein et al., 1991). Bustos et al. (1991) later showed that the β -phaseolin upstream (-795 to +20) sequence had at least five regulatory domains that activated or decreased GUS expression. Spatial information was specified primarily by two upstream activating sequences (UAS1 and UAS2). UAS1 activity was restricted to the cotyledons and shoot meristem while the UAS2 domain extended gene activity to the hypocotyl. Temporal control involved two negative regulatory sequences (NRS1 and NRS2) as well as the positive domain UAS1. The deletion of either negative element caused premature onset of GUS expression.

The 5' upstream region of the rice storage protein type II glutelin gene was examined for its regulatory function in transgenic tobacco (Takaiwa et al., 1991). Various 5' flanking regions of the glutelin gene were fused to the GUS reporter gene and introduced into tobacco by *Agrobacterium*-mediated gene transfer. A region required for the temporal and tissue-specific expression was located between positions -441 and -237. However, proper developmental regulation and low GUS activity was directed by the -237 and -100 constructs. The presence of four direct repeats with enhancer characteristics between -540 and -192 offered the explanation that the sequence between positions -441 and -237 may be involved in the quantitative regulation of the promoter rather than the developmental specificity.

HYPOTHESIS

Genetic transformation is becoming well established and routine in many plant species, including monocots. As the technology progresses, the need for different promoters imparting a variety of expression patterns is becoming more pronounced.

Sugarcane suspension cultures consist of suspension cells which are actively dividing and display no particular tissue specificities. Consequently, one may assume that many of the genes being transcribed in suspension culture are constitutively or developmentally expressed and not tissue specific. The isolation of a promoter element from such a gene may prove useful for the expression of chimeric genes in sugarcane. Because the promoter element is derived from an actively transcribed suspension culture gene, it should contain all the regulatory elements necessary for efficient expression in sugarcane suspension cells.

The purpose of this research is to test the hypothesis that a sugarcane promoter fragment can be isolated from a sugarcane suspension cell genomic library and used to effectively direct the expression of a heterologous reporter gene in sugarcane suspension cells.

CHAPTER 2. METHODS

All methods were performed according to Sambrook et al. (1989) unless otherwise indicated.

TISSUE CULTURE MAINTENANCE

A suspension culture of sugarcane cultivar H50-7209 (Nickel, 1964) was used. The cells were grown in 100 ml of YE medium (White's medium, pH 5.8 supplemented with 50 mM sucrose, 0.1% (w/v) yeast extract, 0.4 mM arginine, and 9 μ M 2,4-D) according to Nickell and Marezki (1969). At 14-day intervals, 9 ml of suspension were transferred to fresh medium and the cultures were grown on a gyratory shaker at room temperature under standard fluorescent lights.

Plates were prepared with YE medium and 1.5% agar. Filter sterilized solutions of G418 and Hygromycin B were added to the medium immediately before pouring the plates. Cultures were grown on plates at room temperature for two to four weeks.

ETHANOL PRECIPITATION OF NUCLEIC ACIDS

Ethanol precipitation of nucleic acids is commonly employed for purification, concentration, and buffer exchange. Ethanol precipitation of DNA was performed by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes ice cold 95% ethanol. The DNA was placed at -20°C for at least 4 hours or -70°C for at least 30 minutes. The DNA was collected by a 20 to 30 minute centrifugation at 12,000g at 4°C. The DNA was washed with 70% ice cold ethanol, air dried, then resuspended in the

desired solution. RNA was precipitated by the same manner except that 2.2 volumes of 95% ethanol were added instead of 2.5 volumes.

LARGE SCALE PLASMID DNA ISOLATION

Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (1979) with modifications. Five hundred milliliters of LB media (1% tryptone, 0.5% yeast extract, 0.5% NaCl) were inoculated with 5 ml of an overnight bacterial culture and grown overnight at 37°C with shaking. Cells were harvested by centrifugation at 10,000g for 10 minutes then resuspended in 18 ml of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA and 2 ml of 10 mg/ml lysozyme. Forty ml of 0.2 N NaOH and 1% SDS were added and the samples were incubated on ice for 10 minutes. Twenty ml of 5 M potassium acetate were added and the samples were kept on ice for 10 minutes. Following centrifugation at 6300g for 15 minutes, the supernatant was poured through 3 layers of cheesecloth and 0.6 volumes of isopropanol were added. The samples were centrifuged for 15 minutes at 4400g. The DNA pellets were air dried and resuspended in 5 ml of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The resuspended DNA was mixed with 8.6 grams of cesium chloride and 200 μ l of 10 mg/ml ethidium bromide, and the volume was adjusted to 10.0 ml with TE. The samples were centrifuged for 18 hours at 25°C in the Beckman Ti70.1 rotor at 55,000 rpm. The plasmid DNA band was removed with an 18 gauge needle. Ethidium bromide was removed by extraction with isoamyl alcohol. The DNA was dialyzed at least 4 hours against TE. Ethanol precipitation and recovery of the DNA was performed as above.

SMALL-SCALE PLASMID DNA ISOLATION

A 10 ml culture of the plasmid culture was grown overnight at 37°C and 1.5 ml of this was centrifuged at 12,000g for 5 minutes. The pellet was resuspended in 100 μ l of TE. Two hundred μ l of 0.2 NaOH containing 1% SDS were gently added and the sample was left on ice for 10 minutes. After the addition of 150 μ l of 5 M potassium acetate, the sample was centrifuged at 12,000g for 10 minutes. The supernatant was transferred to a new tube and 2 μ l of 2 mg/ml RNase were added. The RNase digestion took place at 37°C for 30 minutes, then the RNase and other proteins were removed by two extractions with an equal volume of phenol saturated with 0.1 M Tris-HCl, pH 8.0. The DNA was precipitated by the addition of 1 ml ethanol, incubation for 5 minutes at room temperature followed by centrifugation for 10 minutes at 12,000g. The DNA was washed with 70% ethanol, air dried, and resuspended in 50 μ l TE.

SUGARCANE GENOMIC DNA ISOLATION

High molecular weight genomic DNA was isolated from 7-day old suspension cells of H50-7209 by the following method. One liter of suspension culture was filtered through a Buchner funnel and cells were scraped off the Whatman No. 4 filter paper into a prechilled Sorvall Omni mixer canister. Liquid nitrogen was added to the canister and the cells were ground to a fine powder. Approximately eight volumes (v/w) of prechilled grinding buffer (0.3 M sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂) were added to the powder in a large beaker. The mixture was ground at high speed on ice in a polytron two times for 30 seconds each. Following filtration through three layers of miracloth or 60 μ mesh, the filtrate was centrifuged

10 min at 350g at 4°C. The supernatant was decanted and the pellet briefly drained on ice. The pellet was suspended in approximately 40 ml lysis buffer (20 mM EDTA, 50 mM Tris-HCl, pH8.0, 1.0% Sarkosyl, 300 µg/ml ethidium bromide) on ice in the dark. After the addition of cesium chloride at a rate of 0.95 grams per ml of solution, the lysate was clarified by centrifugation for 30 minutes at 17,400g at 4°C. The supernatant was collected away from the pellet and pellicle and was centrifuged for 48 hours at 48,000 rpm in a Beckman Ti70.1 rotor. The DNA band was removed from the tube using a Pasteur pipet and the ethidium bromide was removed by gentle extraction with water-saturated isoamyl alcohol. The DNA was dialyzed overnight at 4°C against TE, ethanol precipitated, and resuspended in TE.

BACTERIOPHAGE DNA ISOLATION

The procedure for bacteriophage DNA isolation was derived from Yamamoto et al., 1970. An overnight culture of *E. coli* strain Q358 was grown in LB + 0.2% maltose. Three ml of this culture were combined with 7 ml of LB and 5×10^8 pfu of phage at 37°C without shaking. This was added to 1 liter of prewarmed lambda media (1% casamino acids, 0.5% yeast extract, 0.3% NaCl, 0.2% KCl, 25 mM Tris-HCl, pH 7.5, and 10 mM MgCl₂) and incubated at 37°C for 9-12 hours with vigorous shaking. Phage were then isolated from the lysed culture after the addition of sixty grams of NaCl. The culture was centrifuged at 8600g for 20 minutes to remove whole cells and cell debris. Seventy grams of PEG MW8000 were added to the supernatant and it was left at 4°C overnight. The phage particles were collected by centrifugation at 10,000g for 30 minutes and dissolved in 20 ml of SM (0.1 M NaCl, 8 mM MgSO₄, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin). Following a

clarification spin of 12,000g for 10 minutes, the suspension was layered onto a CsCl step gradient consisting of 3 ml of 1.4 density CsCl and 3 ml of 1.6 density CsCl. The samples were centrifuged in the Beckman SW28.1 rotor at 22,000 rpm at 4°C for 2 hours. The bluish-white band at the 1.4/1.6 interface was removed with a Pasteur pipet and centrifuged again in 1.45 g/ml CsCl in SM for 18 hours at 50,000 rpm in the Ti70.1 rotor. The phage band was recovered and dialyzed twice against 100 volumes of 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ for 30 minutes each. The DNA was recovered from the phage particles by extracting twice with equal volumes of phenol saturated with 0.1 M Tris-HCl, pH 8.0. Overnight dialysis at 4°C against TE was performed to remove residual phenol.

SUGARCANE RNA ISOLATION

Sugarcane RNA was isolated by the protocol of Ramagopal (1987). Cells were harvested from 7-day old suspension cultures of H50-7209 by filtration using a Buchner funnel. The cells were ground to a fine powder in liquid nitrogen with a mortar and pestle. Approximately 10 grams of the powder were added to 30 ml of prechilled TNNES buffer (40 mM Tris-HCl, pH 8.0, 100 mM NaCl, 100 mM sodium acetate, 10 mM EDTA, 0.5% SDS) and 30 ml of phenol saturated with TNE buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM NaCl) were added. The mixture was shaken 10 minutes at 300 rpm on a rotary shaker in a fume hood and was then centrifuged at 4°C for 15 minutes at 12,000g to separate the phases. The aqueous phase was transferred to a clean flask while the interface and phenol phases were re-extracted in the same manner with 10 ml of TNNES. The aqueous phases and interface were combined and re-extracted with 40 ml fresh TNE-saturated phenol.

The extractions continued until no interface was present. Chilled ethanol (2.2 volumes) was added to the final aqueous phase and the nucleic acids precipitated overnight at -20°C . The samples were centrifuged for 30 minutes at 4°C at $12,000g$. The pellets were washed two times with 2 M lithium chloride then one time with 0.1 M lithium chloride, 70% ethanol. The total RNA was resuspended in water and stored at -70°C in aliquots.

Messenger RNA was isolated by passing total RNA through an oligo(dT)-cellulose column. The total RNA was mixed with an equal volume of 2X binding buffer (40 mM Tris-HCl, pH 7.6, 1.0 M NaCl, 2 mM EDTA, 0.2% SDS), heat denatured at 65°C for 5 minutes, then quenched on ice before applying to the column. The solution was applied to the column and the eluate was collected, heat-denatured and reapplied to the column. The column was washed with 10 column volumes of 1X binding buffer. Three volumes of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS) was applied to the column and the flow through was collected. In some instances, the sodium chloride concentration of the sample was adjusted to 0.5 M and the sample was reapplied to the column for further purification of mRNA. The mRNA was precipitated by the addition of 0.1 volumes of 3 M sodium acetate, pH 5.2 and 2.2 volumes of 95% ethanol then pelleted by centrifugation for 20 minutes at 4°C and $10,000g$. The pellet was washed with 70% ethanol, dissolved in water, and stored in aliquots at -70°C .

GEL ELECTROPHORESIS

Horizontal agarose gel electrophoresis was used for size-fractionating DNA. Unless otherwise indicated, gels were 1% agarose, 0.5X TBE buffer (0.045 M Tris-

phosphate, 0.002 M EDTA), and 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Gels were 12 \times 14 cm in size. Running buffer was also 0.5X TBE. One-tenth volume loading dye (0.25% bromphenol blue, 0.25% xylene cyanol FF, and 15% Ficoll) was added to the samples before loading into the gel wells. A voltage of 5-20 V/cm was applied and electrophoresis continued until the bromphenol blue had migrated to approximately 4 cm from the anode end of the gel.

RNA was size fractionated through agarose gels containing formaldehyde. Three micrograms of poly(A) RNA or 20 μg of total RNA were dried in the Savant speed vac then resuspended in 50% formamide, 2.2 M formaldehyde, 40 mM MOPS, pH 7.0, 10 mM sodium acetate, and 1 mM EDTA. The sample was heated at 65°C for 10 minutes then quenched on ice. Two μl of 1 $\mu\text{g}/\mu\text{l}$ ethidium bromide and 3 μl of loading dye (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, and 0.05% Xylene Cyanol FF) were added and the sample was electrophoresed on a 1.2% formaldehyde gel (1.2% agarose, 40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 6.7% formaldehyde) at 60 volts until the dye front approached the end of the gel.

Denaturing polyacrylamide gel electrophoresis was employed for the size fractionation of small single-stranded DNA and RNA fragments. The 6% gels consisted of 6% acrylamide, 0.3% *N,N'*-methylenebisacrylamide (bis), 7 M urea, 0.045 M Tris-phosphate, 0.002 M EDTA, 0.06% ammonium persulphate, and 0.06% *N,N,N',N'*-tetramethylethylenediamine (TEMED). The 8% gels had the same formulation except that the acrylamide was 8% and the bis was 0.4%. Electrophoresis was at a constant power of 45 W for the 20 \times 40 cm gels and 60 W for the 35 \times 42 cm gels.

BLUNT-END LIGATION

Following restriction endonuclease digestion, the DNA ends were filled in by the DNA Polymerase I Klenow fragment. Reaction volumes were 20 μ l and consisted of 50 mM potassium phosphate, pH 7.5, 3 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.2 mM dNTPs, and 2 units Klenow fragment. The reactions were incubated 30 minutes at 37°C before the enzyme activity was destroyed by a 10 minute incubation at 75°C. The filled-in DNA was ligated overnight at 16°C in 66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, 66 μ M ATP and 1 unit T4 DNA Ligase in a reaction volume of 20 μ l.

PROBE SYNTHESIS

Random primer DNA probes were prepared using the method developed by Feinberg and Vogelstein (1983). ^{32}P -labelled probes were synthesized using the kit supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN) according to manufacturer's recommendations. Labelling took place at 37°C for 1 hour. The labelled DNA was purified away from unincorporated nucleotides by passage through a Sephadex G50 spin column.

Nonradioactive labelled DNA probes were prepared according to manufacturer's recommendations using the Genius Nonradioactive Labelling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). One hundred nanograms to one μ g linearized DNA was labelled at 37°C for 1-24 hours. Labelled DNA was precipitated by the addition of 2 μ l 4M LiCl, 1 μ l 20 mg/ml glycogen, and 60 μ l ethanol. The DNA was collected by centrifugation at 12,000g for 15 minutes, washed with 70% ethanol, and resuspended in 100 μ l TE, 0.1% SDS at 37°C.

Single-stranded RNA probes were made using the Riboprobe Kit (Promega, Madison, WI) and modifications of the protocol described by Melton et al. (1984). One μg of linearized plasmid DNA containing polymerase binding sites for the T7, T3, or SP6 polymerases was incubated with 5-10 units of the appropriate polymerase in 20 μl of 40 mM Tris-HCl, pH 7.5, 6 mM MgCl_2 , 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 unit RNasin ribonuclease inhibitor, 0.5 mM each of ATP, GTP and UTP, 12 μM CTP, 50 μCi [α - ^{32}P]CTP (3.12 μM). Incubation was for 60 minutes at 37°C. The DNA strand was removed by digestion with 1 unit of RQ1DNase (Promega, Madison, WI) for 15 minutes at 37°C and the probe was purified by ethanol precipitation. Probes were resuspended in 100 μl THE.

cDNA probes were made by incubating 1 μg poly(A) RNA, purified one time on an oligo d(T) column, in 20 μl reverse transcription buffer for 1 hour at 42°C. Reverse transcription buffer consisted of 50 mM Tris, pH 8.3, 8 mM MgCl_2 , 50 mM KCl, 0.004% Actinomycin D, 0.002% oligo d(T) primer, 5 mM each of dCTP, dGTP, and dTTP, 10 mM DTT, 0.4 units/ μl RNase inhibitor, 50 μCi [α - ^{32}P]dATP (16.5 μM), and 10-15 units AMV Reverse Transcriptase. The reaction was stopped by the addition of 1 μl of 0.5 M EDTA. The RNA strand was removed by base hydrolysis in 0.3 N NaOH for 1 hour at 65°C followed by neutralization with 1/6 volume 2M Tris, pH 7.5 and 1/2 volume 1 N HCl. The probe was precipitated by the addition of 1/10 volume 3 M sodium acetate and 2.5 volumes ethanol.

For the labeling of oligomer probes, ten pmoles of oligomer were end-labelled in a 20 μl reaction consisting of 70 mM Tris-HCl, pH 7.6, 0.1 M KCl, 10 mM MgCl_2 , 5 mM DTT, 0.5 mg/ml BSA, 15 μCi [γ - ^{32}P]ATP (10 μM), and 8 units T4 polynucleotide kinase. Incubation was for 45 minutes at 37°C followed by 10

minutes at 65°C. The probe was ethanol precipitated twice then resuspended in 100 μ l water.

SUGARCANE GENOMIC LIBRARY CONSTRUCTION

Reaction conditions for the partial digestion of sugarcane genomic DNA with the restriction endonuclease *Sau3A* were determined by adding 0.004, 0.008, 0.016, 0.031, 0.063, 0.125, 0.250, and 0.500 units enzyme per μ g of DNA and allowing digestion to proceed for 1 hour at 37°C. The conditions favoring production of 10-25 kb sized fragments were determined by electrophoresing the reactions on a 0.4% agarose gel alongside of lambda DNA digested with *HindIII*. A large scale digestion of 230 μ g DNA was prepared using 0.008 units *Sau3A* per μ g DNA. The DNA was fractionated on a step gradient consisting of 4 ml each of 4°C 16%, 13%, 9%, and 5% NaCl in TE. Prior to addition of DNA, the gradients were kept at room temperature for 2-1/2 hours allowing diffusion to approximate a linear gradient. The gradients were centrifuged for 24 hours at 16,000 rpm at room temperature in the SW28.1 rotor. Drip fractions of approximately 0.5 ml were collected and 10 μ l aliquots of each fraction were electrophoresed on a 0.4% agarose gel. Fractions containing 10-25 kb fragments were pooled and the nucleic acid was precipitated with ethanol. The samples were centrifuged at 12,000 rpm for 20 minutes at room temperature and resuspended in TE.

The bacteriophage EMBL4 DNA was prepared by digesting 150 μ g with 3 units/ μ g *BamHI* for 1 hour at 37°C. The DNA was ethanol precipitated then resuspended in 1 ml of TE. The cohesive ends (COS ends) of the phage left and right arms were annealed by addition of 10 μ l 1 M $MgCl_2$ and incubation for 1 hour at

42°C. The DNA was applied to a sodium chloride step gradient and fractions were collected as described previously. The fractions containing phage arm DNA and lacking the phage internal DNA fragment were pooled and dialyzed 4-6 hours at room temperature in TE. The DNA was aliquoted into tubes for the SW28.1 rotor and precipitated by the addition of 0.1 volumes 3 M sodium acetate and 1 volume isopropanol. The tubes were centrifuged for 1 hour at 4°C at 25,000 rpm in the SW28.1 rotor. The pellets were air dried, resuspended in TE, pooled, and quantitated on a 0.4% agarose gel.

Fractionated genomic DNA was added to EMBL4 arms at molar ratios of 1:1, 1:2, and 1:4 for ligation. The 20 μ l ligations took place at 14°C overnight in 20 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM DTT, 50 μ g/ml BSA, 0.5 mM ATP, and 1 unit of T4 DNA ligase.

A 5 μ l aliquot of each ligation reaction was mixed with 50 μ l of packaging extracts (Scalenghe et al., 1981), 35 μ l C and H buffer (40 mM Tris-HCl, pH 8.0, 10 mM spermidine-HCl, 10 mM putrescine-HCl, 7% dimethylsulfoxide), and 1.4 μ l of 0.1 M ATP. The samples were incubated at 37°C for 45 minutes. To 100 μ l of fresh packaging extract, 2.5 μ l of 1 M MgCl₂, 5 μ l DNase I (0.2 mg/ml), and 2.0 μ l of 0.1 M ATP were added and incubated on ice for 15 minutes. Twenty μ l of DNase-treated extract were added to the extract containing the ligation reaction and this was incubated at 37°C for 30-45 minutes. The volume was adjusted to 0.5 ml with SM and the titer was determined.

Plating bacteria were prepared by inoculating 100 ml of LB + 0.2% maltose with *E. coli* strain K802 and growing overnight at 37°C. The cells were collected by centrifugation for 10 minutes at 4000g then resuspended in 40 ml of 0.01 M MgSO₄.

The cells were diluted to an OD₆₀₀ of 2.0 with 0.01 M MgSO₄ and stored at 4°C for up to two weeks.

Phage titer was determined by making serial dilutions in SM. One hundred μ l of each dilution were mixed with 0.2 ml of K802 cells at 37°C for 20 minutes. The samples were mixed with 3 ml of LB soft agar and poured onto LB plates. The plates were incubated at 37°C for approximately 8 hours for the formation of plaques.

The library was amplified by transfecting 0.2 ml of K802 cells with 10,000 pfu and incubating at 37°C for 20 minutes. The sample was divided into two tubes and mixed with 3 ml soft agar for plating. Following an 8 hour incubation at 37°C, the plates were flooded with 6 ml of SM and kept overnight at 4°C. The supernatant and top agar were collected and centrifuged at 4,500g for 10 minutes. The supernatant was transferred to new tubes in 4 ml aliquots and stored at 4°C. The titer of the amplified library was determined as described above.

The *E. coli* strain Q359 was transected by the packaged library and plated at a density of approximately 1000 plaques per plate onto LB plates. Following overnight growth at 37°C, the plaques were transferred to nitrocellulose filters in duplicate by the following method. Sterile nitrocellulose membranes were laid onto the surface of the petri plates and allowed to adhere for 2 minutes. The membranes were then placed, DNA side up, onto a filter paper mat soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 60 minutes then transferred to a mat soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 minutes. The membranes were rinsed in 2X SSPE (0.3 M NaCl, 200 mM NaH₂PO₄, pH 7.4, 20 mM EDTA) and air dried on filter paper. The DNA was fixed onto the membranes in a vacuum oven at 80°C for 2 hours. The membranes were floated on 6X SSC (0.9 M NaCl, 600 mM NaH₂PO₄, pH 7.4, 60 mM EDTA) and submerged for 5 minutes.

They were agitated for 2 hours at 42°C in prewash solution (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS) then prehybridized for 4-6 hours at 42°C in prehybridization solution (50% formamide, 1% ficoll, 1% pvp, 1% BSA, 1% PEG MW8000, 10 mM NaCl, 0.1% denatured sheared calf thymus DNA). The prehybridization solution was expressed and fresh solution containing denatured, radioactively labeled first strand cDNA was added. Hybridization was carried out at 42°C overnight.

The membranes were washed three times for 10 minutes at room temperature in 2X SSC, 0.1% SDS; 2 times 30 minutes at 68°C in 1X SSC, 0.1% SDS; then once in 0.2X SSC, 0.1% SDS at 68°C for 60 minutes.

Hybridization was detected by autoradiography for two days using Kodak XRP-5 film and Lightning Plus intensifying screens. The autoradiographs were aligned with the original petri plates and plugs of agar surrounding the brightest hybridizing plaques were collected for further purification. The plugs were placed in 1 ml of SM at 4°C for overnight phage elution. The titer was determined as above and the phage were replated at a lower density and hybridized again to radioactively labelled first strand cDNA. The process was repeated a third time to ensure that individual hybridizing plaques were isolated.

SOUTHERN HYBRIDIZATION

Southern transfer was by the capillary transfer method. The agarose gel was denatured for 30-60 minutes in 1.5 M NaCl, 0.5 N NaOH with gentle shaking. The gel was rinsed in water then neutralized for 30-60 minutes in 1.5 M NaCl, 1 M Tris-

HCl, pH 7.5. The gel was placed on a support covered with Whatmann 3MM paper and the edges surrounding the gel were covered with plastic wrap or Parafilm.

A piece of nylon membrane was cut to the size of the gel and soaked in water. The wet membrane was placed on the gel then covered with 2 pieces of 3MM paper and a 4 inch stack of paper towels. Transfer buffer was 10X SSC and the transfer proceeded for 8-24 hours. The DNA was fixed to the membrane by baking at 80°C for 30 to 60 minutes.

Prehybridization was for 2-6 hours in approximately 0.25 ml/cm² prehybridization buffer (0.9 M NaCl, 600 mM NaH₂PO₄, pH 7.4, 60 mM EDTA, 0.2% ficoll, 0.2% pvp, 0.2% BSA, 1% SDS, 50 µg/ml fragmented, denatured salmon sperm DNA). The prehybridization solution was expressed and replaced with 0.1 ml/cm² solution containing 1 × 10⁵ cpm/ml of ³²P-labelled probe or 20 ng/ml Digoxigenin-labelled probe. Hybridization was overnight at 65°C.

The blots were washed twice in 6X SSC, 0.5% SDS for 10 minutes at room temperature then twice in 0.1X SSC, 0.5% SDS for 30 minutes at 65°C. Damp blots were wrapped in plastic wrap and placed in a cassette with Kodak XRP-5 X-ray film and Lightning Plus intensifying screens. Exposure was at -70°C for 1 to 3 days (³²P probes) or at room temperature for 5 to 60 minutes (Digoxigenin probes).

NORTHERN HYBRIDIZATION

Formaldehyde gel electrophoresis was carried out as described previously. The RNA was transferred to Nytran nylon membrane (Schleicher and Schuell, Keene NH) by capillary transfer in 20X SSC overnight. The RNA was fixed to the membrane by baking at 80°C for 1 hour.

Prehybridization was in 50% formamide, 0.2% ficoll, 0.2% pvp, 0.2% BSA, 0.5% SDS, 100 $\mu\text{g/ml}$ fragmented, denatured salmon sperm DNA, 5X SSPE for 4 hours at 37°C. The solution was expressed and fresh solution (0.1 ml/cm²) containing 1×10^5 cpm/ml was added. Hybridization was at 37°C for 36-48 hours.

The blots were washed twice for 10 minutes at room temperature in 2X SSPE, 0.1% SDS, twice for 10 minutes at room temperature in 0.1X SSPE, 0.1% SDS, and once for 60 minutes in 0.1X SSPE, 0.1% SDS at 45°C. Autoradiography was for 1 to 3 days at -70°C using Kodak XRP-5 film and Lightning Plus intensifying screens.

DNA SEQUENCING

Plasmid DNA was sequenced by the Sanger dideoxy method (Sanger et al., 1977) using the Sequenase 2.0 Kit (United States Biochemicals, Cleveland, OH). Three to 4 μg of plasmid DNA and 1 μl of 10 $\mu\text{g/ml}$ primer were mixed with an equal volume of 0.4 N NaOH, 0.4 mM EDTA, incubated at 85°C for 10 minutes, then quenched on ice. The DNA was precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol then resuspended in 10 μl of 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂ and 50 mM NaCl. The sample was heated to 65°C for ten minutes to ensure resuspension then put at 37°C for 15 minutes. Labeling occurred by the addition of 1 μl of 0.1 M DTT, 2 μl of 1.5 μM dGTP, dTTP, and dCTP, 0.5 μl of [³⁵S] dATP (10 $\mu\text{Ci}/\mu\text{l}$, 1000 Ci/mmol) and a room temperature incubation of 2-5 minutes. The termination reaction consisted of adding 3.5 μl of DNA solution to 2.5 μl of each termination mix (80 μM dNTPs, 50 mM NaCl, and 8 μM of ddATP, ddGTP, ddCTP, or ddTTP). The tubes were incubated at 37°C for 5

minutes. The reactions were stopped by the addition of 4 μ l stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, and 0.05% Xylene Cyanol FF) and samples were stored at -20°C until electrophoresis on a 6% denaturing acrylamide gel (Sambrook et al., 1989). Prior to electrophoresis, the samples were denatured at 75°C for 5 minutes and quenched on ice. Four μ l of each reaction were loaded on the gel. Following electrophoresis the gels were soaked for fifteen minutes in 5% methanol, 5% acetic acid then dried for 1 hour in a gel drier. Autography was for 24-72 hours at -70°C using Kodak XRP-5 film.

RIBONUCLEASE PROTECTION ASSAY

Thirty μ g of total RNA and 5×10^5 cpm of ^{32}P -labelled riboprobe were resuspended in 30 μ l of 80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA and denatured for ten minutes at 88°C . The samples were immediately transferred to a 45°C waterbath for overnight annealing. Three hundred fifty μ l of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl and 40 μ g/ml RNase were added and the tubes were incubated at 37°C for thirty minutes. Ten μ l of 20% SDS and 10 μ l of 5 mg/ml proteinase K were added and the samples incubated an additional 15 minutes at 37°C . The proteins were removed by extraction with 400 μ l of phenol, chloroform, isoamyl alcohol (49:49:2) and the RNA was precipitated by the addition of 1 μ l of 10 mg/ml tRNA and 1 ml ethanol. After centrifugation at 12,000g for 15 minutes, the RNA was resuspended in 4 μ l loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF) and separated on a 6% denaturing polyacrylamide gel. The molecular weight marker was end-labelled *Hae*III-digested ϕ X174 DNA. This marker covers a range of 72 to

1353 bp. The gel was dried and autoradiography was for 4-24 hours at room temperature using Kodak XRP-5 film.

PRIMER EXTENSION

Thirty μg total RNA were mixed with 1×10^5 cpm end-labelled oligomer probe, ethanol precipitated, and resuspended in 30 μl aqueous hybridization solution (1 M NaCl, 167 mM HEPES, pH 7.5, 3.3 mM EDTA). The samples were placed in a 90°C heat block for 20 minutes, then the heat was turned off and the block returned to room temperature over a period of about four hours. The sample was adjusted to a volume of 200 μl , ethanol precipitated, then resuspended in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl_2 , 5 mM DTT, 50 mM KCl, 32 units RNase inhibitor, 70 μM dNTPs, and 40 units AMV reverse transcriptase. The samples were incubated 1 hour at 37°C then the RNA was removed by incubation with 0.25 μg RNase at 37°C for 30 minutes. The samples were ethanol precipitated, phenol extracted and resuspended in 50% formamide, 10 mM EDTA, 0.03% Bromophenol Blue, 0.03% Xylene Cyanol FF. Electrophoresis was at 45 volts through an 8% denaturing acrylamide gel. A sequencing reaction using the same primer was electrophoresed beside the primer extension reaction as a marker. The gel was dried and autoradiographed for 2-12 hour at room temperature using Kodak XRP-5 film.

PROTOPLAST FORMATION AND ELECTROPORATION

Protoplasts were prepared from 7 or 8 day old suspension cells of H50-7209. Cells were suspended in YEM (YE media adjusted to 0.5 M mannitol) containing 2%

cellulysin (Calbiochem, La Jolla, CA) , 2% driselase (Plenum Scientific Research Inc., Hakensack, NJ), and 0.5% rhozyme HP 150 (Genencor, South San Francisco, CA) and shaken at 30°C for 2-3 hours at 100 rpm. Protoplasts were filtered through a 35 μ mesh screen and washed three times in YEM. Final resuspension was at a density of $1-4 \times 10^6$ per ml in HSB (150 mM KCl, 4 mM CaCl₂, 10 mM HEPES, pH 7.2, and 0.25 M mannitol).

Twenty μ g of supercoiled cesium chloride purified plasmid DNA was added to 300 μ l of protoplasts in 0.4 ml electroporation cells (BioRad, Richmond, CA). Electroporation conditions were two pulses at 200 volts and 500 μ F, using the BioRad Electroporator. Immediately following electroporation the protoplasts were pelleted, resuspended in 1 ml of YEM, and transferred to sterile 35 \times 10 mm petri dishes for an 18-24 hour incubation at room temperature.

GUS FLUORESCENCE ASSAY

GUS activity was determined by the fluorescence assay of Jefferson et al., (1987), with modifications. Protoplasts were collected and resuspended in 250 μ l GUS extraction buffer (50 mM NaH₂PO₄, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM β -mercaptoethanol). Protoplasts were lysed by storage at -70°C and cell debris was removed by two 10 minute centrifugations at 14,000g. Protein concentration was determined by the Bradford method (Bradford, 1976) using the BioRad protein assay kit.

One hundred μ l of extract were mixed with an equal volume of GUS assay buffer (GUS extraction buffer with 2 mM 4-methylumbelliferyl β -D-glucuronide (MUG)) and the samples were incubated at 37°C. Fifty μ l aliquots were removed at

various time points and the reactions were stopped by the addition of 2 ml of 0.2 M sodium carbonate. Fluorescence was measured using the Hoefer TKO100 minifluorometer (San Francisco, CA).

PCR AMPLIFICATION OF PLASMID DNA

The polymerase chain reaction (PCR) was used to amplify specific segments of DNA from plasmids. The reaction was performed according to protocols supported by the manufacturer (Perkin Elmer Cetus, Norwalk, CT) with modifications, and using the *AmpliTag* kit (Perkin Elmer Cetus). The reaction volume was 50 μ l and consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 200 μ M each dNTP, 1.0 μ M each primer, 4 ng template DNA, and 2.5 units of *AmpliTag* DNA polymerase. The components were added in the order given. Prior to the addition of polymerase, the reaction was boiled for 3 minutes then quenched on ice. The polymerase was added and the sample was overlaid with 100 μ l of mineral oil. The reaction profile consisted of an initial 3 minute denaturation step at 94°C, 25 cycles of amplification (one minute at 94°C, one minute at 50°C, and 3.5 minutes at 72°C), and a 7 minute extension at 72°C.

CHAPTER 3. RESULTS

SUSPENSION CELL SENSITIVITY TO HYGROMYCIN AND G418

Sugarcane suspension cell sensitivity to hygromycin B and G418 was determined by plating cells on varying concentration of the antibiotics and monitoring growth. The concentrations of hygromycin B were 0, 5, 10, 50, 100, 300, and 600 $\mu\text{g/ml}$ and the concentrations of G418 were 0, 5, 10, 50, 100, 200, 500 $\mu\text{g/ml}$. Suspension cells were collected in a pipet and the cells were allowed to settle in the pipet. Two hundred μl of the settled cells were spread on each plate and the plates were stored at room temperature for 33 days. Cells grew on the control plates but growth was inhibited on all concentrations of both antibiotics. The test was repeated using a lower concentration range of antibiotics; 0, 0.1, 0.5, 1, 2, 5, 10, and 50 $\mu\text{g/ml}$. Growth on selective media was for 26 days at room temperature. As seen in Figure 1, growth was greatly inhibited at 0.5 $\mu\text{g/ml}$ hygromycin B and completely inhibited at 1 $\mu\text{g/ml}$. The cells were less sensitive to G418 and grew well on 0.5 $\mu\text{g/ml}$. Growth was completely inhibited at 2 $\mu\text{g/ml}$ of this antibiotic.

ISOLATION OF CLONES FOR ABUNDANT TRANSCRIPTS

Plasmid Genomic Library Construction

A genomic library was constructed in the plasmid pUC8 by ligating 10-20 kb genomic DNA fragments to plasmid DNA in molar ratios of 3:1, 5:1, and 10:1. One hundred twenty-nine transformants were screened with labelled first strand cDNA

probe and two colonies hybridized strongly to the probe. Neither hybridized upon secondary screening and an alternative cloning vector was used for library construction.

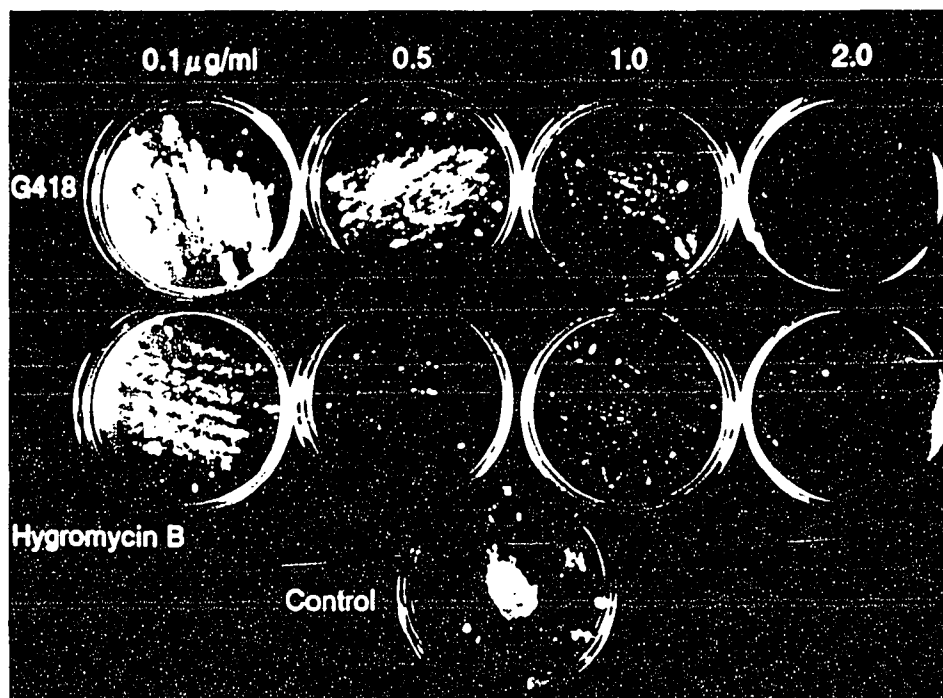


Figure 1. Sensitivity Test of Sugarcane suspension cells to increasing concentrations of G418 and Hygromycin B in YE 1.5% agar plates. The control plate (bottom) has no antibiotics. G418 and Hygromycin B concentrations are from left to right, 0.1, 0.5, 1.0, and 2.0 µg/ml.

Construction of Genomic Library in EMBL4

A genomic library of *Sau3A* partially-digested genomic DNA was constructed in the vector λ EMBL4. The titer of the primary library was 1.8×10^5 pfu/ml. The amplified library had a titer of 3.2×10^5 pfu/ml for a total of 4.5×10^6 clones in the amplified library. Approximately 5000 plaques were screened three times with labelled first strand cDNA. DNA was purified from the 6 plaques which hybridized strongest to the cDNA probe.

The purified DNA was digested with a variety of restriction enzymes, transferred to nylon membrane and probed with labelled cDNA. Initial work focused on clone λ 1 because it hybridized most intensely to the cDNA probe. The *EcoRI* fragments of λ 1 which hybridized to the cDNA probe were subcloned into pUC8 and were referred to as p1-2 and p1-4. A Northern blot of total RNA and poly(A) RNA probed with λ 1, p1-2, and p1-4 indicated that λ 1 contained ribosomal RNA genes. (Figure 2). A Southern blot of the 6 clones digested with *EcoRI* was hybridized with labelled λ 1 (Figure 3). The λ 1 probe hybridized to the genomic insert fragments of λ 1 but did not hybridize to the insert fragments of the remaining five clones. The 18S, 5.8S, and 28S ribosomal genes are found in tandemly repeating clusters with a pre-rRNA repeat unit size of about 8 kb (Long and Dawid, 1980). The lack of hybridization of the remaining clones to λ 1 indicates that the clones were not specific for these ribosomal genes.

Northern blots of poly(A) RNA were hybridized with probes made from the 5 remaining λ clones. λ 2, λ 3, and λ 7 gave strongly hybridizing bands. λ 7 was selected for further subcloning. A restriction map of λ 7 was constructed in preparation for subcloning. Figure 4 illustrates the map of λ 7 and the subclones derived from it.

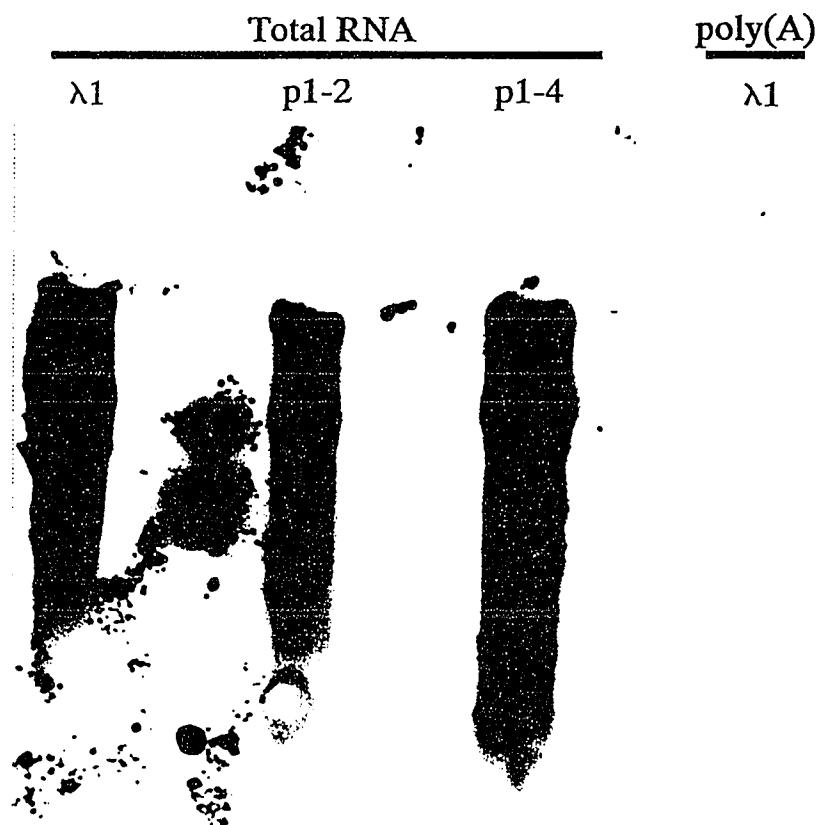


Figure 2. Northern hybridization of total RNA (20 μg) and poly(A) RNA (1 μg) from 7-day old suspension cells. Hybridization probes were random primer labelled $\lambda 1$, p1-2, and p1-4 (1×10^6 cpm/ml).

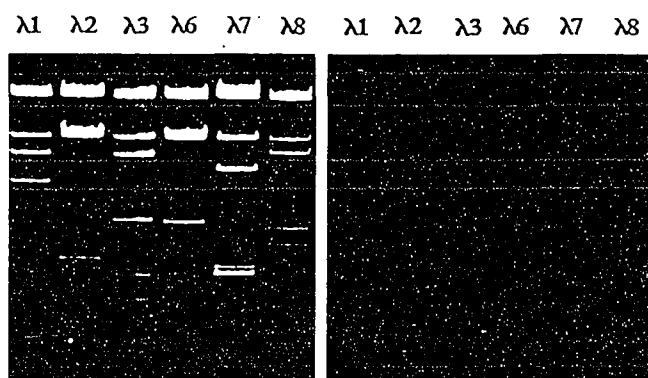


Figure 3. Agarose gel (left) and Southern blot (right) of genomic λ clones ($1 \mu\text{g}$ each) digested with *Eco*RI and hybridized with 1×10^6 cpm/ml of random primer labelled $\lambda 1$.

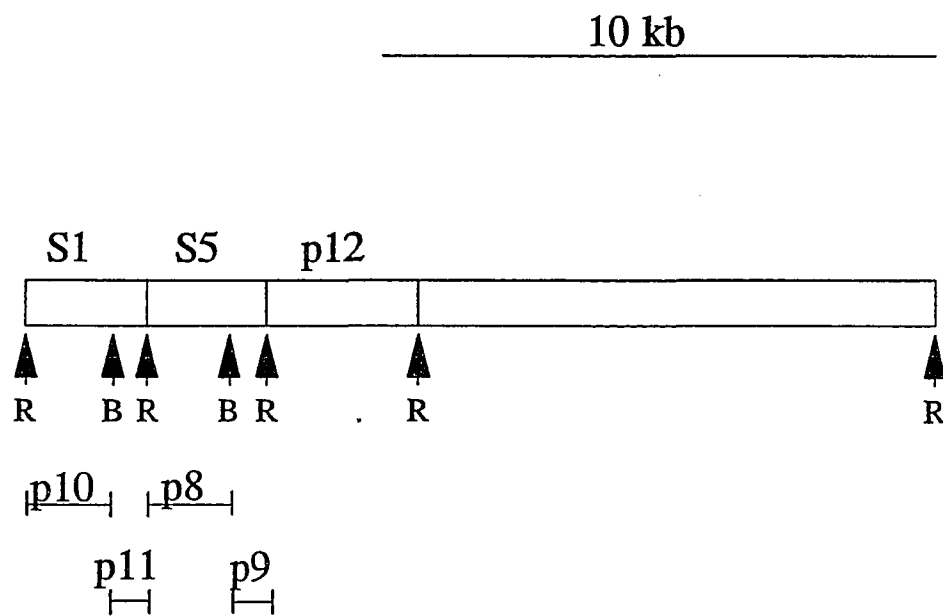


Figure 4. Restriction map of $\lambda 7$ insert (λ arms not included) and the subclones derived from it. R=*EcoRI* and B=*BglIII*.

SUBCLONING

In order to subclone pertinent fragments of $\lambda 7$, regions coding for a transcript were determined by Southern analysis. A Southern blot of *EcoRI*-digested $\lambda 7$ DNA probed with cDNA revealed hybridization in the region of a 2.3 kb doublet and a 2.5 kb fragment (Figure 5). The 2.3 kb bands were subcloned into the plasmid pVZ1 (Henikoff and Eghtedarzadeh, 1987) and are referred to as S1 and S5 (Figure 4). The 2.5 kb fragment was subcloned into the plasmid pGEM-4Z (Promega) and is referred to as p12 (Figure 4).

To ensure that these clones indeed recognized a transcript, slot blots of total RNA from 3, 5, 7, 10, 12, and 14 day suspension cells were probed with labelled S1, S5, and p12 (Figure 6). Strong hybridization was seen at all time points when probed with S5. A relatively strong signal was seen at 3 days when probed with S1 and the hybridization was very weak at all times with the p12 probe. A Northern blot of poly(A) RNA probed with S5 revealed hybridization to a transcript of about 2 kb (Figure 7) while no hybridization was seen with probe S1.

S1 and S5 were digested with *EcoRI* and *BglIII* and the DNA fragments were ligated into pGEM-4Z to produce the subclones p8 through p11 (Figure 4).

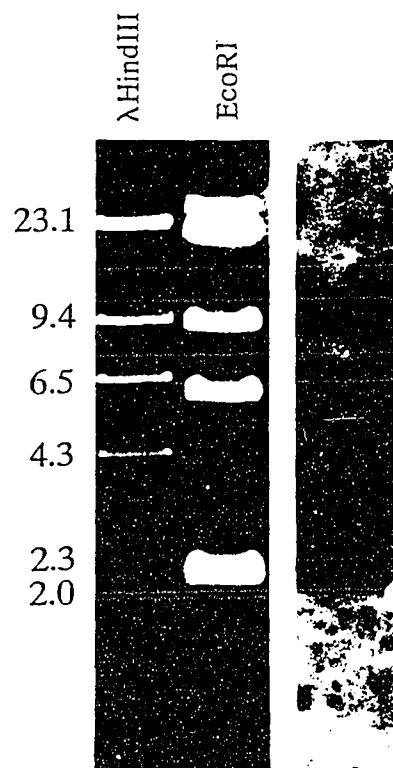


Figure 5. Agarose gel (left) and Southern blot (right) of *EcoRI* digested $\lambda 7$ ($1 \mu\text{g}$) probed with 1×10^6 cpm/ml labelled cDNA from 7-day suspension cells.

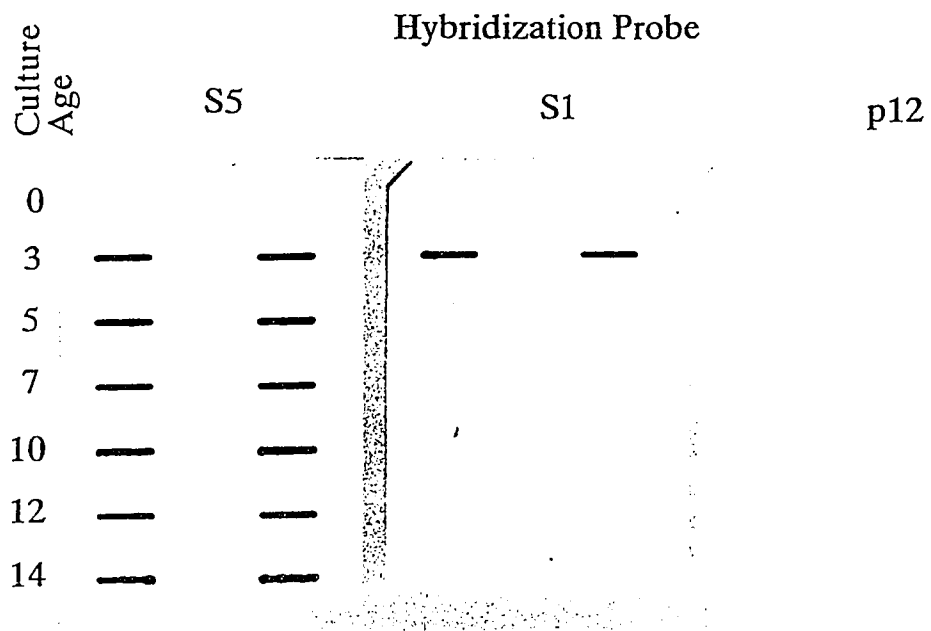


Figure 6. Slot blot hybridization of 20 μ g total RNA from 3, 5, 7, 10, 12, and 14 day old suspension culture cells. Hybridization probes were digoxigenin-labelled S5, S1, and p12 (20 ng/ml).

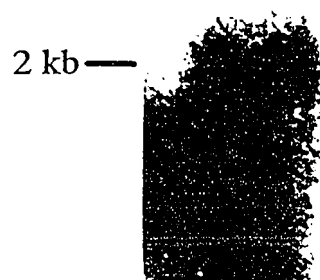


Figure 7. Northern hybridization of 3 μg poly(A) RNA from 7-day suspension cells probed with 1×10^6 cpm/ml random primer labelled S5.

DETERMINATION OF TRANSCRIPTION START SITE

RNase protection assays were performed in order to determine the location and orientation of the transcript. Single stranded riboprobes were synthesized from the plasmids S1, S5, and p8 through p12 (Figure 8). The initial assay using riboprobes A, G, C, and I revealed a fragment of about 70 nt protected by probe I. This pattern was observed for RNA from mature leave as well as suspension cells. Assays using probes A through J revealed that probe D also protected a fragment of about 70 nt. Further analysis using probes D and I confirmed that both probes protected fragments. The protected fragments were both about 70 nt, however, the one protected by D was the smaller of the two and produced a more intense signal indicating a greater abundance of transcript (Figure 9).

The insert fragment of plasmid p9 (Figure 4) was sequenced. Initial sequence information was obtained using the SP6 and T7 primers which bind to plasmid vector sites adjacent to the subcloned DNA in p9. This sequence information was used for the design of additional primers 197, 198, 129, and 164 (Figure 10) which were used to determine the entire sequence of p9. The complete sequence of p9 insert is shown in Figure 11. Shorter riboprobes K through P (Figure 8) were made based on restriction sites deduced from the sequence information (Figure 11). These riboprobes were synthesized using the SP6 and T7 polymerases and p9 plasmid DNA digested with *Mbo*II, *Dde*I, and *Hinf*I. Probes L and M both protected a fragment of about 70 nt while probe K protected a substantially shorter fragment (Figure 12). This indicates that the *Mbo*II site used for generating riboprobe K is located within the 70 nt protected sequence. This could represent an intron-exon junction site or the true 5' end of the transcript. The lack of detectable hybridization

of the probe p12 to total RNA (Figure 6) strongly suggests that this region represents the 5' end of the transcript.

The transcription initiation site was determined by primer extension using primer 130 (Figure 10). This primer is homologous to the nucleotides 36 to 56 of the p9 sequence. The primer extension results indicate that transcription initiation occurs at the C residue at nt 1 (Figure 13). This pattern was observed for 7 day RNA as well as 9 day RNA.

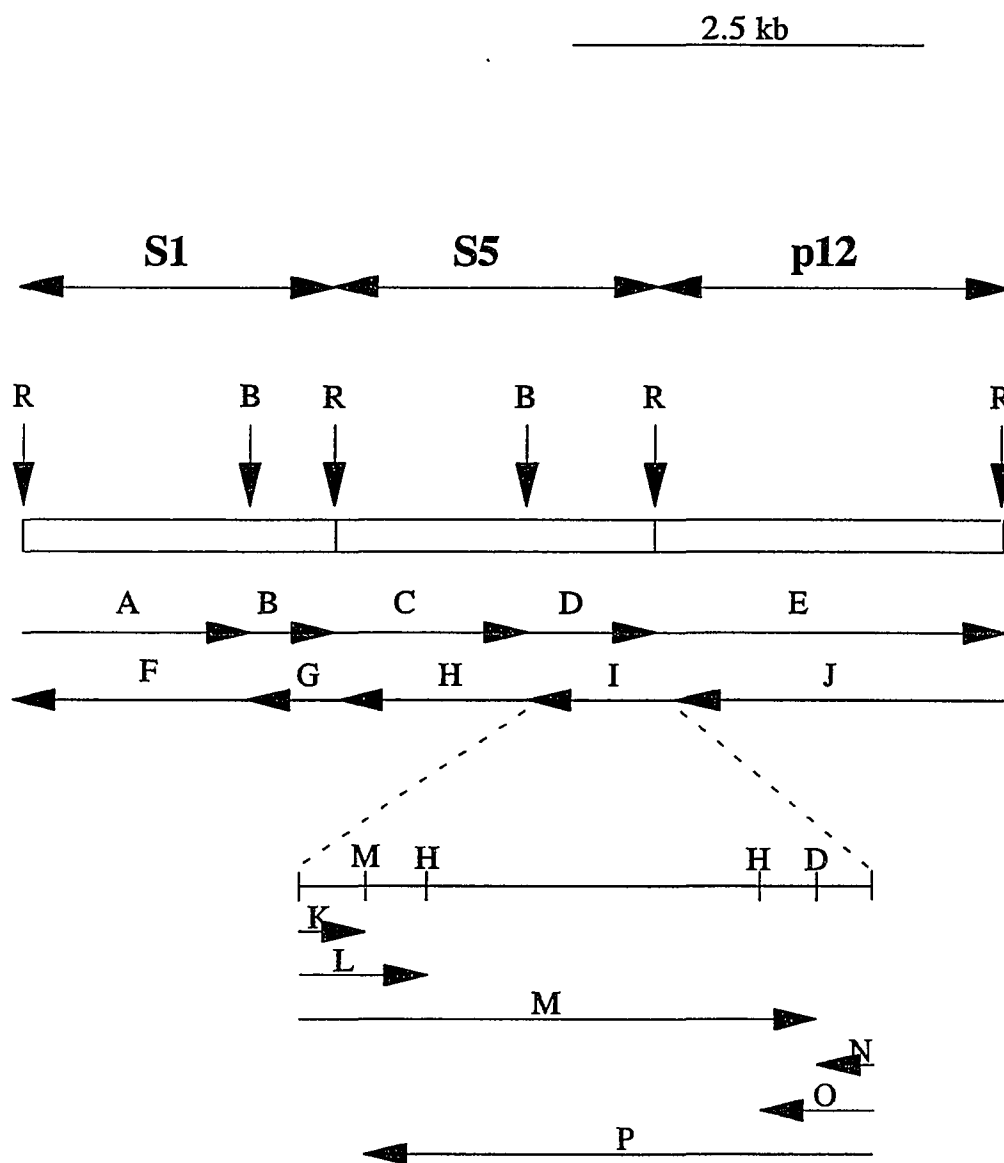


Figure 8. Riboprobes used for RNase protection assays.

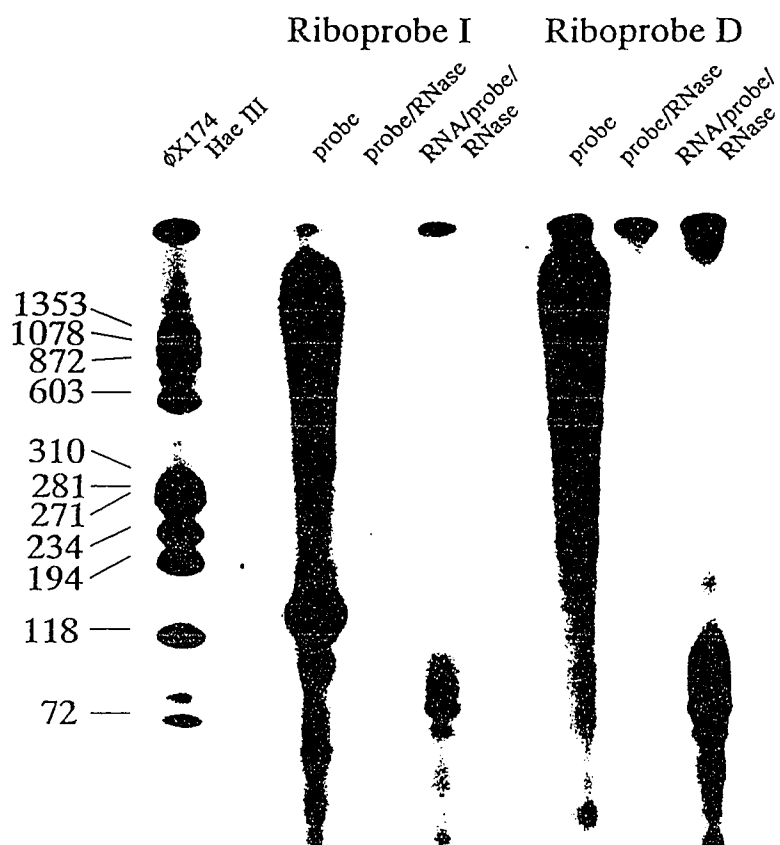


Figure 9. RNase protection assay using riboprobes I and D. The first lane of each assay is 1×10^5 cpm undigested riboprobe. The second lane is 1×10^5 cpm riboprobe digested with RNase. The third lane is 5×10^5 riboprobe hybridized to 30 μ g total RNA then digested with RNase. The molecular weight marker is 1×10^5 cpm of end-labelled ϕ X174 digested with *Hae*III.

<u>Primer</u>	<u>Sequence</u>	<u>Start Point p9 Sequence</u>
Primer 195	GGACATTGCCACCTTCAG	55
Primer 196	CTGAAGCTGGCAATGTCC	72
Primer 197	ATGCCTTTACGATGATCG	-659
Primer 198	CGATCATCGTAAAGGCAT	-676
Primer 129	GACAGTAGTAGCCTCTG	-437
Primer 130	CCTTTATTTATGGATCATCAG	56
Primer 164	CACAGGATCCAGAAGACAGGAAGGACTGAC	9
Primer 165	TGGTAAGCTTCATCAATACCTCTTTTGCTC	-844

Figure 10. Primers used for sequencing and PCR. Primer starting points are given according to nt positions in the p9 sequence (Figure 11).

```

-844      TTCA TCAATACCTC TTTTGCTCTG TCATGGAAAA GGTGCATATC
-800  TGTGTATCT TAACGTGCCA AATTTCTATC ATAGTTCTAT GCTTGGCTTG
-750  ATATAAGCAT GTTCGTATGT CGGGAGGGGT CTAAGCTCTA GCATTCAACC
-700  TGGATACAGT ATGGGGTTTG TACACGATCA TCGTAAAGGC ATAATATATC
-650  ATGATAAACC AAGTTTAAA TATGACATGC ATTGTTATCC ACTGCAAAGA
-600  AAGAGGGGCT CGCTAAGAGT CCATCTTGCT TGTAACACTT TATATCAACA
-550  CTAGCTGTGT GCGTGAACCA TATCATATTG CAATGCAAAC TACTATATGT
-500  TGATCTGGCT AGCCTAGACC ACCATCCATG ATCCATTAGG AAACAAGCTG
-450  TCATATTTCT GCTGACAGTA GTAGCCTCTG TAGTGCAAAT GCAACTGTGC
-400  AAAGCAGGAT GACCATACAT GTTTCTAAAA AAATTAGGAT GGTAACGACA
-350  TTATGTTTTT TCCCTGTAGC AGATTTAATG GCCCTTGAA ATGTTTGGGT
-300  TGATTTTATG CAGCATGCTT GAAATTTAAA GAGTGCTGCC TTGTTTATAC
-250  TTGTACTTGT GCCCATCAGA AAACCTGTGT CACTTCTTC TCTTTGTATG
-200  TTAGAGCTAA CAGGACAACA ATATTTAGTT CATGTCTCCC TGTTTCTCGT
-150  CTCTTTTGGG TTTCATCTCT AGCCTACCCC AACTTGCTTG GGACAAAAGG
-100  CTATGTTGTT GTTGTGTTG TTGTTGTTGC TGCTTGAAAT GATATAAATT
-50   TGATTTTCATG ATGCATAAGG AAAACATTAC TAGTACAAAG TCAGTCCTTC
  1   CTGTCTTCTT TTGAAATGTC GTAAAAAGTT TCTTTCTGAT GATCCATAAA
  51  TAAAGGACAT TGCCACCTTC AGGTAATTTA TGTTCCATTT CAATATTTTA
 101  TGCAGCTGAT GATGTAGTTC TCTACAAG

```

Figure 11. Nucleotide sequence of the p9 insert fragment. Nucleotide 1 represents the transcription start site.

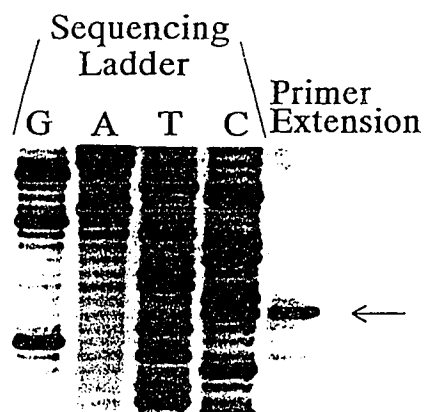


Figure 13. Primer extension analysis of 30 μg total RNA from 9-day suspension culture cells using 1×10^5 cpm of end-labelled primer 130. The sequencing ladder consists of 4 μg of p9 sequenced using the Sequenase 2.0 kit as described in Methods.

CONSTRUCTION OF EXPRESSION VECTORS

The constructs used in this study were derived from the GUS reporter gene of pBI221 (Clontech, Palo Alto, CA). This reporter consists of the CaMV 35S promoter fused to the GUS coding region and the NOS termination signals. The vector is pUC18. Convenient restriction sites flank each element of the reporter gene (Figure 14).

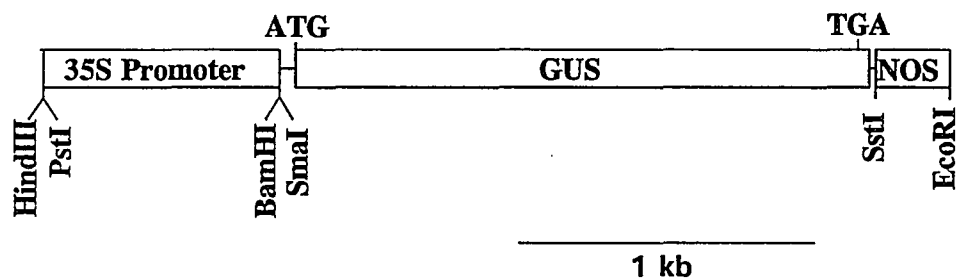


Figure 14. Map of the chimeric GUS reporter gene in pBI221.

Promoterless GUS Construct

A promoterless GUS construct was made by excising the 35S promoter from pBI221. The DNA was digested with *HindIII* and *SmaI* then blunt-ended with Klenow fragment. The DNA was recircularized by blunt-end ligation and the correct

structure was confirmed by restriction digestion and gel electrophoresis. This plasmid is referred to as pGN.

Sugarcane Promoter Construct

The GUS construct containing the sugarcane promoter (SC) was made by replacing the 35S promoter of pBI221 with SC from p9 as follows. The promoter was PCR amplified from p9 using primers 164 and 165. The PCR product was blunt-ended using Klenow fragment then ligated into *Sma*I digested pUC19. Transformed colonies were selected and miniprep DNA was PCR amplified using primer sets 164/165 and 164/129. No amplification was detected using primers 164/165, however two samples produced a product of the correct size with the 164/129 primers. DNA sequence analysis of the two transformants confirmed that 5 bp were missing from the 164 end while 213 bp had been deleted from the 165 end of the PCR product. This resulted in a cloned promoter of 633 bp (nt -632 to +4 of Figure 11). This plasmid was named p13.

pBI221 was treated with *Eco*RI methylase then digested with *Hind*III. The *Hind*III ends were filled using the Klenow fragment and an *Eco*RI site was created by ligation of *Eco*RI linkers. The DNA was digested with *Bam*HI and the large fragment was gel purified. The sugarcane promoter fragment from p13 was gel purified after *Eco*RI and *Bam*HI digestion and ligated to the gel purified pBI221 fragment. A Southern blot of *Eco*RI and *Bam*HI digested DNA was probed with gel purified SC to confirm the presence of the sugarcane promoter (Figure 15). This plasmid was referred to as pSCGN.

High Expression Construct EmuGN

Last et al. (1991) described the synthesis and expression levels obtained when using a synthetic promoter element fused to the GUS coding sequence and NOS termination signals. The promoter element was synthesized by fusing together promoter elements which had previously been shown to impart higher expression levels. The pEmuGN promoter consists of a truncated maize *Adh1* promoter and the *Adh1* intron 1 fused to 6ARE and 4OCS elements. The 6ARE element consists of one ARE enhancer element in the natural orientation preceded by five AREs in the reverse orientation. The 4OCS element is 4 copies of the OCS enhancer. Inclusion of the 4OCS element allows for high level aerobic expression.

All plasmid constructs used for protoplast transformation and promoter analysis are depicted in Figure 16.

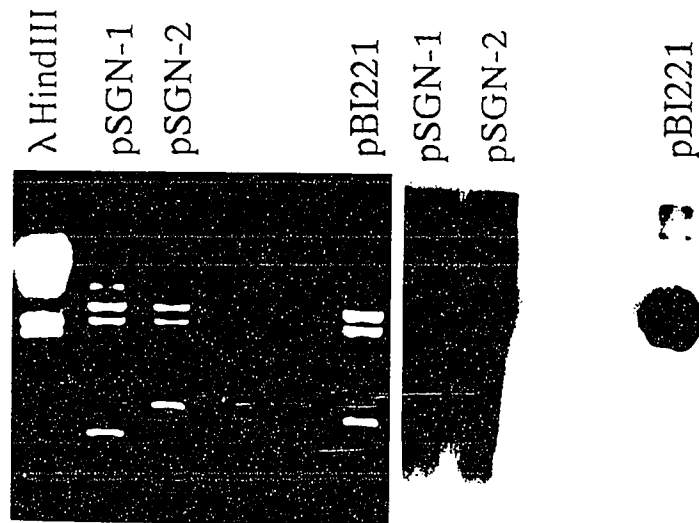


Figure 15. Agarose gel (right) Southern blot (left) of *Eco*RI, *Hind*III, and *Bam*HI digested pBI221 DNA (500 ng) and 200 ng of *Eco*RI and *Bam*HI digested DNA from transformants for pSCGN isolation. Probe was digoxigenin-labelled gel-purified SC (20 ng/ml).

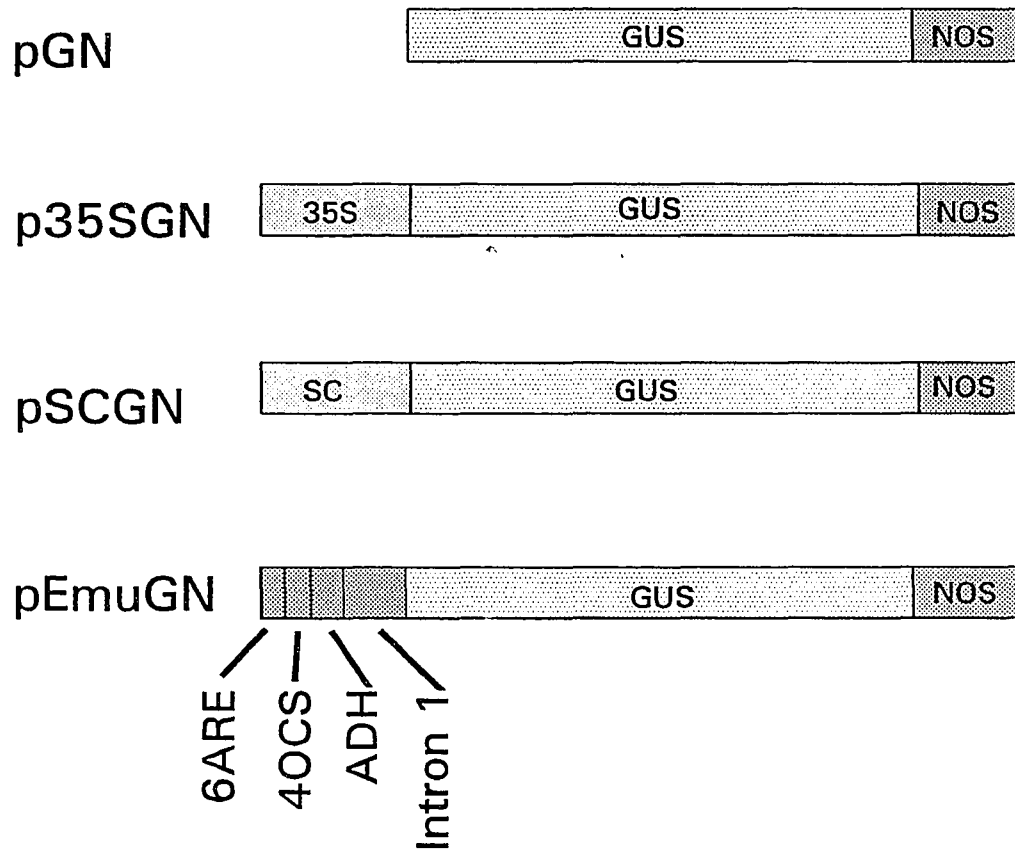


Figure 16. Representation of the reporter gene constructs used for establishing electroporation conditions and promoter analysis.

GUS EXPRESSION ASSAYS

Effective electroporation conditions were optimized using 300 μ l of protoplasts made from 7 or 8 day old suspension cells and 20 μ g of pEmuGN DNA (Last et al., 1991). Two pulses at 200 volts and 500 μ F gave the most consistently high expression (Table 1).

Table 1. Optimization of electroporation conditions. Each treatment was performed in triplicate at 200 volts. Activity is measured as the mean GUS activity \pm S.D.

Treatment	μ F	Pulse #	Activity (pmole MU/mg/min)
A	250	1	8.46 \pm 0.25
B	250	2	14.77 \pm 0.44
C	250	3	27.24 \pm 9.08
D	250	4	29.24 \pm 1.06
E	500	1	14.91 \pm 0.12
F	500	2	33.92 \pm 5.43
G	500	3	12.38 \pm 2.17

Expression levels obtained with the promoterless GUS construct pGN did not differ from electroporation in the absence of DNA and pGN was included as a control treatment in all experiments. Relative strengths of the promoters were determined by assaying GUS activity 18-24 hours after electroporation. The values given are means of at least five replica electroporations per assay (Table 2). In three of the four experiments, the expression level obtained by the 35S promoter was not significantly different from that obtained with the promoterless construct, according to the t-test ($p < 0.05$). In all experiments expression levels obtained using the SC promoter were

significantly greater than levels obtained using the 35S and -35S promoter constructs (t-test; $p < 0.05$).

Table 2. Mean GUS activities (pmole/mg protein/min \pm S.D.) following transient expression in sugarcane protoplasts for four experiments. Relative expression was obtained by subtracting the background level observed with pGN and taking the value of p35SGN as 1.0.

Experiment Number	Plasmid Construct	GUS Activity	Relative Activity
1	pGN	5.37 \pm 0.49	
	p35SGN	6.28 \pm 0.46	1.0
	pSCGN	9.84 \pm 1.01	4.9
2	pGN	5.66 \pm 0.54	
	p35SGN	6.20 \pm 0.42	1.0
	pSCGN	8.96 \pm 0.43	6.1
3	pGN	4.87 \pm 0.47	
	p35SGN	5.16 \pm 0.53	1.0
	pSCGN	6.42 \pm 0.61	5.3
	pEmuGN	53.87 \pm 17.01	53.3
4	pGN	8.90 \pm 0.64	
	p35SGN	9.10 \pm 0.60	1.0
	pSCGN	9.85 \pm 0.26	4.8
	pEmuGN	23.54 \pm 3.01	73.2

CHAPTER 4. DISCUSSION

ANTIBIOTIC RESISTANCE

As seen in Figure 1, growth of the sugarcane cells was inhibited by the antibiotics G418 and hygromycin B, while hygromycin B appears to be a better selection agent for sugarcane. Cell growth was inhibited at 0.5 $\mu\text{g/ml}$ hygromycin B while the cells grew well on plates containing the same concentration of G418. This is in agreement with the report of Hauptmann et al. (1988) which states that cultured cells of most grasses display a high natural resistance to kanamycin. Growth was completely inhibited at 2.0 $\mu\text{g/ml}$ for both antibiotics and selection should be performed at levels greater than this.

EXPRESSION

Clone $\lambda 7$ codes for a sugarcane transcript of approximately two kb in size. Results from the Northern slot blot of total RNA isolated throughout the 14 day suspension growth cycle indicate that the RNA coded by $\lambda 7$ is present throughout the two week growth cycle of suspension cells (Figure 6). Additionally, results of the RNase protection assay indicate that this transcript exists in suspension cell RNA as well as mature leaf RNA.

ANALYSIS OF A SUGARCANE PROMOTER

Approximately 950 bp of promoter region have been sequenced from this sugarcane gene. Several *cis*-acting elements which act as binding sites for nuclear proteins have been identified in the upstream regions of many plant genes (Table 3), but there was no homology to any of these elements in the upstream region of this sugarcane gene. However, a series of repetitive sequences indicative of protein binding sites were identified in the 5' flanking region of this gene. A repetitive sequence of (TGT)₈ was observed at nt -97 to -74, 73 bp upstream of the start site. This sequence is located just 15 bases upstream of the putative TATA element. At nt -396 to -391 and -366 to -361 there are direct repeats of the sequence 5'-AGGATG-3' while at nt -253 to -248 the sequence 5'-ACTTGT-3' is repeated twice. It is possible that some or all of these sequences function as protein binding sites in the regulation of transcription.

Table 3. *Cis*-acting elements reported in plant promoters.

<u>Consensus</u>	<u>Factor</u>	<u>Source</u>	<u>Reference</u>
ACGTCA	HBP-1a,b	H3	Tabata et al., 1991
CCCTCCC	AP2	<i>Sh-1</i>	Maas et al., 1990
CACGTGGC	EmBP-1	ABA	Guiltinam et al., 1990
ACGTGG	GBF	RBSC	Giuliano et al., 1988
GGTTAA	GT-1	<i>rbcS-3A</i>	Green et al., 1987
GCGGTAATT	GT-2	<i>phyA</i>	Dehesh et al., 1990
GGGCCGG	GC-1	<i>cabE</i>	Schindler and Cashmore, 1990
ATAAAAATAATT	AT-1	<i>cabE</i>	Schindler and Cashmore, 1990
CACGTG	CG-1	<i>chs15</i>	Staiger et al., 1989
GATA	GATA	<i>cab22</i>	Gidoni et al., 1989

Characteristics of this sugarcane promoter can be compared with plant promoter features compiled by Joshi (1987) who analyzed the 5'-flanking regions and the leader sequences of 79 published genomic DNA sequences from higher plants. Joshi focused on the region between a putative TATA box and the translation start site.

Primer extension experiments reported here confirmed that transcription initiation occurs at the cytosine residue located at nt 1. The sequence around the transcription initiation site is 5'-TTCCTGT-3' while the deduced consensus for higher plants is 5'-YTCATCA-3', with the A at the transcription initiation site present in 85% of the genes (Joshi, 1987). A survey limited to available grass genes was performed using the Genetics Computer Group Sequence Analysis Software Package, version 7.0 (Devereux et al., 1984). Twenty-one sequences were analyzed from maize, rice, wheat, and sugarcane. Figure 17 shows the consensus sequence derived by Joshi, the sugarcane sequence, and the sequences of the other grass genes in the region of the TATA box and the transcription initiation site. Five of the grass genes initiate transcription with a C residue while 10 of the 21 initiated transcription with a residue other than A. This suggests that the occurrence of an A residue at the transcription initiation site may be more specifically a feature of dicot transcription initiation.

A putative TATA sequence is located at nt -59 and has the sequence 5'-TATAAATTT-3'. Joshi (1987) determined that the consensus sequence for the TATA box and its flanking region in plants is 5'-TATATATAG-3'. This sequence was based on information available for both monocot and dicot plants. Ten of the 21 grass genes in Figure 17, including the sugarcane promoter, had the sequence

TATAAAT. Thus, it appears that recent monocot data weakens the consensus sequence of Joshi.

Joshi (1987) reported the distance between the TATA box and transcription start site as 32 ± 7 nt, with the largest distance of 66 nt found in the maize triose isomerase gene (57). Distances of 58 nt and 51 nt were observed for the maize glycine-rich protein gene and the wheat α -amylase gene, respectively (Figure 17). While the distance of 57 nt observed in the sugarcane sequence is at the limit of the range and greater than average, it represents the best fit to the consensus sequence. Nevertheless, it has not been proven that this sequence represents a functional TATA element.

The CAAT box is a sequence element involved in promoting efficient initiation of transcription predominantly in mammalian systems. If present, it is usually located 40 to 110 bases upstream of the transcription start site and has the consensus sequence 5'-CCAAT-3'. The sequence 5'-ACAAT-3' was observed at nt -184, 180 bp upstream of the transcription initiation site. This sequence is probably not a functional CAAT sequence, based on its location.

Results of the RNase protection assay imply that an intron 5' splice site is present approximately 70 nt from the transcription start site. The sequence 5'-CAG/GTAATT-3' at nt 70 to 78 is a very good match for the 5' splice site consensus sequence of 5'-CAG/GTAAGT-3' (Jackson, 1991). Splicing at this site would produce a 72 nt noncoding exon. Most plant introns are between 70 and 1000 nt in length (Hawkins 1988) and therefore the 3' splice site is probably not present in this clone. Goodall and Filipowicz (1991) report that unlike dicot introns, monocot introns may be G-C rich. GC-rich introns tend to have good matches to the 5' and 3' consensus sequences while the splice sites of AU-rich introns do not necessarily

conform to the consensus sequences. Although incomplete, the available sequence information indicates that this sugarcane intron is AU-rich with 70% of the 56 bases sequenced being A or U. Consequently, sequence information alone may make it difficult to determine the 3' splice site of this intron. A schematic of the promoter and its relationship to $\lambda 7$ is given in Figure 18.

<u>Source</u>	<u>Sequence</u>
Consensus	<u>ACTATATATAG</u> ...32... <u>YTCATCA</u>
Sugarcane	<u>GATATAAATTT</u> ...57... <u>TTCC^UTGT</u>
ZeinZc2 (maize)	<u>GCTATAAATAA</u> ...33... <u>CTCCATC</u>
ZeinZc1 (maize)	<u>GCTATAAATAA</u> ...29... <u>ACTT^TCCTC</u>
Glycine-rich protein (maize)	<u>GCTATAAATCT</u> ...58... <u>TGGC^TTTC</u>
Adh1-1S (maize)	<u>ACTATATAAAT</u> ...40... <u>CTC^ATCT</u>
Adh1-1F (maize)	<u>ACTATATAAAT</u> ...40... <u>CTC^ATCT</u>
Glutelin (rice)	<u>ACTATAAATGC</u> ...28... <u>GTTT^TCCTC</u>
α -amylase (rice)	<u>ACTATAAATAC</u> ...34... <u>TTC^ATCA</u>
RAB21 (rice)	<u>CTTATAAATGG</u> ...30... <u>GCTT^GGCA</u>
α -amylase (wheat)	<u>CCTATAAATAC</u> ...51... <u>GGT^CCAG</u>
Oryzacystatin (rice)	<u>GCTATAAAATC</u> ...35... <u>AAT^ATTCG</u>
Oleosin (maize)	<u>CCTATATATGG</u> ...39... <u>TACT^TCCG</u>
HMW Glutenin (wheat)	<u>CCTATAAAAGC</u> ...33... <u>ATC^ATCA</u>
Gib. resp. protein (wheat)	<u>GCTATAAAGCC</u> ...31... <u>TCC^GCCA</u>
γ -gliadin (wheat)	<u>GCTATAAAAAG</u> ...30... <u>CAT^ACCA</u>
α/β -gliadin (wheat)	<u>GCTATAAAAAG</u> ...37... <u>CTC^ACCC</u>
aldolase (maize)	<u>TTTAAATACAC</u> ...31... <u>CCG^CCCTG</u>
cab (wheat)	<u>TTTAAATAGCT</u> ...29... <u>TTAA^AACC</u>
Sucrose synthase (maize)	<u>TCTATTTATTG</u> ...29... <u>GAGA^AAAC</u>
Cab-1 (maize)	<u>GCTATTTAGCC</u> ...34... <u>TCC^ACCA</u>
PEPC1 (maize)	<u>CCTATTTGAAC</u> ...30... <u>ATC^ACCA</u>

Figure 17. Comparison of the derived TATA and transcription initiation sequences (Joshi, 1987) with those observed for the sugarcane promoter. The numbers represent the nt between the first T of the TATA sequence and the nucleotide prior to the transcription start site.

Joshi observed that most plant genes are consistent with Kozak's scanning hypothesis (Kozak, 1978) which states that translation initiation occurs at the first ATG codon downstream of the transcription start site. Joshi observed only 6 genes with ATGs upstream from the initiation codon. In three lectin encoding genes of *Phaseolus vulgaris*, all the ATGs in the leader sequences are in suboptimum context and only the predicted functional ATG fulfills the condition of longest open reading frame (Hoffman, 1984; Hoffman & Donaldson, 1985). For the remaining three genes, soybean actin gene PSAc3 (Shah et al., 1982), maize actin gene (Shah et al., 1983), and the maize GST 1 gene (Shah et al., 1986), the upstream ATGs are followed by in-frame termination codons before the functional ATG is reached. The leader region of this sugarcane gene contains two ATG initiation codons at nt 16 and 39 followed closely by in-frame termination codons. A third ATG codon at nt 80 is located in the probable intron sequence and would not be present in the mature mRNA. The location of the functional ATG codon is not known. It is possible that the short translation products in the 5' leader region are involved in regulating the rate of gene expression. Putterill and Gardner (1989) and Rogers et al. (1985) observed reduced gene expression in gene constructs containing translation initiation codons upstream of the proper initiation codon.

As determined by Joshi, most plant genes have leader regions which are A-T rich. The sequence information indicates that this sugarcane leader region is 67% A-T from the transcription start site to the end of the available sequence in the first intron. Assuming mRNA processing occurs at the intron splice site, the exon 1 sequence is 65% A-T.

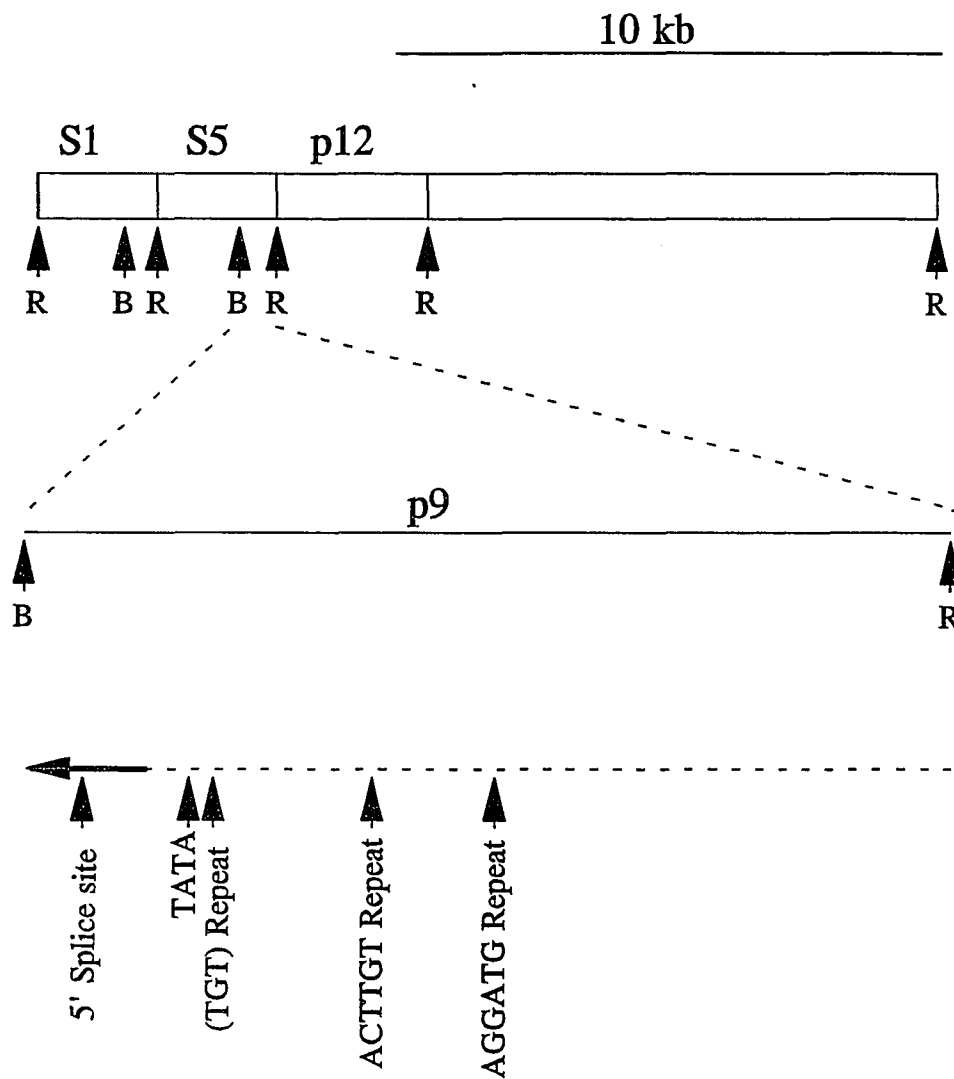


Figure 18. Schematic of the relationship of $\lambda 7$, the p9 subclone, and the sugarcane promoter fragment in p9. The primary transcript is identified by a bold line and the arrow indicates the direction of transcription.

ANALYSIS OF PROMOTER FUNCTION

Many researchers have analyzed the function of promoter elements in monocot transformation but few have looked at the expression of a chimeric gene whose promoter is derived from the genome of the same plant.

McElroy et al. (1990) examined the efficiency of a rice actin promoter in the transformation of rice protoplasts. The rice actin promoter was 5 to 10 times more active than the maize *Adh1* promoter in transformed rice cells. The relative strengths of promoters from like genes (rice *Act1* versus maize *Act1*) or an analysis of the promoter strengths in maize were not investigated.

Callis et al. (1987) compared promoter efficiencies in maize in the presence and absence of introns. The maize *Adh1* promoter (with the intron 1 included) was 6.7 to 7.3 times as effective as the CaMV 35S promoter for the expression of CAT, NPTII, and luciferase in maize.

Vasil et al. (1989) reported expression levels obtained using the maize *Sh1* and 35S promoter elements for the transformation of maize. The *Sh1* promoter element consisted of 2000 bp upstream of the transcription initiation site through the first noncoding exon and the first intron. The highest level of expression with the *Sh1* promoter was 1.6 times greater than that observed with the 35S promoter in maize and 3 to 5 times higher in the heterologous plants *Panicum* and *Pennisetum*. Expression levels were quite variable with the *Sh1* promoter and more consistent with the 35S promoter. Maas et al. (1991) observed that a promoter element containing the maize *Sh1* exon 1 and intron 1 dramatically increased expression in maize and rice. This promoter was however, a fusion of the 35S promoter and the *Sh1* elements rather than a true maize promoter. It has been reported that *Sh1* intron 1 is a stronger promoter element than the *Adh1* intron 1 (Vasil et al., 1989) but this does not reflect

the relative strengths of the intact promoter elements. No side by side comparisons of maize *Sh1*, maize *Adh1*, and CaMV 35S as promoter elements for gene expression in maize have been reported.

Attempts were made to minimize variability in data for the determination of relative promoter strengths. Electroporation efficiencies vary greatly from experiment to experiment and consequently, results were only compared within each experiment. Each electroporation was performed in replicates of five as a means of reducing variability. Inclusion of an internal standard such as cotransformation with a separate reporter gene was not included in these experiments. By incorporating such a standard the variability may be further reduced. Results of the GUS fluorometric assay (Table 2) demonstrate that the sugarcane promoter used in this study is an effective promoter element for heterologous gene expression in sugarcane suspension cells. The expression level obtained using the CaMV 35S promoter was not significantly higher than the background level. The relative GUS activity obtained using the sugarcane promoter was consistently about 5 times that obtained with the 35S promoter element. This compares favorably with results obtained using the maize *Adh1* promoter for heterologous gene expression in maize (Callis et al. 1987). The pEmuGN construct exhibited a much higher level of expression. Last et al. (1991) indicated that pEmuGN results in a 50- to 100-fold increase in GUS expression over p35SGN in sugarcane, which correlates well with the results of this study.

POTENTIAL FOR IMPROVEMENT

While the sugarcane promoter region used in this experiment was sufficient to direct expression of a foreign gene in sugarcane suspension cells, it is likely that

the level of expression can be increased. Inclusion of the first introns of maize *Sh1*, *Adh1*, and *Bz1* and rice *Act1* into the promoter constructs resulted in substantially higher gene expression in other monocots (Vasil et al., 1989, Callis et al., 1987, McElroy et al., 1990). DNA sequence analysis, primer extension, and RNase protection assay results indicate that this sugarcane transcript also contains an intron in the 5' leader region. Therefore it may be advantageous to include this intron in the promoter construct for increased expression. A logical promoter construct would include the putative promoter elements upstream of the transcription initiation site, the noncoding exon 1, the intron 1, and possibly sequences of exon 2 up to the translation initiation codon.

The leader region of the sugarcane gene contains two translation initiation codons followed closely by in-frame termination codons. These sequences may be involved in regulating the rate of gene expression and mutagenesis of these sequences could potentially result in increased gene expression. Putterill and Gardner (1989) monitored the expression of GUS fusion products in plant cells after introducing changes to the leader sequence. Their results adhered to Kozak's scanning model but also suggested the option of translational termination followed by reinitiation at nearby start codons. Constructs containing ATGs upstream and out of frame of the functional ATG displayed diminished GUS expression. Rogers et al. (1985) noted that expression of the NPTII gene was improved when an AUG upstream of the proper start codon was removed.

The method of chimeric gene construction can have a detrimental effect on gene expression. Jones et al. (1985) reported that synthetic linker sequences in plant gene promoters may adversely effect mRNA levels. The plasmid pSCGN contained

polylinker sequence from the cloning vector pUC19 which should be avoided in any future chimeric gene constructs.

In conclusion, an actively transcribed gene has been isolated from a sugarcane suspension culture genomic library. This gene codes for an mRNA of approximately 2 kb which is present throughout the growth cycle of the suspension culture. The promoter element of this gene has been isolated and fused to a reporter gene. This promoter has been shown to direct the expression of the heterologous coding sequence in transiently transformed suspension culture protoplasts. The level of expression obtained with this promoter is similar to that obtained in other monocot systems using similar chimeric gene constructs. Additionally, a higher level of expression may be achieved by inclusion of the nontranslated leader sequence and first intron of the gene. This promoter fragment and further derivatives of it should be useful for future transformation studies in sugarcane suspension cells and where gene expression is desired in leaves of sugarcane plants.

REFERENCES

- Akiyoshi, D.E., Klee, J., Amasino, R.M., Nester, E.W., Gordon, M.P. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA **81**, 5994-5998.
- Altenbach, S.B., Pearson, K.W., Meecker, G., Staraci, L.C., and Sun, S.S.M. (1989). Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methionine-rich protein in transgenic plants. Plant Mol. Biol. **13**, 513-522.
- Alton N.K., and Vapnek, D. (1979). Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. Nature **282**, 864-869.
- An, G., Watson, B.D., Stachel, S., Gordon, M.P. and Nester, E.W. (1985). New cloning vehicles for transformation of higher plants. EMBO J. **4**, 277-284.
- Armstrong, C., Petersen, W., Buchholz, W., Bowen, B., and Sulc, S. (1990). Factors affecting PEG-mediated stable transformation of maize protoplasts. Plant Cell Rep. **9**, 335-339.
- Aryan, A.P., An, G., and Okita, T.W. (1991). Structural and functional analysis of promoter from gliadin, an endosperm-specific storage protein gene of *Triticum aestivum* L. Mol. Gen. Genet. **225**, 65-71.
- Baba, A., Hasezawa, S., and Syono, K. (1986). Cultivation of rice protoplasts and their transformation mediated by *Agrobacterium* spheroplasts. Plant Cell Physiol. **27**, 463-471.
- Barry, G.F., Rogers, S.G., Fraley, R.T., and Brand, L. (1984). Identification of a cloned cytokinin biosynthetic gene. Proc. Natl. Acad. Sci. USA **81**, 4776-4780.
- Baulcombe, D.C., Saunders, G.R., Bevan, M.W., Mayo, M.A., and Harrison, B.D. (1986). Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature **321**, 446-449.
- Baumlein, H., Boerjan, W., Nagy, I., Panitz, R., Inze, D., and Wobus, U. (1991). Upstream sequences regulating legumin gene expression in heterologous transgenic plants. Mol. Gen. Genet. **225**, 121-128.
- Beck, E., Ludwig, G., Auerwald, E.A., Reiss, B., and Schaller, H. (1982). Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene **19**, 327-336.
- Bekkaoui, F., Pilon, M., Laine, E., Faju, D.S.S., Crosby, W.L., and Dunstan, D.I. (1988). Transient gene expression in electroporated *Picea glauca* protoplasts. Plant Cell Rep. **7**, 481-484.

- Bellini, C., Chupeau, M., Guerche, P., Bastra, G., and Chupear, Y. (1989). Transformation of *Lycopersicon peruvianum* and *Lycopersicon esculentum* mesophyll protoplasts by electroporation. *Plant Science* **65**, 63-75.
- Benfey, P.N., and Chua, N.-H. (1989). Regulated genes in transgenic plants. *Science* **244**, 174-181.
- Benfey, P.N., Ren, L., and Chua, N.-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. *EMBO J.* **8**, 2195-2202.
- Benoist, C., and Chambon, P. (1981). *In vivo* sequence requirements of the SV40 early promoter region. *Nature* **290**, 304-310.
- Bevan, M., and Flavell, R. (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* **304**, 184-187.
- Bevan, M., Barnes, W.M., and Chilton, M.D. (1983). Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucl. Acids Res.* **11**, 369-385.
- Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**, 8711-8721.
- Bielmann, A., Pfitzner, A., Goodman, H., and Pfitzner, U. (1991). Functional analysis of the pathogenesis-related 1a protein gene minimal promoter region. *Eur. J. Biochem.* **19**, 415-421.
- Birnboim, H.C., and Doly, J. (1979). A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. *Nucl. Acids Res.* **7**, 1513-1523.
- Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41**, 521-530.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Brisson, N., Paszkowski, J., Penswick, J., Gronenborn, B., Potrykus, I., and Hohn, T. (1984). Expression of a bacterial gene in plants by using a viral vector. *Nature* **310**, 511-514.
- Bruce, W.B., Christensen, A., Klein, T., Fromm, M., and Quail, P.H. (1989). Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. *Proc. Natl. Acad. Sci. USA* **86**, 9692-9696.
- Bruce, W.B., and Quail, P.H. (1990). *cis*-acting elements involved in photoregulation of an oat phytochrome promoter in rice. *Plant Cell* **2**, 1081-1089.

- Bustos, M., Guiltinan, M., Jordano, J., Begum, K., Kalkan, F., and Hall, T. (1989). Regulation of β -glucuronidase expression in transgenic tobacco plants by an A/T-rich, *cis*-acting sequence found upstream of a French bean β -phaseolin gene. *Plant Cell* **1**, 839-853.
- Bustos, M., Begum, D., Kalkan, F., Batraw, M., and Hall, T. (1991). Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. *EMBO J.* **10**, 1469-1479.
- Bytebier, B., Deboeck, F., Greve, H., Van Montagu, M., and Hernalsteens, J. (1987). T-DNA organization in tumor cultures and transgenic plants of the monocotyledon *Asparagus officinalis*. *Proc. Natl. Acad. Sci. USA* **84**, 5345-5349.
- Callis, J., Fromm, M., and Walbot, V. (1987). Introns increase gene expression in cultured maize cells. *Genes Dev.* **1**, 1183-1200.
- Chaleff R.S., and Ray, T.B. (1984). Herbicide-resistant mutants from tobacco cell cultures. *Science* **223**, 1148-1151.
- Chee, P.P., Klassy, C., and Slightom, J. (1986). Expression of a bean storage protein "phaseolin minigene" in foreign plant tissues. *Gene* **41**, 47-57.
- Chen, W.H., Gartland, K.M.A., Davey, M.R., Sotak, R., Gartland, 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 Rep.* **6**, 297-301.
- Chen Z.-L., Pan N.-S., and Beachy, R.N. (1988). A DNA sequence element that confers seed-specific enhancement to a constitutive promoter. *EMBO J.* **7**, 297-302 .
- Cheung, A.Y., Bogorad, L., Van Montagu, M., and Schell, J. (1988). Relocating a gene for herbicide tolerance: A chloroplast gene is converted into a nuclear gene. *Proc. Natl. Acad. Sci. USA* **85**, 391-395.
- Christou, P., Murphy, J., and Swain, W.F. (1987). Stable transformation of soybean by electroporation and root formation from transformed callus. *Proc. Natl. Acad. Sci. USA* **84**, 3962-3966.
- Christou, P., McCabe, D.E., and Swain, W.F. (1988). Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiol.* **87**, 671-674.
- Christou, P., McCabe, D.E., Martinell, B.J. and Swain, W.F. (1990). Soybean genetic transformation-commercial production of transgenic plants. *Trends Biotechnol.* **8**, 145-151.
- Chupeau, M., Bellini, C., Guerche, P., Maisonneuve, B., Vastra, G., and Chupeau, Y. (1989). Transgenic plants of lettuce (*Lactuca sativa*) obtained through electroporation of protoplasts. *Bio/Technology* **7**, 503-508.

- Comai, L., Facciotti, Hiatt, W.R., Thompson, G., Rose, R.E., and Stalker, D.M. (1985). Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. *Nature* **317**, 741-743.
- Colot, V., Robert, L.S., Kavanagh, T.A., Bevan, M.W., and Thompson, R.D. (1987). Localisation of sequences in wheat endosperm protein genes which confer tissue specific expression in tobacco. *EMBO J.* **6**, 3559-3564.
- Cuozzo, M., O'Connell, K., Kaniewski, W., Fang, R., Chua, N., and Tumer, N. (1988). Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Bio/Technology* **6**, 549-557.
- Daniell, H., Vivekananda, J., Nielsen, B., Ye, G., Tewari, K., and Sanford, J. (1990). Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proc. Natl. Acad. Sci. USA* **87**, 88-92.
- Datta S., Peterhans A., Datta K., and Portykus I. (1990). Genetically engineered fertile Indica-rice recovered from protoplasts. *Bio/Technology* **8**, 736-740.
- Davey, M.R., Cocking, E.C., Freeman, J., Pearce, N., and Tudor, I. (1980). Transformation of *Petunia* protoplasts by isolated *Agrobacterium* plasmids. *Plant Sci. Lett.* **18**, 307-313.
- De Block, M., Herrera-Estrella, L., Van Montagu, M., Schell, J., and Zambryski, P. (1984). Expression of foreign genes in regenerated plants and in their progeny. *EMBO J.* **3**, 1681-1689.
- De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Rao Movva, N., Thompson, C., Van Montagu, M., and Lee Mans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J.* **6**, 2513-2518.
- De Block, M., Brouwer, D.D., and Tenning, P. (1989). Transformation of *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. *Plant Physiol.* **91**, 694-701.
- De Clercq, A., Vandewiele, M., Van Damme, J., Guerche P., Van Montagu, M., Vandekerckhove, J., and Krebbers, E. (1990). Stable accumulation of modified 2S albumin seed storage proteins with higher methionine contents in transgenic plants. *Plant Physiol.* **94**, 970-979.
- De Greve, J., Dhaese, P., Seurinck, J., Lemmers, M., Van Montagu, M., and Schell, J. (1983). Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid encoded octopine synthase gene. *J. Mol. Appl. Genet.* **2**, 499-511.
- Dehesh, K., Bruce, W.B., and Quail, P.H. (1990). A *trans*-acting factor that binds to a GT-motif in a phytochrome gene promoter. *Science* **250**, 1397-1399.

- Delannay, X., LaVallee, B., Proksch, R., Fuchs, R., Sims, S., Greenplate, J., Marrone, P., Dodson, R., Augustine, J., Layton, J., and Fischhoff, D. (1989). Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. *Kurstaki* insect control protein. *Bio/Technology* **7**, 1265-1269.
- De la Pena, A., Lorz, H., and Schell, J. (1987). Transgenic plants obtained by injecting DNA into young floral tillers. *Nature* **325**, 274-276.
- Deshayes, A., Herrera-Estrella, L., and Cabocho, M. (1985). Liposome-mediated transformation of tobacco mesophyll protoplasts by an *Escherichia coli* plasmid. *EMBO J.* **4**, 2731-2737.
- Devereux, J.R., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**, 387-395.
- Dierks, P., Van Ooyen, A., Cochran, M.D., Dobkin, C., Reiser, J., Weissmann, C. (1983). Three regions upstream from the cap site are required for efficient and accurate transcription of the rabbit β -globin gene in mouse 3T6 cells. *Cell* **32**, 695-706.
- Doerner, P., Stermer, B., Schmid, J., Dixon, R., and Lamb, C. (1990). Plant defense gene promoter-reporter gene fusions in transgenic plants: Tools for identification of novel inducers. *Bio/Technology* **8**, 845-848.
- Drocourt, D., Calmels, T., Reynes, J., Baron, M., and Tiraby, G. (1990). Cassettes of the *Streptoalloteichus hindustanus ble* gene for transformation of lower and higher eukaryotes to phleomycin resistance. *Nucl. Acids Res.* **18**, 4009.
- Dudler, R., and Travers, A. (1984). Upstream elements necessary for optimal function of the hsp 70 promoter in transformed flies. *Cell* **38**, 391-398.
- Efstratiadis, A., Posakony, J.W., Maniatis, T., Lawn, R.M., O'Connell, C., Spritz, R.A., DeRiel, J.K., Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C., Proudfoot, N.J. (1980). The structure and evolution of the human β -globin gene family. *Cell* **21**, 653-668.
- Eichholtz, D.A., Rogers, S.G., Horsch, R.B., Klee, H.J., Hayford, M., Hoffmann, N.L., Bradford, S.B., Fink, C., Flick, J., O'Connell, K.M., and Fraley, R.T. (1987). Expression of mouse dihydrofolate reductase gene confers methotrexate resistance in transgenic petunia plants. *Somatic Cell Mol. Genet.* **13**, 67-76.
- Ellis, J., Llewellyn, D., Dennis, E., and Peacock, W. (1987). Maize *Adh-1* promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco. *EMBO J.* **6**, 11-16.

- Elliston, K., Messing, J. (1989). The molecular architecture of plant genes and their regulation. In: *Plant Biotechnology*. Butterworths Publishers, Boston, pp 115-139.
- Enomoto, S., Itoh, H., Ohshima, M., and Ohashi, Y. (1990). Induced expression of a chimeric gene construct in transgenic lettuce plants using tobacco pathogenesis-related protein gene promoter region. *Plant Cell Rep.* **9**, 6-9.
- Everett, R.D., Baty, D., and Chambon, P. (1983). The repeated GC-rich motifs upstream from the TATA box are important elements of the SV40 early promoter. *Nucl. Acids Res.* **11**, 2447-2464.
- Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.
- Fischhoff, D.A., Bowditch, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G., and Fraley, R.T. (1987). Insect tolerant transgenic tomato plants. *Bio/Technology* **5**, 807-813.
- Fitch, M.M.M., Manshardt, R.M., Gonsalves, D., Slightom, J.L., and Sanford, J.C. (1990). Stable transformation of papaya via microprojectile bombardment. *Plant Cell Rep.* **9**, 189-194.
- Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P., Flick, J., Adams, S., Bittner, M., Brand, L., Fink, C., Fry, J., Galluppi, G., Goldberg, S., Hoffmann, N., and Woo, S. (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA* **80**, 4803-4807.
- Fraley, R.T., Rogers, S.G., Horsch, R.B., Eichholtz, D.A., Flick, J.S., Adams, S., Bittner, M., Brand, L., Fink, C., Fry, J., Galluppi, J., Goldberg, S., Hoffmann, N., and Woo, S. (1985). The SEV system: A new disarmed Ti plasmid vector system for plant transformation. *Bio/Technology* **3**, 629-635.
- Fromm, M., Taylor, L., and Walbot, V. (1985). Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proc. Natl. Acad. Sci. USA* **82**, 5824-5828.
- Fromm, M., Taylor, L., and Walbot, V. (1986). Stable transformation of maize after gene transfer by electroporation. *Nature* **319**, 791-793.
- Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., and Klein, T.M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Bio/Technology* **8**, 833-838.
- Gasser, C., Simonsen, C., Schilling, J., and Schimke, R. (1982). Expression of abbreviated mouse dihydrofolate reductase genes in cultured hamster cells. *Proc. Natl. Acad. Sci. USA* **79**, 6522-6526.

- Genilloud, O., Garrido, M., and Moreno, F. (1984). The transposon *Tn5* carries a bleomycin resistance determinant. *Gene* **32**, 225-233.
- Gerlach, W., Llewellyn, D., and Haseloff, J. (1987). Construction of a plant disease resistance gene from the satellite RNA of tobacco ringspot virus. *Nature* **328**, 802-805.
- Gidoni, D., Brosio, P., Bond-Nutter, D., Bedbrook, J., Dunsmuir, P. (1989). Novel *cis*-acting elements in *Petunia Cab* gene promoters. *Mol. Gen. Genet.* **215**, 337-344.
- Gillies, S.D., Morrison, S.L., Oi, V.T., and Tonegawa, S. (1983). A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. *Cell* **33**, 717-728.
- Giuliano, G., Pichersky, E., Malik, V.S., Timko, M.P., Scolnik, P.A., and Cashmore, A.R. (1988). An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. *Proc. Natl. Acad. Sci. USA* **85**, 7089-7093.
- Goodall, G., and Filipowics, W. (1991). Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants. *EMBO J.* **10**, 2635-2644.
- Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* **2**, 603-618.
- Gould, J., Devey, M., Hasegawa, O., Ulian, E., Peterson, G., and Smith, R. (1991). Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. *Plant Physiol.* **95**, 426-434.
- Graham, J., McNicol, R., Kumar, A. (1990). Use of the GUS gene as a selectable marker for *Agrobacterium*-mediated transformation of *Rubus*. *Plant Cell, Tissue and Organ Culture* **20**, 35-39.
- Graves, A., and Goldman, S. (1986). The transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens*. *Plant Mol. Biol.* **7**, 43-50.
- Green, P.J., Kay, S.A., and Chua, N.H. (1987). Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the *rbcS-3A* gene. *EMBO J.* **6**, 2543-2549.
- Grimsley, N.H., Hoh, T., Davies, J.W., and Hoh, B. (1987). *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. *Nature* **325**, 177-179.
- Gritz, L., and Davies, J. (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**, 179-188.

- Gruss, P., and Khoury, G. (1980). Rescue of a splicing defective mutant by insertion of an heterologous intron. *Nature* **286**, 634-637.
- Gruss, P., Dhar, R., Khoury, G. (1981). Simian virus 40 tandem repeated sequences as an element of the early promoter. *Proc. Natl. Acad. Sci. USA* **78**, 943-947.
- Guerche, P., Charbonnier, M., Jouanin, L., Tourneur, C., Paszkowski, J., and Pelletier, G. (1987). Direct gene transfer by electroporation in *Brassica napus*. *Plant Science* **52**, 111-116.
- Guerineau, F., Brooks L., Meadows, J., Lucy, A., Robinson, C., and Mullineaux, P. (1990). Sulfonamide resistance gene for plant transformation. *Plant Mol. Biol.* **15**, 127-136.
- 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 characterization of transcripts. *Cell* **30**, 763-773.
- Gultinam, M.J., Marcotte, Jr., W.R., and Quatrano, R.S. (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. *Science* **250**, 267-271.
- Hamer, D., and Leder, P. (1979). Splicing and the formation of stable RNA. *Cell* **18**, 1299-1302.
- Harrison, B., Mayo, M., and Baulcome, D. (1987). Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. *Nature* **328**, 799-802.
- Hauffe, K., Paszkowski, U., Schulze-Lefert, P., Hahlbrock, K., Dangl, J., and Douglas, C. (1991). A parsley 4CL-1 promoter fragment specifies complex expression patterns in transgenic tobacco. *Plant Cell* **3**, 435-443.
- Haughn, G.W., Smith, J., Mazur, B., and Somerville, C. (1988). Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. *Mol. Gen. Genet.* **211**, 266-271.
- Hauptmann, R.M., Ozias-Akins, P., Vasil, V., Tabaeizadeh, Z., Rogers, S.G., Horsch, R.B., Vasil, I.K., and Fraley, R.T. (1987). Transient expression of eletroporated DNA in monocotyledonous and dicotyledonous species. *Plant Cell Rep.* **6**, 161-168.
- 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 transformation in the Graminae. *Plant Physiol.* **86**, 602-606.
- Hawkins, J.D. (1988). A survey on intron and exon length. *Nucl. Acids Res.* **16**, 9893-9908.

- Hayashimoto, A., Li, Z., and Murai, N. (1990). A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. *Plant Physiol.* **93**, 857-863.
- 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 acetyltransferases. *Plant Phys.* **86**, 1216-1222.
- Helmer, G., Casadban, M., Bevan, M., Kayes, L., and Chilton, M. (1984). A new chimeric gene as a marker for plant transformation: the expression of *Escherichia coli* β -galactosidase in sunflower and tobacco cells. *Bio/Technology* **2**, 520-527.
- Hemenway, C., Fang, R., Kaniewski, W., Chua, N., and Tumer, N. (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO J.* **7**, 1273-1280.
- Henikoff, S., and Eghtedarzadeh, M.K. (1987). Conserved arrangement of nested genes at the drosophila *Gart* locus. *Genetics* **117**, 711-725.
- Hernalsteens, J., Thia-Toong, L., Schell, J., and Van Montagu, M. (1984). An *Agrobacterium*-transformed cell culture from monocot *Asparagus officinalis*. *EMBO J.* **3**, 3039-3041.
- Herrerra-Estrella, L., De Block, M., Messens, E., Hernalsteens, J., Van Montagu, M., and Schell, J. (1983). Chimeric genes as dominant selectable markers in plant cells. *EMBO J.* **2**, 987-995.
- Hoekema, A., Hirsch, P., Hooykaas, P., and Schilperoort, R. (1983). A binary plant vector strategy based on separation of the *vir*- and T-region of the *Agrobacterium tumefaciens* Ti plasmid. *Nature* **303**, 179-180.
- Hoffman, L.M. (1984). Structure of a chromosomal *Phaseolus vulgaris* lectin gene and its transcript. *J. Mol. Appl. Genet.* **2**, 447-453.
- Hoffman, L.M., Donaldson, D.D. (1985). Characterization of two *Phaseolus vulgaris* phytohemagglutinin genes closely linked on the chromosome. *EMBO J.* **4**, 883-889.
- Hoffman, L.M., Donaldson, D.D., Bookland, R., Rashka, K., and Herman, E.M. (1987). Synthesis and protein body deposition of maize 15-kd zein in transgenic tobacco seeds. *EMBO J.* **6**, 3213-3221.
- Hooykaas-Van Slogteren, G., Hooykaas, P., and Schilperoort, R. (1984). Expression of Ti-plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. *Nature* **311**, 763-764.
- Horn, M., Shillito, R., Conger, B., and Harmes, C. (1988). Transgenic plants of orchardgrass (*Dactylis glomerata* L.) from protoplasts. *Plant Cell Rep.* **7**, 469-472.

- Horsch, R.B., Fraley, R.T., Rogers, S.G., Sanders, P.R., Lloyd, A., and Hoffmann, N. (1984). Inheritance of functional foreign genes in plants. *Science* **223**, 496-498.
- Horsch, R.B., Fry, J.E., Hoffmann, N., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. *Science* **277**, 1229-1231.
- Horsch, R.B., and Klee, H.J. (1986). Rapid assay of foreign gene expression in leaf discs transformed by *Agrobacterium tumefaciens*: Role of T-DNA borders in the transfer process. *Proc. Natl. Acad. Sci. USA* **83**, 4428-4432.
- Hu, C., Chee, P., Chesney, R., Zhou, J., and Miller, P. (1990). Intrinsic GUS-like activities in seed plants. *Plant Cell Rep.* **9**, 1-5.
- Huang, Y., and Dennis, E. (1989). Factors influencing stable transformation of maize protoplasts by electroporation. *Plant Cell, Tissue and Organ Culture* **18**, 281-296.
- Ishige, R., Ohshima, M., and Ohashi, Y. (1991). Transformation of Japanese potato cultivars with the β -glucuronidase gene fused with the promoter of the pathogenesis-related 1a protein gene of tobacco. *Plant Science* **73**, 167-174.
- Jackson, I.J. (1991). A reappraisal of non-consensus mRNA splice sites. *Nucl. Acids Res.* **19**, 3795-3798.
- Jefferson, R.A., Burgess, S.M., and Hirsh, D. (1986). β -Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* **83**, 8447-8451.
- 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 J.* **6**, 3901-3907.
- Joersbo, M., and Brunstedt, J. (1990). Direct gene transfer to plant protoplasts by mild sonication. *Plant Cell Rep.* **9**, 207-210.
- Jones, J.D.G., Dunsmuir, P., and Bedbrook, J. (1985). High level expression of introduced chimeric genes in regenerated transformed plants. *EMBO J.* **4**, 2411-2418.
- Jones, J.D.G., Svab, Z., Harper, E.C., Hurwitz, C.D., and Maliga, P. (1987). A dominant nuclear streptomycin resistance marker for plant cell transformation. *Mol. Gen. Genet.* **210**, 86-91.
- Jones, J., Ooms, G., and Jones, M.G.K. (1989). Transient gene expression in electroporated *Solanum* protoplasts. *Plant Mol. Biol.* **13**, 503-511.
- Joshi, C.P. (1987). An inspection of the domain between putative TATA box and translation start site in 79 plant genes. *Nucl. Acids Res.* **15**, 6643-6653.

- Kaniewski, W., Lawson, C., Sammons, B., Haley, L., Hart, J., Delannay, X., and Turner, N. (1990). Field resistance of transgenic Russet Burbank potato to effects of infection by potato virus X and potato virus Y. *Bio/Technology* **8**, 750-754.
- Kay, R., Chan, A., Daly, M., and McPherson, J. (1987). Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* **236**, 1299-1302.
- Klee, H., White, F., Iyer, V., Gordon, M., and Nester, E. (1983). Mutational analysis of the virulence region of an *Agrobacterium tumefaciens* Ti plasmid. *J. Bacteriol.* **153**, 878-883.
- Klee, H., Yanofsky, M., and Nester, E. (1985). Vectors for transformation of higher plants. *Bio/Technology* **3**, 637-642.
- Klee, H.J., Muskopf, Y.M., and Gasser, C.S. (1987). Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Mol. Gen. Genet.* **210**, 437-442.
- Klein, T.M., Wolf, E.D., Wu, R., and Sanford, J.C. (1987). High-velocity microprojectiles for delivery of nucleic acids into living cells. *Nature* **327**, 70-73.
- Klein, T., Fromm, M., Gradziel, T., and Sanford, J. (1988a). Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. *Bio/Technology* **6**, 559-536.
- Klein, T., Fromm, M., Weissinger, A., Tomes, D., Schaaf, S., Sletten, M., and Sanford, J. (1988b). Transfer of foreign genes into intact maize cells using high-velocity microprojectiles. *Proc. Natl. Acad. Sci. USA* **85**, 4305-4309.
- Klein, T., Harper, E., Svab, Z., Sanford, J., Fromm, M., and Maliga, P. (1988c). Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. *Proc. Natl. Acad. Sci. USA* **85**, 8502-8505.
- Klein, T.M., Kornstein, L., Sanford, J.C., and Fromm, M.E. (1989). Genetic transformation of maize cells by particle bombardment. *Plant Physiol.* **91**, 440-444.
- Koehler, F., Golz, C., Eapen, S., Kohn, H., and Schieder, O. (1987a). Stable transformation of moth bean *Vigna aconitifolia* via direct gene transfer. *Plant Cell Rep.* **6**, 313-317.
- Koehler, G., Golz, C., Eapen, S., and Schieder, O. (1987b). Influence of plant cultivar and plasmid-DNA on transformation rates of tobacco and moth bean. *Plant Science* **53**, 87-91.

- Koncz, C., and Schell, J. (1986). The promoter of T-L-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**, 383-396.
- Kozak, M. (1978). How do eucaryotic ribosomes select initiation regions in messenger RNA. *Cell* **15**, 1109-1123.
- Kozaki, A., Sakamoto, A., Tanaka, K., and Takeba, G. (1991). The promoter of the gene for glutamine synthetase from rice shows organ-specific and substrate-induced expression in transgenic tobacco plants. *Plant Cell Physiol.* **32**, 353-358.
- Laimins, L.A., Tschlis, P., Khoury, G. (1984). Multiple enhancer domains in the 3' terminus of the Prague strain of Rous sarcoma virus. *Nucl. Acids Res.* **12**, 6427-6442.
- Lamppa, G., Nagy, F., and Chua, N.-H. (1985). Light-regulated and organ-specific expression of a wheat Cab gene in transgenic tobacco. *Nature* **316**, 750-752.
- Last, D., Brettell, R., Chamberlain, D., Chaudhury, A., Larkin, P., Marsh, E., Peacock, W., and Dennis, E. (1991). pEmu: an improved promoter for gene expression in cereal cells. *Theor. Appl. Genet.* **84**, 584-588.
- Lawton, M.A., Dean, S.M., Dron, M., Kooter, J.M., Kragh, K.M., Harrison, M.J., Yu, L., Tanguay, L., Dixon, R.A., and Lamb, C.J. (1991). Silencer region of a chalcone synthase promoter contains multiple binding sites for a factor, SBF-1, closely related to GT-1. *Plant Mol. Biol.* **16**, 235-249.
- Leboul, J., and Davies, J. (1982). Enzymatic modification of hygromycin B in *Streptomyces hygroscopicus*. *J. Antibiot.* **35**, 527-528.
- Lee, K.Y., Townsend, J., Tepperman, J., Black, M., Chui, C.F., Mazur, B., Dunsmuir, P., and Bedbrook, J. (1988). The molecular basis of sulfonylurea herbicide resistance in tobacco. *EMBO J.* **7**, 1241-1248.
- Lindsey, K. and Jones, M.G.K. (1987). Transient gene expression in electroporated protoplasts and intact cells of sugar beet. *Plant Mol. Biol.* **10**, 43-52.
- Lindsey, K., and Jones, M.G.K. (1989). Stable transformation of sugar beet protoplasts by electroporation. *Plant Cell Rep.* **8**, 71-74.
- Lindsey, K., and Jones, M.G.K. (1990). Electroporation of cells. *Physiologia Plantarum* **79**, 168-172.
- Loesch-Fries, L., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S., and Halk, E. (1987). Expression of alfalfa mosaic virus RNA4 in transgenic plants confers virus resistance. *EMBO J.* **6**, 1845-1851.
- Long, E.O., and Dawid, I.B. (1980). Repeated genes in eukaryotes. *Ann. Rev. Biochem* **49**, 727-764.

- Lonsdale, D., Onde, S., and Cuming, A. (1990). Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. *J. Exp. Bot.* **41**, 1161-1165.
- Lorz, J., Baker, B., and Schell, J. (1985). Gene transfer to cereal cells mediated by protoplast transformation. *Mol. Gen. Genet.* **199**, 178-182.
- 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.
- Maas, C., Schaal, S., and Werr, W. (1990). A feedback control element near the transcription start site of the maize *Shrunken* gene determines promoter activity. *EMBO J.* **9**, 3447-3452.
- 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 1 enhances reporter gene expression up to 1000-fold. *Plant Mol. Biol.* **16**, 199-207.
- Maier, U.G., Brown, J.W.S., Toloczyki, C., and Feix, G. (1987). Binding of a nuclear factor to a consensus sequence in the 5' flanking region of zein genes from maize. *EMBO J.* **6**, 17-22.
- Maretzki, A., Sun, S.S., Nagai, C., and Bidney, D., Houtchens, K.A., and Dela Cruz, A. (1990). Development of a transformation system for sugarcane. *Abs. VII Int. Cong. Plant Tiss. Cell Cult.*, June 1990, Amsterdam.
- Marris, C., Gallois, P., Copley, J., and Kreis, M. (1988). The 5' flanking region of a barley B hordein gene controls tissue and developmental specific CAT expression in tobacco cells. *Plant Mol. Biol.* **10**, 359-366.
- Marton, L., Wullems, G.J., Molendijk, L., and Schilperoort, R.A. (1979). In vitro transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. *Nature* **277**, 129-131.
- Matsuki, R., Onodera, H., Taeko, Y., and Uchimiya, H. (1989). Tissue-specific expression of the *rolC* promoter of the Ri plasmid in transgenic rice plants. *Mol. Gen. Genet.* **220**, 12-16.
- Matzke, A., and Matzke, M. (1986). A set of novel Ti plasmid-derived vectors for production of transgenic plants. *Plant Mol. Biol.* **7**, 357-365.
- McCabe, D., Swain, W., Martinell, B., and Christou, P. (1988). Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology* **6**, 923-926.
- McElroy, D., Zhang, W., Cao, J., and Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* **2**, 163-171.

- McKnight, S.L. (1982). Functional relationships between transcriptional control signals of the thymidine kinase gene of Herpes simplex virus. *Cell* **32**, 355-365.
- Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, R., Zinn, K., and Green, M.R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**, 7035-7056.
- Meyer, P., Walgenbach, E., Bussmann, K., Hombrecher, G., and Saedler, H. (1985). Synchronized tobacco protoplasts are efficiently transformed by DNA. *Mol. Gen. Genet.* **201**, 513-518.
- Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G., Chua, N-H. (1985). A short conserved sequence is involved in the light-inducibility of a gene encoding ribulose 1,5-bisphosphate carboxylase small subunit of pea. *Nature* **315**, 200-204.
- Morikawa, H., and Yamada, Y. (1985). Capillary microinjection into protoplasts and intranuclear localization of injected materials. *Plant Cell Physiol.* **26**, 229-236.
- Morikawa, H., Iida, A., Matsui, C., Ikegami, M., and Yamada, Y. (1986). Gene transfer into intact plant cells by electroinjection through cell walls and membranes. *Gene* **41**, 121-124.
- Mullins, M.G., Tang, F.C.A., and Facciotti, D. (1990). *Agrobacterium*-mediated genetic transformation of grapevines: Transgenic plants of *Vitis rupestris* scheele and buds of *Vitis vinifera* L. *Bio/Technology* **8**, 1041-1045.
- Nagy, F., Morelli, G., Fraley, R., Rogers, S., and Chua, N.H. (1985). Photoregulated expression of a pea *rbcS* gene in leaves of transgenic plants. *EMBO J.* **4**, 3063-3068.
- Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G., and Sala, F. (1987). Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. *Plant Mol. Biol.* **8**, 363-373.
- Neumann, E., Shaefer-Ridder, M., Wang, Y., and Hofschneider, P.H. (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* **1**, 841-845.
- Nickell, L.G. (1964). Tissue and cell cultures of sugarcane - another research tool. *Hawaiian Sugar Planter's Record* **57**, 223-239.
- Nickell, L.G., and Maretzki, A. (1969). Growth of suspension cultures of sugarcane cells in chemically defined media. *Physiol. Plant.* **22**, 117-125.

- Nims, R.C., Halliwell, R.S., and Rosberg, D.W. (1967). Disease development in cultured cells of *Nicotiana tabacum* L. var. Samsun NN injected with tobacco mosaic virus. *Cytologia* **32**, 224-235.
- Oard, J.H., Paige, D., and Dvorak, J. (1989). Chimeric gene expression using maize intron in cultured cells of breadwheat. *Plant Cell Rep.* **8**, 156-160.
- Odell, J., Nagy, F., and Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**, 810-812.
- 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.
- Olive, M.R., Walker J.C., Singh, K., Dennis, E.S., and Peacock W.J. (1990). Functional properties of the Anaerobic Responsive Element of the maize *Adh1* gene. *Plant Mol. Biol.* **15**, 593-604.
- Otten, L.A.B.M., and Schilperoort, R.A. (1978). A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochim. Biophys. Acta* **527**, 497-500.
- Ou-Lee, T., Turgeon, R., and Wu, R. (1986). Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter, after electroporation of protoplasts of rice, wheat, and sorghum. *Proc. Natl. Acad. Sci. USA* **83**, 6815-6819.
- Ow, D.W., Wood, K.V., DeLuca, M., De Wet, J.R., Helinski, D.R., and Howell, S.H. (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234**, 856-859.
- Paszkowski, J., Shillito, R., Saul, M., Mandak, V., Hohn, T., Hohn, B., and Potrykus, I. (1984). Direct gene transfer to plants. *EMBO J.* **3**, 2717-2722.
- Peacock, W.J., Wolstenholme, D., Walker, J.C., Singh, K., Llewellyn, F.J., Ellis, J.G., and Dennis, E.S. (1987). Developmental and environmental regulation of the maize alcohol dehydrogenase 1 (*Adh1*) gene: promoter-enhancer interaction. In: plant gene systems and their biology. Alan R. Liss, New York, pp 263-277.
- Pederson, K., Devereux, J., Wilson, D., Sheldon, E., and Larkins, B. (1982). Cloning and sequence analysis reveal structural variation among related zein genes in maize. *Cell* **29**, 1015-1026.
- Perez, P., Tiraby, G., Kallerhoff, J., and Perret, J. (1989). Phleomycin resistance as a dominant selectable marker for plant cell transformation. *Plant Mol. Biol.* **13**, 365-373.

- Perlak, F., Deaton, R., Armstrong, T., Fuchs, R., Sims, S., Greenplate, J., and Fischhoff, D. (1990). Insect resistant cotton plants. *Bio/Technology* **8**, 939-943.
- Potrykus, I., Paszkowski, J., Saul, M.W., Petruska, J., and Shillito, R.D. (1985). Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. *Mol. Gen. Genet.* **199**, 169-177.
- Powell-Abel, P., Nelson, R., De, B., and Hoffman, N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-743.
- Putterill, J.J., and Gardner, R.C. (1989). Initiation of translation of the β -glucuronidase reporter gene at internal AUG codons in plant cells. *Plant Science* **62**, 199-205.
- Queen, C., and Baltimore, D. (1983). Immunoglobulin gene transcription is activated by downstream sequence elements. *Cell* **33**, 717-728.
- Raineri, D., Bottino, P., Gordon, M., and Nester, E. (1990). *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). *Bio/Technology* **8**, 33-38.
- Ramagopal, S. (1987). Differential mRNA transcription during salinity stress in barley. *Proc. Natl. Acad. Sci. USA* **84**, 94-98.
- Reeves, C.D., and Okita, T.W. (1987). Analysis of α/β -type gliadin genes from diploid and hexaploid wheats. *Gene* **52**, 257-266.
- Reich, T.J., Iver, V.N., and Miki, B.L. (1986). Efficient transformation of alfalfa protoplasts by the intranuclear microinjection of Ti plasmids. *Bio/Technology* **4**, 1001-1004.
- Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D., and Detmer, J.J. (1988). Genetically transformed maize plants from protoplasts. *Science* **240**, 204-207.
- Riggs, C., and Bates, G. (1986). Stable transformation of tobacco by electroporation. *Proc. Natl. Acad. Sci. USA* **83**, 5602-5606.
- Rogers, S.G., Fraley, R.T., Horsch, R.B., Levine, A.D., Flick, J.S., Brand, L.A., Fink, C.L., Mozer, T., O'Connell, K., and Sanders, P.R. (1985). Evidence for ribosome scanning during translation initiation of mRNAs in transformed plant cells. *Plant Mol. Biol. Rep.* **3**, 111-116.
- Rolfe, S., and Tobin, E. (1991). Deletion analysis of a phytochrome-regulated monocot *rbcS* promoter in a transient assay system. *Proc. Natl. Acad. Sci. USA* **88**, 2683-2686.
- Salomon, F., Deblaere, R., Leemans, J., Hernalsteens, J.-P., Van Montagu, M., and Schell, J. (1984). Genetic identification of functions of TR-DNA transcripts in octopine crown galls. *EMBO J.* **3**, 141-146.

- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory)
- Sanger, F., Nicklen, S., and Coulson, R.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- Scalenghe, F., Turco, E., Edstrom, J.E., Pirrotta, V., and Melli, M. (1981). Microdissection and cloning of DNA from a specific region of *Drosophila melanogaster* polytene chromosomes. *Chromosoma* **82**, 205.
- Schafer, W., Gorz, A., and Kahl, G. (1987). T-DNA integration and expression in a monocot crop plant after induction of *Agrobacterium*. *Nature* **327**, 529-531.
- Scherthner, J.P., Matzke, M.A., and Matzke, A.J.M. (1988). Endosperm-specific activity of a zein gene promoter in transgenic tobacco plants. *EMBO J.* **7**, 1249-1255.
- Schindler, U., and Cashmore, A.R. (1990). Photoregulated gene expression may involve ubiquitous DNA binding proteins. *EMBO J.* **9**, 3415-3427.
- Schroder, G., Waffenschmidt, S., Weiler, E., and Schroder, J. (1984). The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. *Eur. J. Biochem.* **138**, 387-391.
- Seguin, A. and Lalonde, M. (1988). Gene transfer by electroporation in betulaceae protoplasts: *Alnus incana*. *Plant Cell Rep.* **7**, 367-370.
- Shah, D.M., Hightower, R.C., and Meagher, R.B. (1982). Complete nucleotide sequence of a soybean actin gene. *Proc. Natl. Acad. Sci. USA* **79**, 1022-1026.
- Shah, D.M., Hightower, R.C., and Meagher, R.B. (1983). Genes encoding actin in higher plants: Intron positions are highly conserved but coding sequences are not. *J. Mol. Appl. Genet.* **2**, 111-126.
- Shah, D.M., Hironaka, C.M., Wiegand, R.C., Harding, E.I., Krivi, G.G., and Tieweier, D.C. (1986). Structural analysis of a maize gene coding for glutathione-S-transferase involved in herbicide detoxification. *Plant. Mol. Biol.* **6**, 203-211.
- Shillito, R., Saul, M., Paszkowski, J., Muller, M., and Potrykus, I. (1985). High efficiency direct gene transfer to plants. *Bio/Technology* **3**, 1099-1103.
- Shimamoto, K., Terada, R., Izawa, T., Fujimoto, H. (1989). Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* **38**, 274-276.
- Shirsat, A., Wilford, N., Croy, R., and Boulter, D. (1989). Sequence responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. *Mol. Gen. Genet.* **215**, 326-331.

- Simonsen, C.C., and Levinson, A.D. (1983). Isolation and expression of an altered mouse dihydrofolate reductase cDNA. *Proc. Natl. Acad. Sci. USA* **80**, 2495-2499.
- Spena, A., Hain, R., Ziervogel, U., Saedler, J., and Schell, J. (1985). Construction of a heat-inducible gene for plants. Demonstration of heat-inducible activity of the *Drosophila hsp70* promoter in plants. *EMBO J.* **4**, 2739-2743.
- Spencer, T., Gordon-Kamm, W., Daines, R., Start, W., and Lemaux, P. (1990). Bialaphos selection of stable transformants from maize cell culture. *Theor. Appl. Genet.* **79**, 625-631.
- Staiger, D., Kaulen, H., and Schell, J. (1989). A CACGTG motif of the *Antirrhinum majus* chalcone synthase promoter is recognized by an evolutionarily conserved nuclear protein. *Proc. Natl. Acad. Sci. USA* **86**, 6930-6934.
- Stalker, D.M., McBride, K.E., and Malyj, L.D. (1988). Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. *Science* **242**, 419-423.
- Streber, W.R., and Willmitzer, L. (1989). Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D. *Bio/Technology* **7**, 811-816.
- Tabata, T., Nakayama, T., Mikami, K., and Iwabuchi, M. (1991). HBP-1a and HBP-1b: leucine zipper-type transcription factors of wheat. *EMBO J.* **10**, 1459-1467.
- Takaiwa, J., Oono, K., and Kato, A. (1991). Analysis of the 5' flanking region responsible for the endosperm-specific expression of a rice glutelin chimeric gene in transgenic tobacco. *Plant Mol. Biol.* **16**, 49-58.
- Tautorius, T.E., Bekkaoui, F., Pilon, M., Datla, R.S.S., Crosby, W.L., Fowke, L.C., and Dunstan, D.I. (1989). Factors affecting transient expression in electroporated black spruce (*Picea mariana*) and jack pine (*pinus banksiana*) protoplasts. *Theor. Appl. Genet.* **78**, 531-536.
- Toriyama, K., Arimoto, Y., Uchimiya, H. and Hinata, K. (1988). Transgenic rice plants after direct transfer into protoplasts. *Bio/Technology* **6**, 1072-1074.
- Toyoda, H., Matsuda, Y., Hirai, T. (1985). Resistance mechanism of cultured plant cells to Tobacco Mosaic virus (TMV). Efficient microinjection of tobacco mosaic virus into tomato callus cells. *Ann. Phytopath. Soc. Japan* **51**, 32-38.
- Toyoda, H., Yamaga, T., Matsuda, Y., and Ouchi, S. (1990). Transient expression of the β -glucuronidase gene introduced into barley coleoptile cells by microinjection. *Plant Cell Rep.* **9**, 299-302.
- Tsukada, M., Kusano, T., and Kitagawa, U. (1989). Introduction of foreign genes into tomato protoplasts by electroporation. *Plant Cell Physiol.* **30**, 599-603.

- Tumer, N., O'Connell, K., Nelson, R., Sanders, P., Beachy, R., Fraley, R., and Shah, D. (1987). Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. *EMBO J.* **6**, 1181-1188.
- Twell, D., Yamaguchi, J., Wing, R., Ushiba, J., and McCormick, S. (1991). Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. *Genes Dev.* **5**, 496-507.
- Uchimiya, H., Hirochika, H., Hashimoto, H., Hara, A., Masuda, T., Kasumimoto, T., Harada, H., Ideda, J., and Yoshioka, M. (1986). Co-expression and inheritance of foreign genes in transformants obtained by direct DNA transformation of tobacco protoplasts. *Mol. Gen. Genet.* **205**, 1-8.
- Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M., and Leemans, J. (1987). Transgenic plants protected from insect attack. *Nature* **328**, 33-37.
- Vaeck, M., Arlette, R., and Höfte, J. (1989). *Cell Cult. Somatic Cell Genet. Plants* (Schell J and Vasil IK, eds), pp. 425-438, Academic Press.
- Van den Elzen, P., Townsend, J., Lee, K.Y., and Bedbrook, J. (1985). A chimeric hygromycin resistance gene as a selectable marker in plant cells. *Plant Mol. Biol.* **5**, 299-302.
- Van Dun, C., Bol, J., and van Vloten-Doting, L. (1987). Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology* **159**, 299-305.
- Van Dun, C., Overduin, B., van Vloten-Doting, L., and Bol, J. (1988). Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross protect against alfalfa mosaic virus infection. *Virology* **164**, 383-389.
- Van Dun, C., and Bol, J. (1988). Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. *Virology* **167**, 649-652.
- Vandekerckhove, J., Van Damme, J., Van Lijsebettens, J., Botterman, J., De Block, M., Vandewiele, M., De Clercq, A., Leemans, J., Van Montagu M., and Krebbers, E. (1989). Enkephalins produced in transgenic plants using modified 2S seed storage proteins. *Bio/Technology* **7**, 929-932.
- Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I., Schilperoort, R.A., and Schell, J. (1974). Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. *Nature* **252**, 169-170.
- Vasil, V., Clancy, M., Ferl, R.J., Vasil, I.K., and Hannah, L.C. (1989). Increased gene expression by the first intron of maize *Shrunken-1* locus in grass species. *Plant Physiol.* **91**, 1575-1579.

- Vodkin, L., Rhodes, P., and Goldberg, R. (1983). A lectin gene insertion has the structural features of a transposable element. *Cell* **34**, 1023-1031.
- Waldron, C., Murphy, E.B., Roberts, J.L., Gustafson, G.D., Armour, S.L., and Malcom, S.K. (1985). Resistance to hygromycin-B. *Plant Mol. Biol.* **5**, 103-108.
- 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 Mol. Biol.* **11**, 433-439.
- Watson, B., Currier, T.C., Gordon, M.P., Chilton, M.-D., and Nester, E.W. (1975). Plasmid required for virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* **123**, 255-254.
- Yadav, N., Vanderleyden, J., Bennet, D., Barnes, W., and Chilton, M.-D. (1982). Short direct repeats flank the T-DNA of a nopaline Ti plasmid. *Proc. Natl. Acad. Sci. USA* **79**, 6322-6326.
- Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L., and Treiber, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its applicaiton to large-scale virus purification. *Virology* **40**, 734-744.
- Yanofsky, M., Porter, S., Young, C., Albright, L., Gordon, M., and Nester, E. (1986). The *virD* operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. *Cell* **47**, 471-477.
- Zambryski, P., Depicker, A., Kruger, D., and Goodman, H. (1982). Tumor induction by *Agrobacterium tumefaciens*: an analysis of the boundaries of T-DNA. *J. Mol. Appl. Genet.* **1**, 361-370.
- Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., and Schell, J. (1983). *Ti* plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* **2**, 2143-2150.
- Zhang, H., and Wu, R. (1988). Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. *Theoretical and Applied Genetics* **76**, 835-840.
- Zhang, H.M., Yang, H., Rech, E.L., Golds, T.J., Davis, A.S., Mulligan, B.J., Cocking, E.C., and Davey, M.R. (1988). Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. *Plant Cell Reports* **7**, 379-384.