DEVELOPMENT OF NOVEL ANTIBACTERIAL AND ANTIVIRAL TRANSGENE VECTORS AND TECHNIQUES FOR THEIR APPLICATION AND ANALYSIS IN SUGARCANE

by

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ABSTRACT

Sugarcane is challenged by a number of phytopathogenic bacteria and viruses that are best managed by the development of resistant varieties. Genetic engineering is a promising strategy in such breeding efforts, as it allows novel mechanisms of resistance not available in any parent germplasm to be introduced into the crop.

DNA sequences encoding cystatin from papaya (*Carica papaya*), and pleurocidin from the winter flounder (*Pleuronectes americanus)* were envisaged as transgenes in this work due to their theoretical potential to increase sugarcane resistance to viruses and pathogenic or opportunistic bacteria, respectively. Cystatin is a cysteine proteinase inhibitor. Cysteine proteinases are used by potyviruses to cleave the polyprotein gene product, an essential step in the viral life cycle. Constitutive expression of cystatin may therefore lend the host plant resistance to a range of potyviruses, including the economically important pathogen sugarcane mosaic virus (SCMV). Pleurocidin is an amphipathic, α-helical, cationic peptide, with broad-spectrum anti-bacterial activity at physiological pH. By binding to the cell membranes of both Gram positive and Gram negative bacteria, pleurocidin disrupts the membrane potential, causing it to become more permeable, especially to cations, leading to death of the bacterial cell. Initial microbiological bioassays showed that pleurocidin has inhibitory and bactericidal effects on the organisms which cause leaf scald (*Xanthomonas albilineans*), gumming disease (*Xanthomonas campestris* pv. *vasculorum*) and post-harvest sucrose conversion in sugarcane, as well as inhibitory effects against *Leifsonia xyli* ssp. x*yli*, which causes ratoon stunting disease (RSD).

For transformation vector construction, the cystatin and pleurocidin coding sequences were altered so that their start codons were in the most favourable consensus context for expression in monocotyledonous plants. In the case of pleurocidin, an extracellular peroxidase signal sequence was attached. The prepared sequences were spliced into the vector pUBI510 in which the gene of interest is driven by the CaMV 35S promoter linked in tandem to a derivative of the maize ubiquitin promoter. The constructs generated were named pUBI510-cys3 and pUBI510-pleuro8 respectively. The plasmid structures were confirmed using restriction endonuclease analysis and DNA sequencing.

Since the transformation of sugarcane is known to be inefficient, two routes of morphogenesis for the production of somatic embryos were compared in the transformation procedure. These were (1) indirect embryo production via callus and (2) the direct and indirect production of embryos from transverse sections of leaf roll. Field grown sugarcane varieties N12 and NCo376 were the source of explant material. Plasmids pUBI510-cys3 and pUBI510-pleuro8 were respectively co-delivered by microprojectile bombardment with the antibiotic resistance selection plasmid pUBIKN containing the neomycin phosphotransferase gene (*npt-II*). Cultures were maintained in the dark on selection medium containing various concentrations of the antibiotic geneticin (G418) for several weeks before being allowed to regenerate in the light. Plantlets coming through selection were hardened off in the glasshouse when approximately 100mm high.

Primer pairs for amplification of the cystatin insert were designed in various ways. The primer pair which ultimately proved most useful was designed to be complementary to the 5' and 3' ends of the papaya cystatin nucleotide sequence. Primer Premier analysis of a sorghum cystatin sequence provided additional possible primers. A further pair for potential future use was devised based on complementarity to conserved regions on maize cystatins 1 and 2, sorghum, rice, and papaya cystatins. The nucleotide sequence was constructed using the most common monocotyledon codon permutations for each amino acid. Pleurocidin primers were designed to be complementary to 5' and 3' regions of the nucleotide sequence encoding the pleurocidin pre-pro-protein. PCR and RT-PCR protocols for the detection of transgenes and transcript production in putative transgenic plants were developed using these primers.

No plants survived selection via the callus route, although some were regenerated via direct embryogenesis. Putative transformed plants were analysed using PCR to test for the presence of integrated transgenes and Southern hybridization to determine transgene copy number. Both types of transgene were reproducibly detectable by PCR in DNA from some immature plants, but results were negative in DNA from those same plants when mature. Southern hybridization analysis detected the cystatin transgene in DNA from immature plants but no transgenes were detected in up to 20 μg DNA from mature plants. Single copy constructions of the transgenes in backgrounds of non-transformed DNA were detectable by both PCR and Southern hybridization analysis. Overall, PCR, RT-PCR and Southern hybridization results indicated that the plants regenerated fell into two categories: non-transformed plants that had survived selection (escapes) and chimaeric individuals with a component of both transformed

and non-transformed cells, in which the transgene had probably become diluted during plant development under non-selective conditions.

A method for extracting leaf exudates was tested, in conjunction with a cysteine proteinase assay to detect the presence of cystatin transgenes in the intracellular spaces of sugarcane leaves of confirmed transformants. Although it could not be applied within the scope of this project, this assay will prove useful in future work.

PREFACE

The experimental work described in this dissertation was carried out in the laboratories of the South African Sugar Association Experiment Station (SASEX) under the supervision of Dr Stuart Rutherford and Dr Barbara Huckett of SASEX and Professor Paula Watt of the University of Natal, Durban.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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LIST OF ABREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic Acid
Avr-genes	Avirulence Genes
Amp	β-Lactamase Gene
bar	Phosphinothricin Aceltyltransferase
b-32	Maize Ribosome-Inactivating Gene
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower Mosaic Virus
Cry 1Ac, Cry 1B, Cry 2A	Synthetic *Bacillus thuringiensis*-Toxin Genes
CsC	Chestnut Seed Cystatin
CTAB	Cetyltrimethylammonium Bromide
D4E1	Synthetic Antimicrobial Peptide
DTT	Dithiothreitol
EDTA	Ethyline Diamine Tetra-Acetic Acid
G418	Geneticin Antibiotic
GM	Genetically Modified
GNA	Snowdrop Lectin Gene

gus	β-Glucuronidase
HR	Hypersensitive Response
JGMV	Johnson Grass Mosaic Virus
LB	Luria Bertani
LPS	Lipopolysaccharides
lux	Firefly Luciferase
man	Mannopine Synthase
MCS	Multiple Cloning Site
Mes	(2 – [N-Morpholino] Ethanesulfonic Acid) Hydrate
MBC	Minimum Bacteriocidal Concentration
MDMV	Maize Dwarf Mosaic Virus
MIC	Minimum Inhibitory Concentration
N-CBZ-Gly-Gly-Arg-AMC	N-Benzyloxycarbonyl- Gly-Gly-Arg-Amino-4-Methylcoumarin
N-CBZ-Phe-Arg-AMC	N-Benzyloxycarbonyl-Phe-Arg-Amino-4-Methylcoumarin
nos	Nopaline Synthase

nptII	Neomycin Phosphotransferase II
N-*t*-Boc-Gly-Lys-Arg-7-AMC	N-*t*-Butyl-Oxycarbonyl- Gly-Lys-Arg-7-Amino -4 Methylcoumarin
OcI and OcII	Oryza Cystatin I and II
OcIΔD86 and CC-I	Synthetic Cystatin Genes
ocs	Octopine Synthase
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PIG	Particle Inflow Gun
PMC	Potato Multicystatin
PMSF	Phenylmethylsulfonyl Fluoride
PR-proteins	Pathogen Related Proteins
PSORT	Reasoning Algorithm for Predicting Protein Subcellular Localization
PVP	Polyvinylpyrrolidone
R-genes	Resistance Genes
ROS	Reactive Oxygen Species
RSD	Ratoon Stunting Disease

RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAR	Systemic Acquired Resistance
SCMV	Sugarcane Mosaic Virus
SDS	Sodium Dodecyl Sulfate
SM	Standard Medium
SrMV	Sorghum Mosaic Virus
Ti	Tumour-Inducing
Tris	Trichloroacetic Acid
Ubi-1	Maize Ubiquitin Derived Promoter
UMDNJ	University of Medicine and Dentistry of New Jersey

CHAPTER 1: INTRODUCTION

1.1 COMMERCIAL CROP PRODUCTION: A GLOBAL PERSPECTIVE

Challenges facing agriculture in the modern era are not new. Land area suitable for growing crops is limited and is said to be decreasing due to the spread of development, soil erosion and degradation (Kishore and Shewmaker, 1999). Similarly water quantity and quality, and supplies of non-renewable resources such as phosphorus and potassium are declining (Kishore and Shewmaker, 1999). Insect pests and bacterial, fungal and viral pathogens account for enormous global yield losses every year. This is no surprise since an estimated 67 000 species of insects and even greater numbers of pathogens are capable of damaging or causing disease in crops (Herrera-Estrella, 1999). The combined effect of insect pests and pathogens is estimated to reduce crop yields by more than 50% (Herrera-Estrella, 1999). Plants contend with harsh environmental conditions such as droughts, floods and extreme temperature, which also contribute to yield losses. Boyer (1982) calculates that only 10-20% of potential yield is harvested in major crops such as maize, wheat, rice and peanuts.

Despite these challenges, agricultural intensification, the use of pesticides and herbicides, improved irrigation and tillage techniques and genetically superior crops have allowed crop production to increase (Kishore and Shewmaker, 1999). The population continues to grow at an estimated rate of 160 people per minute and is expected to be more than 8 billion by the year 2050 (Hoisington *et al.*, 1999) thus placing an ever growing demand on crop production. Further, the largest proportion of the population live in developing countries where agricultural productivity is low (Herrera-Estrella, 1999). Although current agricultural practices have increased productivity there is concern that such practices may not be sustainable. In addition, herbicides, pesticides and fertilisers are potentially degrading to arable land and the surrounding environment. Further degradation is caused by improper irrigation and tillage, which also promote the leaching of nutrients from the soil.

Considering these facts, agricultural productivity must continue to increase (Callow *et al.*, 1997) especially in developing countries where the potential for increased productivity is

greatest and where increased crop production is most needed (Herrera-Estrella, 1999). At the same time agricultural methods must be sustainable with a limited impact on the environment. Consequently every tool, technology or resource that may assist in achieving these goals must be pursued with due diligence.

1.2 BIOTECHNOLOGY: A RESOURCE FOR CROP PRODUCTION

Though improvements in crop production may still be made through better farming practices, it is thought by many that sustainable and successful agriculture in the future will require an integration of conventional farming, plant breeding and biotechnological methods such as genetic engineering (Borlaug, 2000). Biotechnology involves microbiology, molecular biology and biochemistry (Naik, 2001). Conceptual and methodological advances in these fields led to advances in recombinant DNA technologies (Bent and Yu, 1999) such that the rapid screening of germplasm for single genes or characters is now possible (Callow *et al.*, 1997; Bent and Yu, 1999). Advances in the micropropagation of plants have also occurred and it is generally accepted that any plant given the correct protocol can be propagated *in vitro* and most commercially grown crops have well established protocols for micropropagation (George, 1993). Together these advances have made genetic engineering as we know it today possible (Callow *et al.*, 1997) and therefore have implications for commercial crop production, particularly in the area of breeding.

The modern definition of 'genetic engineering/transformation' is: "the formation of new combinations of heritable material by any virus bacterial plasmid or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur, but in which they are capable of continued propagation" (Walker and Cox, 1988). In the past the term was more generally applicable to selective plant breeding and other forms of genetic manipulation on plants and animals (Bent and Yu, 1999). Modern genetic engineering of plants differs in that it allows the incorporation of specific and well characterised genes from any source into the genome of any plant (Hererra-Estrella, 2000).

Plant breeding has been a key element in successful agricultural programs in the past and will continue to be important in the future. However, successful plant breeding programs in the future will more than likely include the use of genetic transformation.

One of the greatest advantages of genetic engineering is that it allows the transfer of genetic material across species, phyla and kingdom barriers. Consequently the pool of potential genetic diversity for plant breeders has become exponentially enlarged. Megadatabases on the internet, which catalogue known genes, contain only a fraction of the genes that exist. Manipulation of base pair sequences generates still more diversity. There is conceivably a gene or gene family to confer the necessary trait to address every possible agricultural concern. Genetic engineering gives plant breeders access to all of these genes for incorporation into their crop of interest. In addition, genetic engineering may reduce the time required to produce new varieties of crops and reduce the rate of varietal decline.

1.3 THE CURRENT STATUS OF SUGARCANE PRODUCTION

The sugarcane species *Saccharum officinarum* is a thick species of cane with high sucrose levels, given the term 'noble'-cane because it was said to 'ennoble' wild canes with which it was crossed to produce cultivable hybrids (Barnes, 1974; Babu, 1979). It has a lower fibre content and thicker stalk than other species of *Saccharum* but is not very resistant to disease or harsh conditions (Barnes, 1974; Babu, 1979). Heavy tillering, deep root systems and tolerance to drought are associated with *Saccharum barberi* and *sinense* (Barnes, 1974; Babu, 1979). The species most variable and unsuitable for commercial production, *Saccharum spontaneum,* is considered inferior to the other species except in disease resistance and hardiness (Barnes, 1974; Babu, 1979). In general high levels of natural disease resistance seem to be associated with high fibre, low sucrose and thin stalks. Thus the natural species of *Saccharum* are either too thin and low in sucrose content or susceptible to disease to be cultivated commercially. Modern varieties of commercially cultivated sugarcane are therefore hybrids (Naik, 2001) produced through selective breeding to incorporate the best traits of the parent species.

Commercially grown sugarcane is vegetatively propagated. This is partly because progeny forming from true sugarcane seed is numerous (several thousand) and highly variable. Using setts of cane as "seeding" material ensures that all plants are genetically identical and therefore possess the traits for which the particular variety was chosen. Secondly, in many climates sugarcane does not naturally produce viable seed (Barnes, 1974; Babu, 1979).

A trend that has become evident is that new varieties of sugarcane decline in vigor and yield after a few years of commercial cultivation. The decline may take place gradually or suddenly and is thought to be due to changes such as an increase in the size of the pathogen population, brought about by the presence of the variety over an extended period. This type of problem is encouraged by the fact that sugarcane is grown as a monoculture and two to three ratoon crops are harvested (Barnes, 1974). To counter varietal decline, new varieties are continually bred and put into circulation (Barnes, 1974).

1.4 THE PROJECT: AIMS AND OBJECTIVES

Sugarcane in South Africa is a host to several bacteria, which cause economically important diseases. Gumming Disease, Leaf Scald Disease (LSD) and Ratoon Stunting Disease (RSD) are three of the most important diseases of sugarcane, in South Africa and in other cane growing countries. The diseases are caused by *Xanthomonas campestris* pv. *vasculorum*, *Xanthomonas albilineans* and *Leifsonia xyli* ssp. *xyli* respectively. *Leuconostoc mesenteroides* is a non-pathogenic bacterium that reduces the sucrose yield by converting sucrose in the stalk to fructose and dextran. Together with the viral disease, Sugar Cane Mosaic Virus (SCMV), these bacteria represent the potential for massive economic loss for sugarcane growers and millers. Therefore the ultimate aim of the larger project in which this study was devised was to produce and analyse, on a molecular and phenotypic level, transformed sugarcane plants with increased resistance to bacterial and viral pathogens. Pleurocidin and cystatin were chosen as transgenes, pleurocidin for its antimicrobial properties (Cole *et al.*, 1997; Cole *et al.*, 2000) and cystatin predominantly for its antiviral properties (Arai, 1993; Gutierrez-Campos *et al.*, 1999).

The production of transformed plants requires that the transgenes can be transferred to and expressed in the host cells and that the cells can regenerate whole plants. Thus the first objective in this project was to generate constructs containing the transgenes of interest suitable for expression in sugarcane. In addition the transgenes were targeted for export into the apoplast and it was necessary to manipulate the signal sequence of the transgenes to ensure that this would occur.

The use of cystatin as a transgene is well documented (Urwin *et al.*, 1995; Vain *et al.*, 1998; Gutierrez-Campos *et al.*, 1999; Urwin *et al.*, 2000; Delledone *et al.*, 2001) although not in sugarcane. Cystatin has been reported to be effective in increasing the resistance of transformed host plants to viruses belonging to the genus *Potyvirus* (Gutierrez-Campos *et al.*, 1999) to which genus SCMV also belongs. However, the use of pleurocidin as a transgene has not been reported and its effect on bacterial sugarcane pathogens was unknown. Another objective therefore was to test the efficacy of pleurocidin against some common, yet economically important, bacterial sugarcane pathogens.

It was important to establish that positively transformed plants could be tested for the presence of transgene product. Since both transgenes had been targeted for export to the apoplast, the extraction of apoplastic fluid without injuring plant cells became an important objective of this work. The fluid would potentially form the basis of subsequent assays to determine the presence or absence of both pleurocidin and cystatin. Bacterial growth inhibition assays could be used to determine the presence or absence of pleurocidin; however, it was important to develop a method to test for cystatin. Cystatin expressed in the apoplast would affect endogenous levels of cysteine proteinase so it was necessary to establish that cysteine proteinases were present in sugarcane apoplastic fluid and to develop a cysteine proteinase assay.

The biolistic method of transformation was used in this work since it has proven to be one of the most effective approaches for sugarcane transformation (Bower and Birch, 1992; Gambley *et al.*, 1993; Bower *et al.*, 1996; Snyman *et al.*, 1996; Hansom *et al.*, 1998; Snyman *et al.*, 2000; Snyman *et al.*, 2001). Most commonly, the target material for sugarcane transformation has been embryogenic callus (Snyman, 2001). Although good results have been obtained using this method (Bower *et al.*, 1996) the reported transformation efficiency varies widely (Snyman *et al.*, 2000; Snyman *et al.*, 2001). In addition to continual development of this more common protocol, of particular interest at SASEX is a sugarcane transformation protocol which uses transverse sections of immature leaf roll (also known as leaf discs) as target tissue for gene delivery. In this protocol transgenic plants are regenerated by direct somatic embryogenesis as opposed to indirect somatic embryogenesis in the more common protocol. Because of this the protocol offers potential advantages over the more common protocol in terms of time, labor and cost (Snyman *et al.*, 2000; Snyman *et al.*, 2001). One objective of the work in this project was therefore the comparison of transformation

using either leaf discs or callus for gene delivery with regeneration by direct or indirect somatic embryogenesis respectively. A further objective was the preliminary, molecular analysis of putative transgenic plants produced from bombarded leaf discs by direct somatic embryogenesis.

CHAPTER 2: REVIEW OF LITERATURE

2.1 ORIGIN, CHARACTERISTICS AND AGRONOMY OF SUGARCANE

There has been a great deal of debate concerning the origins of sugarcane species (Barnes, 1974; Babu, 1979). This is not surprising considering that sugarcane is an ancient crop and has been referred to in documents dating to civilizations long before the Christian era (Barnes, 1974). The prevailing theory is that at least two species, *Saccharum sinense* and *S. barberi*, and possibly a third, *S. officinarum*, originated in India (Babu, 1979) and gave rise to the species *S. spontaneum* and *S. robustum* (Babu, 1979; Naik, 2001). *Saccharum robustum* and *S. spontaneum* are wild canes whereas the other species are domesticated or cultivated canes (Babu, 1979). This fact originally led to confusion about whether *S. officinarum* gave rise to *S. robustum* or vice versa (Grassl, 1977; Jones, 1985). Despite the fact that *S. officinarum* is not found in the wild state, it is a well-established and ancient species and, unlike *S. robustum*, is genetically stable (Babu, 1979). It has been suggested that *S. officinarum* may have given rise to *S. barberi* and *S. sinense* in India but was subsequently destroyed in this part of the world by an outbreak of red-rot disease to which it was not resistant (Naik, 2001). However, before this event occurred sugarcane had been transported west from India by Alexander the Great in around 325 B.C and was also carried to Haiti by Columbus in 1493 (Naik, 2001). Thus *S. officinarum* was at one time thought to have originated in New Guinea (Brandes, 1956).

Another theory, proposed by Mukherjee (1957) is that the species *officinarum, barberi* and *sinense* of the genus *Saccharum* may have arisen as hybrids from interbreeding species in the genera; *Erianthus, Sclerostachya* and *Narenga.* These species have subsequently been reclassified; *Sclerostachya* A. Camus, *Ripidium* Trin, and *Miscanthus* Anderss (Daniels *et al.*, 1975). *S. spontaneum* and *S. robustum* are wild species and may be hybrids resulting from interbreeding of the original three species.

Sugarcane is a C_4 grass of the order Graminae (Barnes, 1974) which also includes many of the world's most important cereals, e.g., wheat and maize (Jones, 1985). Grasses are viewed as a group of plants with highly conserved genomes, sharing many genes and gene families in common. Some genes have been identified in similar linkage arrangements, which appear to operate in the same place and manner in rice, sorghum, barley, wheat and sugarcane (Irvine, 1999). Though closely related to other graminaceous species structurally (Barnes, 1974) sugarcane has a higher photosynthetic potential than other plants, accumulates as much as 20% sucrose in the stalk (Babu, 1979) and grows at an average rate of 35 – 40 $g/m^2/day$. Sugarcane grows between the latitudes of 35° N and 35° S (Babu, 1974) at altitudes ranging from sea level to several thousand meters (Barnes, 1974).

Sugarcane is cultivated on around 12 million hectares (ha.) of land, roughly 1% of the total cultivated area, worldwide (Naik, 2001). Today, 650 million tonnes of sugar are produced yearly in just under 60 countries from sugarcane (Naik, 2001) while 320 million tons were produced annually in the seventies (Barnes, 1974). Roughly 60% of the world production of sugar is from sugarcane, the other 40% coming from sugar beet (Barnes, 1974; Babu, 1979) in temperate regions (Marlander, 2000). Sugarcane is also used in the production of alcohol, paper, woods, animal feeds (Arencibia *et al.*, 2001) and bagasse, a by-product of milling of cane is used to make glass bottles (Barnes, 1974). India is the largest producer of sugar, growing sugarcane on 3.9 million ha. of land (Naik, 2001). However, Hawaii and Australia have for many years been the countries with the highest cane yield per hectare and sucrose recovery respectively (Barnes, 1974; Babu, 1979; Naik, 2001).

In South Africa sugarcane is grown between 35° 45' N and 28° 15' S (Babu, 1979) predominantly in the Natal coastal margin and Midlands regions (Goodman *et al.*, 1998). The conditions range from nearly tropical in the north to more temperate, sub-tropical in the south with a variety of soils present; sandy, alluvial, clayey, sandy loams and clay loams (Babu, 1979). Sugarcane was introduced into Natal in 1848 and spread quickly in the region among farmers due to its high degree of disease resistance compared with other crops being grown at the time (Agriculture in South Africa, 1989). South Africa produces about 22 million tons of cane per year with a sucrose recovery in the region of 10% (Lionnet, 2001). Two to three ratoons are harvested and crops are harvested at 12 –14 months or 22-24 months of age (Babu, 1979).

2.2 MAJOR SUGARCANE PATHOGENS

Sugarcane is host to several bacterial, viral, and fungal diseases, as well as diseases caused by phytoplasmas, nematodes and insects (Rott *et al.*, 2000). Four important bacterial diseases and one viral disease are discussed in further detail here due to their relevance to the present work.

2.2.1 *Xanthomonas campestris* pv. *vasculorum* (Cobb, 1894) - Gumming Disease

Various attempts have been made to classify the strains of the pathogen *X. campestris* pv. *vasculorum,* (Saumtally and Dookun, 2000). Based on DNA-DNA hybridization studies the pathogen was reclassified *X. axonopodis* pv. *vasculorum* and *X. vasicola* pv. *vasculorum* (Vauterin *et al.*, 1995). In Mauritius, three races have been identified, race 1 attacking the noble canes and races 2 and 3 attacking the hybrid cultivars (Mauritius Sugar Industry Research Institute, 1983). Geographically the pathogens have been separated by restriction fragment length polymorphism (RFLP) analysis into a Southern African and Eastern African strain (Qhobela and Claflin, 1992). Five strains were distinguished among strains of the pathogen from different countries, by fatty acid analysis (Dookun *et al.*, 2000). Group B in this study corresponded to race 1 and *X. axonopodis* pv. *vasculorum*, group C to *X. vasicola* pv. *vasculorum*, and group D to races 2 and 3. Group E was associated with the occasionally seen red stripe symptoms of gumming disease and group A was weakly pathogenic on sugarcane, corresponding to strains from *Thysanoleana maxima* (Dookun, 2000; Saumtally and Dookun, 2000).

Gumming disease is a vascular disease which occurs in cane growing countries worldwide though it is reported to have been eradicated in Australia and has not been reported in Barbados, Guademala or Martinique for many years (Saumtally and Dookun, 2000). The disease is spread by wind-blown rain and infection occurs when the serrated edges of leaves rub against each other. The bacterium can also be spread mechanically by infected cane knives and mechanical harvesters (Saumtally and Dookun, 2000). The foliar phase of the disease is characterised by the occurrence of yellow and sometimes distinctly red foliar stripes, originating at a point on the leaf blade and progressing towards the leaf sheath and

stalk. The older portions of the stripes become necrotic. In susceptible varieties the foliar phase progresses to a systemic phase characterised by chlorosis of new leaves, tillers and young ratoons (Peros and Lombard, 1992), stalk deformation and transverse splits in young stalks. In addition, red discolouration at the nodes and the formation of gum pockets throughout the stalk and in the growing point occur internally. Severe infections are known to cause the death of the mature stalk (Saumtally and Dookun, 2000).

Gumming disease has been shown to decrease stalk length and diameter, and to reduce cane and sugar yield, with systemic infection reducing yield as much as 45% (Autrey *et al.*, 1986). Although the breeding of resistant cultivars has reduced the economic importance of gumming disease (Saumtally and Dookun, 2000) epidemics of the disease are common following cyclonic weather conditions, which promote growth in the size of the bacterium population. This is particularly true when the cyclonic period is followed by a cool, dry season.

2.2.2 *Xanthomonas albilineans* – Leaf Scald Disease

Leaf scald disease (LSD) is caused by the bacterium *Xanthomonas albilineans*, and is considered to be one of the most economically important diseases of sugarcane (Egan, 1972a; Mohamed, *et al.*, 1996; Rott and Davis, 1996). Several strains of *X. albilineans* are known to exist and have been identified using serological variability (Rott *et al.*, 1994a), random-amplified polymorphic DNA (RAPD) markers (Permaul *et al.*, 1996), bacteriophage reaction, fatty acid, colony and cell morphology, whole cell proteins and methyl esters (Rott and Davis, 1996). Strains of *X. albilineans* have as a result been assigned to three serovars and four genetic groups (Rott and Davis, 1996). Although variation in virulence among strains is suspected, this has never been proven (Mohamed *et al.*, 1996). The presence of the bacterium has been confirmed by its isolation in 59 geographic locations (Rott and Davis, 1996). Several studies have shown that *X. albilineans* is distinguishable from other *Xanthomonas* species (reviewed in Birch, 2001).

Leaf scald disease is a vascular disease of sugarcane with symptoms superficially similar to those of gumming disease. The disease is characterised by chronic, acute and latent phases (Rott and Davis, 2000). White pencil lines occur parallel to the midrib in infected leaves in

the chronic phase of the disease (Birch, 2001). These lines become red with age and necrosis is often observed spreading from the leaf margins in infected leaves (Birch, 2001). Emerging leaves exhibit extensive chlorosis (Rott and Davis, 2000) due to albicidin toxins, produced by the pathogen, which block plastid development (Birch, 2001). Other symptoms include the growth of well-developed basal shoots, formation of cavities in the stalk, stunting, wilting and death (Rott and Davis, 2000; Birch, 2001). The acute form of the disease is characterised by rapid wilting (Rott and Davis, 200) and death of symptomless, apparently healthy cane (Birch, 2001). Entire fields of cane can be wiped out in a few weeks when this form of the disease occurs (Rott and Davis, 2000; Birch, 2001). Acute leaf scald often occurs during or following periods of environmental stress (Rott and Davis, 2000) but attempts to induce the disease by recreating these conditions in the greenhouse have proved unreliable (Birch, 2001).

Further complicating the picture is the occurrence of latency of infection in cane that has been colonised by the pathogen (Birch, 2001). This results in symptomless, but infected cane (Egan, 1972b; Steindl, 1972; Rott *et al.*, 1994b; Mohamed *et al.*, 1996, Saumtally *et al.*, 1996; Birch, 2001). The pathogen can be present in the cane for weeks or even months with no symptoms being evident (Rott *et al.*, 1994a), a phenomenon that has made the control of the disease difficult. It is also thought to have facilitated the spread of the disease to cane growing countries throughout the world through the importation of infected cane (Rott *et al.*, 1994a).The most effective means of controlling the disease in these countries to date has been the cultivation of resistant hybrids (Egan, 1972b; Koike, 1972; Mohamed *et al.*, 1996; Birch, 2001).

2.2.3 *Leifsonia xyli* ssp. *xyli* – Ratoon Stunting Disease

RSD is one of the most serious diseases in the sugarcane industry. It is caused by the xylem-limited bacterium, *Leifsonia xyli* ssp. *Xyli* (Evtushenko *et al.*, 2000) and was first recognised in Australia in 1944-45 (Comstock *et al.*, 1996). The bacterium, originally classified *Clavibacter xyli* ssp. *xyli* (Davis *et al.*, 1980) has been reported in most cane growing countries around the world (Steib 1972; Comstock *et al.*, 1996).

Yield losses due to RSD are commonly reported as being between 5 and 10% worldwide but can be as high as 30% (Comstock *et al.*, 1996) or 67% (Koike *et al.*, 1982) in any given year. In Florida in 1988-89 losses attributed to RSD were estimated at 36.8 million U.S. dollars (Davis and Bailey, 2000). In South Africa samplings indicate that approximately 12% of commercial cane fields contain RSD and that yield losses directly related to RSD are around 1% (Bailey and McFarlane, 1999). However RSD causes no distinctive external symptoms (Koike *et al.*, 1982; Comstock *et al.*, 1996) other than slow growth and stunting. Therefore, when an entire field is infected the symptoms are easily overlooked (Davis and Bailey, 2000). Thus the actual effect of RSD may be greater than the quoted figures suggest.

The disease is spread through the use of infected cane knives or harvesters and through the planting of infected seed cane (Steib 1972; Koike *et al.*, 1982; Comstock *et al.*, 1996). Hot water or hot air treatments have been used to control the disease (Steib 1972; Koike *et al.*, 1982; Comstock *et al.*, 1996). These treatment are simple and effective for disinfecting seed cane, but are expensive and reduce the germinability of the cane (Davis and Bailey, 2000). In addition the bacterium is known to survive in the soil and can re-infect sterilised cane (Comstock *et al.*, 1996). Varietal differences in the resistance of cane to RSD are known to exist (Steib, R.J., 1972; Koike *et al.*, 1982; Comstock *et al.*, 1996; Mills *et al.*, 2001) and the most effective means of control the disease is through the cultivation of resistant hybrids (Barnes, 1974).

2.2.4 *Leuconostoc mesenteroides* – Post Harvest Sucrose Deterioration

The lactic acid bacterium *Leuconostoc mesenteroides* converts sucrose to dextran and fructose (Ul-Quader *et al.*, 2001) by sucrose phosphorylase mediated phosphorolysis of sucrose (Kawasaki *et al.*, 1996). The bacterium occurs naturally in sugarcane and is non-pathogenic (CS/NF/DOS/7/ADD 3 FINAL, 2000). It nevertheless causes significant losses in sucrose yield of sugarcane. The severity of infection and sucrose deterioration increases when cane is damaged by frost, machines, disease or pests, delays occur between harvest, grinding and shipping, and when cane is wet or muddy (Cuddihy *et al.*, 1999). Bacterial populations build up quickly in cut cane and most of the conversion of sucrose to occurs between harvesting and milling (Cuddihy *et al.*, 1999). Dense populations of *Leuconostoc mesenteroides* and

other bacteria occur 6 inches from the cut ends of the cane as little as 2 hours after the cane has been cut (Cuddihy *et al.*, 1999). It is estimated that for each 0.1% increase in dextran in cane juice, a factory grinding 10 000 tons of cane per day with a recovery of 88%, would lose 3.87 tons of sugar per day (Cuddihy *et al.*, 1999).

2.2.5 Sugar Cane Mosaic Virus

Mosaic is one of the most prevalent viral diseases of sugarcane, and is present in all cane growing countries except Mauritius and Guyana (Koike *et al.*, 1982). It was initially believed to be caused by a many-strained, single virus. Presently it is recognised as four distinct potyviruses; Johnson grass mosaic virus (JGMV), maize dwarf mosaic virus (MDMV), sugarcane mosaic virus (SCMV) and sorghum mosaic virus (SrMV) (Shukla, 1994). Potyviruses are the largest group of plant viruses, with 180 possible or definite members, infecting a wide range of host plants. They are transmitted by a range of aphid vectors but are non-persistent in the aphids themselves (Revers, 1999; Fellers, 1998; Shukla *et al.*, 1994). Two of these strains, SCMV and SrMV are responsible for mosaic in sugarcane (Grisham, 2000).

Generally yield losses due to mosaic are highly dependant on the sugarcane variety and the strain of the virus (Grisham, 2000). In a 14-year study in Louisiana mosaic caused yield losses ranging from 7%-21% in a range of commercially grown hybrids (Grisham, 2000). However the virus can cause devastating reductions in sucrose yield as demonstrated in the 1980s when an epiphytotic of SCMV resulted in yield losses of up to 40 % in the Isis region of Southern Queensland (Goodman *et al.*, 1998). In South Africa, severe infections are reported to reduce sucrose yield by 42% (Goodman *et al.*, 1998).

Mosaic causes the occurrence of a mosaic pattern of contrasting shades of green on the leaf blade, due to varying levels of chlorophyll in the cells. Depending on the sugarcane variety the chlorotic regions may have diffuse or sharply defined edges and may be accompanied by necrosis in some instances (Grisham, 2000). Certain variety/virus strain combinations are characterised by the occurrence of a red/brown discoloration of the midrib. The symptoms vary in intensity depending on the sugarcane variety but are generally more visible in young

leaves particularly near the basal portion of the leaves. Internally the virus may cause necrosis of cells and a slight reddening of the nodal regions (Grisham, 2000).

Serial hot water treatments and apical meristem culture have been used as methods of controlling mosaic (Benda, 1972). Micropropagation of virus-free material is used to increase the number of uninfected plants (Grisham, 2000). The cultivation of resistant hybrids is considered to be the most effective method of controlling the disease. Although classic breeding programs have been able to produce resistant plants, negative aspects associated with the resistance, e.g., poor sucrose yield, have limited their use (Fellers, 1998).

2.3 NATURAL PLANT DEFENSE AGAINST PATHOGENS

Despite living in an environment where potential pathogens are abundant most interactions between plants and pathogens do not result in disease (Staskawics, 2001). Pathogens capable of sustained reproduction on/in a plant do not necessarily cause disease; plants possess preformed physical and chemical barriers which prevent infection (Hammond-Kosack and Jones, 1996; Osbourn, 1996; Chico *et al.*, 2002) as well as highly complex, regulated defense mechanisms which are induced by pathogens (Staskawics *et al.*, 1995; Hammond-Kosack and Jones, 1996; Staskawics, 2001) which reduce the occurrence and intensity of disease.

2.3.1 Constitutive Plant Defenses

Plant defense mechanisms often take the form of constitutive physical or chemical barriers that prohibit pathogen infection (Hammond-Kossack and Jones, 1996; Osbourn, 1996). A number of molecules with antimicrobial activity are expressed constitutively in plants either in active or precursor form (Osbourn, 1996). For example, phenols, phenolic glycosides, unsaturated lactones, sulphur compounds including elemental sulphur (Williams *et al.*, 2002) saponins, cyanogenic glycosides and glucosinolates have all been shown to be antifungal agents (Osbourn, 1996). Cyanogenic glycosides and glucosinolates are examples of molecules that are expressed as pre-cursors and converted to active form by enzyme activity (Osbourn, 1996). Preformed antimicrobial molecules are often expressed predominantly in the outer cell

layers of plant organs, are normally stored in vacuoles or organelles and are released and converted to active form when pathogens invade a cell (Osbourn, 1996).

Certain types of antimicrobial molecules also have other roles in the plant, which explains why they are expressed in the absence of pathogens. Cystatins for example are important enzymes regulating protein turnover in seeds of *Triticum eastivum* (Kuroda *et al.*, 2001) and are commonly expressed, along with thionins (Epple *et al.*, 1997a), at early stages of seed maturation. Thionins have known antibacterial activity while cystatins have been found to possess antifungal activity in chestnut seeds (Pernas *et al.*, 1999). Other examples are an antimicrobial protein with homology to plant lipid transfer proteins isolated from onion seeds (Cammue *et al.*, 1995) and a seed-specific antimicrobial protein from pokeweed (Liu *et al.*, 2000).

Structural barriers also exist which prohibit pathogen entry and subsequent infection of cells. The cell wall in its native state is a substantial barrier to many pathogens restricting their entry to points of injury (Osbourne, 1996). When pathogen challenge is successful several biochemical events bring about structural changes in the cell wall to strengthen and make it more resistant. A burst of reactive oxygen species (ROS) production occurs very shortly after pathogen infection has occurred (Bent, 1996). It is proposed that certain ROS serve to strengthen the cell wall itself and the binding between adjacent cell walls. Deposition of callose at sites of pathogen infection is also thought to increase resistance to pathogens (Beffa *et al.*, 1996).

2.3.2 Induced Plant Defenses

In addition to pre-formed physical and chemical defenses, plants are capable of a strong, disease-preventing response, upon recognition of a specific pathogen. Resistance resulting from this type of response is known as race-specific resistance (Hammond-Kosack and Jones, 1996) gene-for-gene resistance and hypersensitive resistance (Bent, 1996). This type of resistance occurs when plants recognise specific pathogen signals or elicitors. Pathogen genes coding for proteins which lead to this type of resistance in resistant host plants are called avirulence (*Avr*) genes (Bent, 1996; Staskawics, 2001). Recognition of *Avr* gene products by

the host plant induces expression of specific resistance (*R*) genes and results in disease resistance (Staskawics, 2001). However *Avr* genes do not affect the virulence of pathogens with respect to non-resistant hosts (Bent, 1996). Gene-for-gene resistance and the relationship between *R*-genes in the host and *Avr*-genes in the pathogen was first described by Flor (1947). It has been observed that gene-for-gene resistance is characterised by a hypersensitive response which is usually accompanied by programmed cell death in the immediate vicinity of the site of pathogen challenge (Bent, 1996).

The hypersensitive response (HR) involves the death of host cells within a few hours of pathogen attack (Agrios, 1988). and the formation of necrotic lesions both at the primary site of pathogen challenge and in distal regions of the plant where pathogens are not present (Hammond-Kosack and Jones, 1996; Jackson and Taylor, 1996; Morris *et al.*, 1998). Cells close to veins have been shown to be particularly vulnerable to death. That finding is consistent with the general consensus that the role of HR-cell death may be to restrict pathogen access to nutrients and to prevent systemic infection (Hammond-Kosack and Jones, 1996; Jackson and Taylor, 1996).

Other features closely accompanying HR include the induced synthesis of antimicrobial compounds and enzymes such as chitinases and glucanases (Bent, 1996), elemental sulphur (Williams *et al.*, 2002) and copper amine oxidase (Rea *et al.*, 2002), generation of signaling molecules and reactive oxygen species such as O_2 and H_2O_2 (reviewed in Bent, 1996). Many of the compounds produced have a direct inhibitory effect on pathogens, e.g., glucanases and chitinases break down fungal cell walls (Osbourne, 1996). In addition, H_2O_2 directly inhibits bacterial pathogens (Morris *et al.*, 1998) and is thought to strengthen cell walls by inducing cross linking of cell wall glycoproteins and polymerisation of lignin precursors in the intracellular spaces (Sticher *et al.*, 1997). During HR-cell death, cells decompartmentalise and release antimicrobial vacuolar compounds which further assist in the breakdown of the cell (Osbourn, 1996).

The HR induces the expression of three groups of defense proteins (Kregar and Strukelj, 1999). The first of these include structural proteins and enzymes that strengthen and repair or alter cell walls. A second group of proteins, the pathogenesis related proteins (PR-proteins) are low molecular weight proteins which accumulate extracellularly and are resistant to proteolysis and acidic pH (Kregar and Strukelj, 1999). The defining characteristic of PR-

proteins is that they are induced by pathogens in tissues where they are not normally expressed (Van Loon and Van Strein, 1999). The PR-proteins have been classed into 14 families, including enzymes such as chitinases, glucanases, peroxidases and 'ribonuclease-like' proteins, antimicrobial peptide defensins, thionins and lipid transfer proteins (Maldonado *et al.*, 2002) proteinase inhibitors and PR-1, the function of which is still unknown (Van Loon and Van Strein, 1999). The third group of defense proteins possess antimicrobial activity. These proteins include enzymes involved in the synthesis of oxidised phenolics, tannins and phytoalexins, toxic proteins and inhibitors of proteinases and amylases (Kregar and Strukelj, 1999).

Also associated with the HR (Staskawics *et al.*, 1995) is a heightened state of pathogen resistance known as systemic acquired resistance (SAR) (Ryals *et al.*, 1996). SAR is brought about through the expression of a spectrum of genes (Morris *et al*, 1998) which are defined as being genes whose expression correlates well with the onset and maintenance of the resistance state (Ryals *et al.*, 1996). Several SAR proteins are also classified as pathogen related proteins (PR-proteins) (Bostock *et al.*, 2001). SAR-gene products include, ß-1,3-glucanases, class II and III chitinases, hevein-like proteins, thaumatin-like proteins, osmotins and others (reviewed in Ryals *et al*, 1996). At least nine families of SAR genes are recognised (Jackson and Taylor, 1996) but their expression is pathogen and host specific (Oostendorp *et al.*, 2001).

Proteinase inhibitors and antimicrobial peptides fall into the category of PR-proteins (Van Loon and Van Strein, 1999) and SAR-proteins (Ryals *et al.*, 1996). These will be discussed in greater detail in this review.

2.3.3 Proteinase Inhibitors

Proteinase inhibitors are low molecular weight proteins which form complexes with and inactivate proteinases (Elden, 1999). These proteins are regulators of endogenous proteases (Kregar and Strukelj, 1999) but are also recognised as important defense proteins of plants (Lawrence and Koundal, 2002) and have been shown to inhibit the proteolytic, digestive enzymes of insects (Elden, 1999; Ussuf *et al*, 2001). Proteinase inhibitors are normally present in high concentrations in plant storage organs such as seeds and tubers and are also

induced by pathogens in the vegetative parts of the plant where they are normally only found at low levels and often associated with vacuoles cell walls and occasionally the Golgi apparatus (Kregar and Strukelj, 1999). Four classes of proteinase inhibitors exist. These are inhibitors of serine, cysteine, aspartic and metalloproteinases (Kregar and Strukelj, 1999). The best known of these are the serine and cysteine proteinase inhibitors while very few aspartic and metalloproteinase inhibitors of plant origin are known (Kregar and Strukelj, 1999).

Proteinase inhibitors have been shown to accumulate in plants as a result of injury (Ryan, 1981; Ryan,1990). Ryan (1990) showed that this response was a result of either mechanical injury or insect or pathogen attack. Botella *et al.* (1996) and Bolter *et al.* (1998) have both shown that wounding (mechanical or herbivore attack) or the application of methyl-jasmonate, the fatty acid methyl ester of jasmonic acid which is produced by plants in response to injury (Solomon *et al.*, 1999), induced cysteine proteinase inhibitor activity. Bolter (1998) also showed that serine and aspartate proteinase inhibitor activity were induced by the same stimuli. Potato multicystatin (PMC) and multi-domain cystatin isolated from tomato (Walsh and Strickland, 1993 and Wu and Haard, 2000) occur in relatively high quantities in the leaves (up to 0.6 mg/g fresh weight in the case of PMC). These inhibitors are 85- and 88-kDa respectively and each possess eight functional papain-binding sites. While the tomato cystatin was extracted after the leaves had been sprayed with methyl jasmonate, presumably to increase the level of cystatin present, PMC was observed to accumulate in the leaves under conditions which are known to induce accumulation of other proteinase inhibitors linked to plant defense. Although proteinase inhibitors must certainly regulate proteolysis they are normally present in concentrations exceeding that which would be necessary if this were their only function (Bolter *et al.*, 1998). Thus it has been suggested that they are part of the plants defense against herbivory (Johnson *et al.*, 1989, Solomon *et al.*, 1999; Pernas *et al.*, 2000).

a) Cystatins

Characteristics and Natural Occurrence

Cystatins are a group of cysteine proteinase inhibitors (Barret, 1981) specifically inhibiting sulfhydryl proteinase activity (Barret, 1987). The amino acid sequence 'QVVAG' (Turk and

Bode, 1991) and characteristic amino acids such as the *gly* near the N-terminus and the *pro-trp* near the C-terminus of the mature peptide (Brown and Dziegielewska, 1997) are highly conserved among all of the members of the cystatin family. With the exception of Family 1 cystatins, which posses no cysteine residues or disulphide bonds (Katunuma and Kaninami, 1995) most cystatins posses 4 cysteine residues which cause the formation of disulphide loops in the mature proteins (Katunuma and Kaninami, 1995; Brown and Dziegielewska, 1997). Family 2 cystatins and Family 3 cystatins possess highly homologous amino acid sequences and Family 3 cystatins possessing three copies of a Family 2 type sequence (Katunuma and Kaninami, 1995).

Cystatins have been isolated from rat, bovids, chicken, humans, and several others animals (Brown and Dziegielewska, 1997). The first definite and best-characterised plant cystatin was isolated by Kieko Abe and co-workers (Abe *et al.*, 1987a). They isolated the cystatin from rice and subsequently named it oryzacystatin (Abe *et al.*, 1987b). A second cystatin from rice, oryzacystatin II, was later isolated (Kondo *et al.*, 1990) and shown to have different specific activity than oryzacystatin I. Cystatins have since been isolated from many plants (Ryan, 1990) including soybean and potato (Rowan *et al.*, 1990) maize (Abe *et al.*, 1992) cowpea (Fernandes *et al.*, 1993) papaya (Odani *et al.*, 1996) chestnut seeds (Pernas *et al.*, 1999) lima bean (Lawrence and Nielsen, 2001) wheat (Kuroda *et al.*, 2001) chestnut stems (Connors *et al.*, 2002) and sugarcane (Soares-Costa *et al.*, 2002). The plant cystatins all lack cysteine residues and belong to family 1 cystatins and it has been suggested that they be called the phytocystatins (Kondo *et al.*, 1991). Interestingly all family 1 cystatins are considered to be intracellular inhibitors whereas family 2 cystatins are extracellular inhibitors.

By the late 1980s the cystatin "superfamily" (Brown and Dziegielewska, 1997) included the cystatins, stefins (family 1) and the kininogens (family 3). More recently however the fetuins found in many mammals and the Habu snake, histidine-rich glycoprotein (mammalian plasma protein), the cystatin-related proteins, the variant/divergent cystatins from snakes and flies and the invariant chain (a protein involved in the assembly of class II MHC molecules) have also been added to the superfamily (reviewed in Brown and Dziegielewska, 1997).

Biological Functions

Despite being widely represented in the plant and animal kingdoms, the biological role of cystatin is not well understood. It is clear cystatins must play a role in the regulation of proteolysis but it has been suggested that they may also play a role in plant defense (Solomon *et al.*, 1999).

Many insects, especially those in the Coleopteran (Murdock, 1987) and Hemipteran (Kuroda *et al.*, 1996) orders, have been shown to possess alkaline guts and make use of cysteine proteinases for protein hydrolysis. The gut proteinase activity of these insects can be inhibited by several cysteine proteinase inhibitors and not by serine proteinase inhibitors (Murdock *et al.*, 1987, Weiman and Nielsen, 1988). Edmonds *et al.* (1996) showed that the synthetic cystatin E-64 and chicken egg-white cystatin inhibited the gut contents of the southern corn root worm and that oryzacystatin I and E-64 caused decreases in larval survival and weight gain when incorporated into their diet. Similar results were obtained for several Coleopterans and Hemipterans using oryzacystatin (Kuroda *et al.*, 1996). Hilder *et al.* (1987) suggested that plant proteinase inhibitors act as antifeedants, reducing the capacity of certain insects to make use of dietary proteins, delaying development and reducing fecundity. More recently oryzacystatin expressed in transgenic tomato conferred resistance to *Globadera pallida*, a parasitic nematode (Urwin *et al.*, 1995). Vain *et al.* (1998) produced transgenic rice, expressing a cysteine proteinase inhibitor that was also resistant to certain parasitic nematodes.

Aside from the ability of cystatins to inhibit the gut proteinase activity as well as the growth and fecundity of certain insects and nematodes, a chestnut seed cystatin (CsC) has been shown to possess antifungal and acaricide activity (Pernas *et al.*, 1999; Pernas *et al.*, 2000). A recombinant cystatin identical to CsC was later shown to fully inhibit the growth of phytopathogenic fungi *Colletotrichum graminicola*, *Septoria nodorum*, and *Botrytis cinerea* at a concentration of 9 μM. Interestingly, the cystatin did not affect the growth of the non-phytopathogenic fungi; *Trichoderma viride* or show any inhibitory effects on the phytopathogenic bacteria tested (Pernas *et al.*, 2000). The activity of the chestnut cystatin was comparable to that of thionin and other known antibiotics. However, in the same experiment,

neither egg cystatin nor E-64 had any effect on the fungi suggesting that all cystatins do not have the same functions (Pernas *et al.*, 2000).

2.3.4 Antimicrobial Peptides

Antimicrobial peptides are small peptides (26-50 amino acid residues) which are involved in the innate defense systems of all species (Hancock and Scott, 2000) and exert broad spectrum microbicidal activity (Cole *et al.*, 2000) against bacteria and fungi (Hancock and Scott, 2000). Many of these peptides are also capable of killing eukaryotic cells, such as malignant and normal mammalian cells (Lehrer *et al.*, 1993; Ganz and Lehrer, 1998) parasites such as nematodes and planaria, and enveloped viruses, such as HIV and herpes simplex virus. However, these peptides are normally selective in killing microbes instead of eukaryotic cells (Zasloff, 1992; Hancock and Scott, 2000). The peptides are either constitutively expressed or are induced by microbes and their products in parts of the plant or animal where they are likely to come into contact with environmental pathogens (Hancock and Scott, 2000). In animals this includes the exterior surfaces of the body, epithelial surfaces (Diamond *et al.*, 1996; Bals *et al.*, 1998) bone marrow and testes (Hancock and Scott, 2000). They are widespread in plants, being found in the seeds (Cammue *et al.*, 1995; Thevissen *et al.*, 2000a; Thevissen, *et al.*, 2000b; van der Biezen, 2001) rosettes, flowers and siliques (Epple *et al.*, 1997a Epple, *et al.* 1997b) stems, axillary, leaves and floral buds (Penninckx *et al.*, 1996) and storage organs such as tubers (Segura *et al.*, 1999; Berrocal-Lobo *et al.*, 2002). Although widespread, they tend to be found in the outer cell layers of plant organs (Broekaert *et al.*, 1995; Osbourn 1996; Segura *et al.*, 1999) either in the intercellular spaces (Terras *et al.*, 1995) or in vacuoles or organelles.

a) Structural Properties of Antimicrobial Peptides

At physiological pH, certain antimicrobial peptides are cationic with a net charge of between +2 and +6 (Hancock, 1997) due to numerous lysine and arginine residues (Hancock and Diamond, 2000). The 3-dimensional structure of these peptides is such that they possess a hydrophobic face made up of non-polar amino acids and a hydrophilic face made up of a high

proportion of polar, positively charged amino acids and they are therefore referred to as amphipathic. Hancock and Diamond (2000) suggest an additional double-wing structure consisting of two positively charged (hydrophilic) regions positioned on either side of a hydrophobic core (Hancock and Scott, 2000). There are over 500 naturally occurring cationic, amphipathic antimicrobial peptides (Barra *et al.*, 1998; Hancock and Scott, 2000; van der Biezen, 2001) and these are grouped into at least four structural classes; beta(β)-sheet peptides, alpha(α)-helices, extended helices (similar to the cattle neutrophil peptide indolicidin) and loop structures (Boman, 1995; Hancock, 1997). The two latter classes are much rarer than the first two (Boman, 1995) and will not be discussed in this review. Considering the similarities in the tertiary structure of the peptides, there is a surprising degree of dissimilarity in the number and sequence of amino acids among and within the classes (Boman, 1995; Hancock, 1997; Barra *et al.*, 1998).

b) Classes of Antimicrobial Peptides

β-Sheet Peptides: Defensins as Representatives

Defensins are the most well known example of the β-sheet type peptides. The β-sheet peptides are characterised as being cysteine rich (Broekaert *et al.*, 1995), possessing between 4 and 8 residues per peptide (van der Biezen, 2001), and containing two or more disulphide bonds which stabilise the 3-dimensional folding pattern (Boman, 1995). The first defensins were isolated from rabbit macrophages in 1980 (Lehrer, 1993; Boman, 1995). Defensins have since been found in plants (Broekaert *et al.*, 1995; Terras *et al.*, 1995; Penninckx *et al.*, 1996; Epple *et al.*, 1997; Thevissen *et al.*, 2000a; Thevissen *et al.*, 2000b) molluscs, insects, crustaceans, snakes and other mammals including humans (Lehrer *et al.*, 1993). Plant defensins are more effective against fungi than against bacteria (Broekaert *et al.*, 1995) and do not possess cytotoxic activity against plant or animal cells (Boman, 1995). This contrasts sharply with mammalian and insect defensins which have strong activity against gram positive bacteria, and can be cytotoxic to mammalian cells but possess limited activity against fungi (Boman, 1995). The antimicrobial activity of defensins in general is very sensitive to ionic effects and salt concentration (Boman, 1995). Defensins are expressed intracellularly and in mammals they are stored in granules (Boman, 1995; Barra *et al.*, 1998) while in insects they are secreted

into the hemolymph upon induction by bacterial infection (Boman, 1995). Plant defensins are found in the seeds and the vegetative tissues where they are both constitutively expressed and induced upon pathogen infection. In either case they are normally localised in the outer layers of the organs in which they are expressed (Broekaert *et al.*, 1995).

α-Helical Peptides: Cecropins as Representatives

The most abundant class of antimicrobial peptides at present are linear peptides which contain no cysteine residues and assume an alpha-helical structure (Boman, 1995; van der Biezen, 2001). Many of these peptides possess a proline hinge which is now thought to be very important in determining the ability of the peptide to kill microbial cells (Park *et al.*, 2000). This group includes the cecropins, magainins, bombinins, temporins (Barra *et al.*, 1998; Park *et al.*, 2000) buforins, dermaseptin (Park *et al.*, 2000) clavanins (Lee *et al.*, 1997) and melittins (van der Biezen, 2001). Although there is a great deal of variation in length and amino acid sequence, all of these peptides are cationic and contain alternating hydrophilic and hydrophobic segments (Nicolas and Mor, 1995) a property which allows the formation of near perfect amphipathic α-helices (Boman and Hultmark, 1987).

Cecropins are the most well characterised peptides in the class and were first found in the cecropia moth, *Hyalophora cecropia* (Hultmark, *et al.*, 1980). Subsequently it has become clear that cecropins are common in many insects and mammals (Lee *et al.*, 1989) amphibians and crustaceans (Hancock, 1997) and more recently in the tunicate, *Styela clava* (Zhao *et al.*, 1997) and *Helicobacter pylori* (Putsep *et al*, 1999). Cecropins are inducible by pathogen infection (Boman and Hultmark, 1987; Boman, 1991). Cecropins have activity against gram positive and gram negative bacteria (Boman and Hultmark, 1987; Boman, 1991; Kjuul *et al.*, 1999) and some fungi (Cavallarin *et al.*, 1998; van der Biezen, 2001). Although cecropins do not possess cytotoxic or hemolytic activity, melittins do (Boman and Hultmark, 1987; van der Biezen, 2001).

c) Mechanism of Action

The cationic, amphipathic structure of antimicrobial peptides facilitates electrostatic attraction to negatively charged bacterial membranes, fungal cell walls and surfaces of enveloped viruses (van der Biezen, 2001). Membranes are considered to be the target of antimicrobial peptides (Boman and Hultmark, 1987; Boman, 1995) and several studies have been conducted to elucidate the mechanisms by which these peptides kill (Weiprecht *et al.*, 1997; Aguilera *et al.*, 1999; Matsuzaki *et al.*, 1999; Park *et al.*, 2000; Orlov *et al.*, 2002). It has become clear that polyanionic lipopolysaccharides (LPS) of microbial membranes are important interfaces for interactions with antimicrobial peptides (Hancock, 1997; Aguilera *et al.*, 1999; Matsuzaki *et al.*, 1999; Hancock and Scott, 2000) and recognition factors which elicit the induction of antimicrobial peptides by mammalian cells (Diamond *et al.*, 1996). Antimicrobial peptides competitively displace divalent cations such as Mg^{2+} and Ca^{2+} which have a neutralising effect on LPS and subsequently cause a disruption of the outer microbial membrane (Hancock 1997; Hancock and Scott, 2000). This results in an altering of the membrane permeability. Cationic molecules, specifically those bound to the disrupted outer membrane pass through the membrane, termed 'self-promoted uptake' (Hancock and Scott, 2000) and interact with the negatively charged phospholipid membrane creating channels or pores in the membrane termed 'aggregate channels' (Hancock and Scott, 2000), 'supramolecular peptide/lipid complexes' (Matsuzaki *et al.*, 1999) or 'toroidal channels' (Hancock and Scott, 2000). The channels themselves cause the death of cells due to permeability problems (Zasloff, 1992; Aguilera *et al.*, 1999; Hancock and Scott, 2000). However some peptides kill without cell lysis and are presumed to target intracellular molecules, such as nucleic acids, or organelles (Park *et al.*, 2000). The physical nature of the killing mechanism of antimicrobial peptides decreases the frequency of bacterial resistance (Hancock and Scott, 2000)

Since structural properties of the peptides are responsible for their microbe killing activity, the amino acid composition is very important in determining the potency of a peptide. Melittins and cecropins belong to the same class of antimicrobial peptides but melittins possess hemolitic and cytotoxic activity against animal and plant cells while cecropins do not (Cavallarin *et al.*, 1998). Further evidence for this fact has been elucidated through various experiments using synthetic and natural antimicrobial peptides.

2.4 APPLICATION OF BIOTECHNOLOGY TO PEST CONTROL IN MODERN CROP PRODUCTION

2.4.1 Plant Tissue Culture and Micropropagation

Tissue culture is a general term for the growth of cells, tissue and organs in a sterile medium outside the original organism. Plant tissue culture is an important aspect of plant biotechnology and genetic engineering (Vasil, 1996; Vasil, 1999). Indeed the efficient regeneration of normal and fertile plants from single cells is a basic requirement for genetic improvement of plants (Vasil, 1994). Since the 1980s efficient protocols for the regeneration of plants from cultured cells have been developed for many species of plants (Vasil, 1996; Soh and Bhojwani, 1999).

According to a review by Vasil (1999) certain key discoveries made the progress in plant tissue culture possible. The first of these was the realisation that individual cells of an organism are totipotent, i.e. have the ability to form whole organisms. This concept stems from the Cell Theory of Schleiden (1938) and Schwann (1939) which states that individual cells of an organism 'have the capacity for independent life'. The idea sparked attempts at tissue culture as early as the 1900s (Haberlandt, 1902). These initial attempts were unsuccessful and more detailed studies began in earnest in the 1930s. Secondly, in the early 1930s the first naturally occurring auxin, indole-3-acetic acid, was discovered and its effects on plant growth were studied and documented. The use of this auxin greatly assisted the early attempts at tissue culture and by 1939 plant roots and cells could be grown in an unlimited fashion in culture and organogenesis of roots and shoots had been induced in carrot and tobacco cultures respectively. A second class of plant growth regulators, the cytokinins, was later discovered by Skoog and Miller (1957) and it was found that these growth regulators controlled shoot morphogenesis. Specifically it was shown that the auxin-cytokinin ratio was an important factor determining shoot differentiation. Relatively higher cytokinin concentrations promote shoot differentiation while relatively higher auxin concentrations promote root differentiation (Soh and Bhojwani, 1999). The first example of plants being obtained from single plants occurred in 1965 (Soh and Bhojwani, 1999). Empirical

investigation of the effects of hormones on plant tissues in culture has subsequently enabled the control and manipulation of vegetative growth of plants in culture.

Morphogenesis is the development of form and structure associated with developmental changes in the life cycle of an organism (Soh and Bhojwani, 1999). In relation to plants morphogenesis is the development of tissues and organs such as leaves, shoots, flowers, and roots (Soh and Bhojwani, 1999). Cells of an explant in culture can be induced to form shoots, roots or somatic embryos which possess both shoot and root poles (George, 1993). Such cells are termed organogenic, morphogenic or embryogenic signifying their capacity to undergo a particular route of morphogenesis (George, 1993). Besides the development of specific tissues and organs, cells in culture can be induced to grow in a dedifferentiated, unspecialised and unorganised state forming amorphous masses or clumps known as callus (George, 1993). Callus cells do not necessarily lose their morphogenicity and can be induced to form specific organs or somatic embryos (George, 1993). When morphogenesis is induced in the cells of an explant in the absence of an unorganised growth stage, the morphogenesis is termed direct morphogenesis whereas that occurring in cells of callus is termed indirect morphogenesis (George, 1993).

The production of somatic embryos from sugarcane tissue with potential for regeneration of whole plants is said to be routine (Moore, 1999) and sugarcane tissue culture is performed in many countries worldwide (Naik, 2001). Callus can be produced from shoot and root apical meristems, young leaves, root band tissue from young nodes, immature inflorescence and pith parenchyma (reviewed in Naik, 2001). There are four distinct types of sugarcane callus (Ho and Vasil, 1983; Taylor *et al.*, 1992) of which, type 3 is embryogenic and visually recognizable from the other types by its white nodular appearance (Snyman *et al.*, 1996). Sugarcane has been regenerated from protoplasts, cell suspension cultures and embryogenic callus (Birch *et al.*, 1996; Bower *et al.*, 1996; Snyman *et al.*, 1996; McCallum *et al.*, 1998; Snyman *et al.*, 1998; Snyman *et al.*, 2001) and leaf discs (Irvine and Benda, 1985; Grisham and Bourg, 1989; Gambley *et al.*, 1993, Snyman *et al.*, 2000; Snyman *et al.*, 2001). Sugarcane transformation is most commonly performed using embryogenic callus with regeneration via indirect morphogenesis (Birch *et al.*, 1996; Bower *et al.*, 1996; Snyman *et al.*, 1996; McCallum *et al.*, 1998; Snyman *et al.*, 1998; Snyman *et al.*, 2001) but has also been achieved using leaf discs with regeneration via direct morphogenesis (Gambley *et al.*, 1993, Snyman *et al.*, 2000; Snyman *et al.*, 2001). Although regeneration from embryogenic callus via indirect

morphogenesis has been successful, it is considered more labour intensive than regeneration from leaf discs via direct embryogenesis (Snyman *et al.*, 2001). In addition sugarcane can be regenerated more quickly by direct embryogenesis (14-22 weeks) than by indirect embryogenesis (24-36 weeks; Snyman *et al.*, 2000). This is considered advantageous in terms of the reduced time and labour investment and because the reduced time in culture decreases the potential for occurrence of somaclonal variation (Moore, 1999)

The ability to grow/culture plant material *in vitro* and to regenerate whole plants from this material is a basic requirement for the development of genetically modified plants. Growth of plant material *in vitro* allows the researcher to prepare the plant material adequately for the delivery of transgenes and to ensure that those cells which are most likely to regenerate have a high probability of receiving the transgene during the gene-delivery process. Without an adequate regeneration protocol successfully transformed cells may not develop fully grown transformed plants. Therefore the importance of tissue culture methods for the production of transformed plants cannot be overstated.

2.4.2 Genetic Engineering

a) Introduction

The potential benefits of genetically modified (GM) crops have been recognised for over thirty years (Bent and Yu, 1999). These crops are seen by many as being essential for meeting human food and fibre requirements in the future (Borlaug, 2000) and are potentially mass producers of specific desirable proteins, amino acids and enzymes, antigens, vitamins and energy (Tripathi, 2000). The production of GM crops with so called "production traits" reduces the cost of crop production and potentially improves the ability of growers to manage all manner of pests (Barton and Dracup, 2000). In recent years some of these potential benefits have become a reality and in the United States at least, the commercial production of GM crops has become commonplace (Owen, 2000).

Twenty species of GM crops were grown on 40 million ha of land worldwide in 1999 and GM crops generated a 1.64 billion dollar market in 1998 (Owen, 2000). In 2001, GM crops were grown on 52.6 million ha (James, 2001) and were claimed to have increased farm income in

the US alone by 1 billion dollars (Adam, 2002). The two major commercial GM applications to date have been insect tolerant crops, engineered to express the δ-endotoxin gene from *Bacillus thuringiensis*, and glyphosate and glufosinate herbicide resistant crops (Bent and Yu, 1999; Owen, 2000). Over 1000 glyphosate resistant varieties are now available (Owen, 2000). In the United States glyphosate resistant soybean was grown on 0.4 million ha in 1996 and 11.3 million ha in 1998. In 1999 in Iowa 50-60% of the soybean crop was transgenic. While only 1.6-2 million ha were planted with glyphosate resistant corn, the use of insect tolerant Bt-corn brought the transgenic component of corn grown in the United States in 1999 to about 50% of the total (Owen, 2000).

Bt-potato, cotton and maize and herbicide tolerant soybean, cotton and maize have been the most successful commercially produced varieties but a number of other commercially and non-commercially produced GM crops exist. Insect resistant varieties of tobacco, tomato, walnut, sugarcane and rice (Herrera-Estrella, 2000) and herbicide tolerant oilseed rape, sugar beet and wheat have also been produced (Borlaug, 2000). Besides insect and herbicide tolerant varieties, at least 20 species of plants have been produced with resistance to more than 30 viral diseases by using variations of pathogen-derived resistance and a great deal of work is also being done in breeding plants resistant to bacterial and fungal diseases (Borlaug, 2000). Table 2.1 shows examples of plants that have been genetically modified for increased pest/pathogen resistance

Table 2.1: Transformation studies published predominantly between 1998 and 2002, with an application towards inducing or increasing pest/pathogen tolerance or resistance in plants.

Trait	Technology	Gene	Host	Reference
Bacterial Disease Resistance				
	Agrobacterium-mediated transformation	Modified cecropin MB39	Tobacco – *Nicotiana tabacum*	Huang *et al.*, 1997
	Agrobacterium-mediated transformation	Modified cecropin-melittin cationic peptide chimaera MsrA1	Potato – *Solanum tuberosum*	Osusky *et al.*, 2000

Table 2.1 (cont.)

Trait	Technology	Gene	Host	Reference
	Agrobacterium-mediated transformation	Bacterial sarcotoxin IA	Tobacco – *Nicotiana tabacum*	Mitsuhara *et al.*, 2000
	Agrobacterium-mediated transformation	Modified cecropin MB39	Apple – *Malus domestica*	Liu *et al.*, 2001
	Agrobacterium-mediated transformation	Cationic peroxidase *spi 2*	Tobacco – *Nicotiana tabacum*	Elfstrand *et al.*, 2002
	Agrobacterium-mediated transformation	Polyphenol oxidase	Tomato – *Lycopersicon esculentum*	Li and Steffens, 2002
Viral Disease Resistance				
	-	β-1,3-glucanase from tobacco in antisense direction	Tobacco – *Nicotiana tabacum* and *N. sylvestris*	Beffa *et al.*, 1996
	-	Potato mop-top virus coat protein	Tobacco - *Nicotiana benthamiana*	Reavy *et al.*, 1997
	-	Plum pox potyvirus coat protein	Plum – *Prunus domestica*	Ravelonandro *et al.*, 1997
	Agrobacterium-mediated transformation	Potato mop-top virus coat protein	Potato – *Solanum tuberosum*	Barker *et al.*, 1998
	Agrobacterium-mediated transformation	Potyvirus Proteinases	Tobacco – *Nicotiana tabacum*	Fellers *et al.*, 1998
	Microprojectile bombardment	Nucleocapsid protein gene from tomato spotted wilt virus	Peanut – *Arachis hypogaea*	Yang *et al.*, 1998
	Agrobacterium-mediated transformation	Coat protein gene from bean yellow mosaic virus	Clover – *Trifolium subterraneum*	Chu *et al.*, 1999
	Agrobacterium-mediated transformation	Rice cysteine proteinase inhibitor (Oryzacystatin I)	Tobacco – *Nicotiana tabacum*	Gutierrez-Campos *et al.*, 1999
	Agrobacterium-mediated transformation	Coat protein genes of two strains of cucumber mosaic virus	Tomato – *Lycopersicon esculentum*	Kaniewski *et al.*, 1999
	Agrobacterium-mediated transformation	Coat protein of sweet potato feathery mottle potyvirus	Tobacco - *Nicotiana benthamiana*	Sonoda *et al.*, 1999
	Microprojectile Bombardment	Wheat streak mosaic virus replicase (NIb)	Wheat – *Triticum aestivum*	Sivamani *et al.*, 2000

Table 2.1 (cont.)

Trait	Technology	Gene	Host	Reference
	Agrobacterium-mediated transformation	Arabis mosaic nepovirus coat protein	Grapevine – *Vitis rupestris* and Tobacco – *Nicotiana benthamiana*	Spielmann *et al.*, 2000
	Agrobacterium-mediated transformation	Alfalfa mosaic virus coat protein	Alfalfa – *Medicago trunculata*	Jayasena *et al.*, 2001
Fungal Disease Resistance				
	Agrobacterium-mediated transformation	Bean chitinase	Tobacco – *Nicotiana tabacum*	Broglie *et al.*, 1991
	Agrobacterium-mediated transformation	Vacuolar β-1,3-glucanase from *Nicotiana plumbaginifolia*	Tobacco – *Nicotiana tabacum* and Potato – *Solanum tuberosum*	Libantová *et al.*, 1998
	Microprojectile bombardment	Antifungal class II chitinase from barley-seed	Wheat – *Triticum aestivum*	Bliffield *et al.*, 1999
	Microprojectile bombardment	Stilbene synthase from *Vitis vinifera*	Wheat – *Triticum aestivum* and Barley	Leckbland *et al.*, 1999
	Microprojectile bombardment	CDNA of antifungal protein KP4 from a *Ustilago maydis*-infecting virus	Wheat – *Triticum aestivum* L.	Clausen *et al.*, 2000
	-	Alfalfa defensin (alfAFP)	Potato – *Solanum tuberosum*	Gao *et al.*, 2000
	Agrobacterium-mediated transformation	Rice chitinase (RCC2)	Grapevine – *Vitis vinifera*	Yamamoto *et al.*, 2000
Insect Tolerance				
	Agrobacterium-mediated transformation	Cowpea trypsin inhibitor	Tobacco – *Nicotiana tabacum*	Hilder *et al.*, 1987
	Agrobacterium-mediated transformation	Sweet potato trypsin inhibitor	Cauliflower – *Brassica oleracea* var. *botrytis*	Ding *et al.*, 1998
	Agrobacterium-mediated transformation	Synthetic cry9Aa2	Tobacco – *Nicotiana tabacum*	Gleave *et al.* 1998
	Agrobacterium-mediated transformation	Cry1C	Broccoli – *Brassica oleracea* ssp. *Italica*	Cao *et al.*, 1999

Table 2.1 (cont.)

Trait	Technology	Gene	Host	Reference
	Microprojectile bombardment	Xa21 gene from rice and an agglutinin gene from *Galanthus nivalis*	Rice – *Oryza sativa*	Tang *et al.*, 1999
	Microprojectile Bombardment	Synthetic Cry1B	Rice – *Oryza sativa*	Breitler *et al.*, 2000
	Agrobacterium-mediated transformation	Soybean serine proteinase inhibitors; Kti_3, C-II and PI-IV	Tobacco – *Nicotiana tabacum*, and Potato – *Solanum tuberosum*	Marchetti *et al*, 2000
	Microprojectile Bombardment	Bt fusion gene derived from cry1Ab and cry1Ac	Rice – *Oryza sativa*	Tu *et al.*, 2000
	Agrobacterium-mediated transformation	Synthetic cry1Ab	Rice – *Oryza sativa*	Shu *et al.*, 2000
	-	Synthetic cry1Ac	Soybean – *Glycine max* L.	Walker *et al.*, 2000
	Microprojectile bombardment	*Bt cry*2Aa2 operon expressed in chloroplasts	Tobacco – *Nicotiana tabacum*	De Cosa *et al.*, 2001
	Agrobacterium-mediated transformation	Cysteine proteinase inhibitor from *Arabidopsis thaliana*	White Poplar – *Populus alba* L.	Delledonne *et al.*, 2001
	Agrobacterium-mediated transformation	Synthetic Cry9Aa	Tobacco – *Nicotiana tabacum*, Potato – *Solanum tuberosum*, Cauliflower and Turnip Rape	Kushinov, *et al.*, 2001

Other areas of research include the production of plants with improved food quality value through the expression of specific amino acids, proteins (Wang *et al.*, 2001) fatty acids (Rangasamy and Ratlegde, 2001) or waxes. Also, supplementation of common crops with antigens and vitamins, e.g. vitamin A in rice (Richter *et al.*, 2000) will make these substances more accessible to people living in developing countries (Herrera-Estrella, 2000). Transgenic poplar trees with altered lignification have been field tested for a period of four years and have been shown to increase the efficiency of pulp production for paper-making purposes (Pilate *et al.*, 2002; Chiang, 2002). Enhanced phytonutrient content, juice quality and vine life have been achieved in transgenic tomato through the expression of increased levels of the polyamines spermine and spermidine (Mehta *et al.*, 2002). Expression of the enzyme

glutamate dehydrogenase in crops has the possible application of increasing fertiliser use efficiency (Borlaug, 2000). Further, a great deal of work has been done in recent years on increasing the tolerance of plants to environmental stresses such as soil alkalinity, aluminium and iron toxicity (Borlaug, 2000) drought, heat and water stress (Grover *et al.*, 1998). Finally a transformation protocol, the amplicon-plus system, has been reported by Mallory *et al.* (2002) to promote high-level expression of transgenes in plants.

b) Plasmid Vectors as Transformation Units

Common to all methods of transformation is the use of plasmids as vectors for the insertion of foreign DNA. Plasmids are extrachromosomal genetic elements, which are normally double stranded, closed circular DNA molecules (Maniatis *et al.*, 1982). They occur naturally in a variety of bacterial species (Maniatis *et al.*, 1982) and possess genes conferring traits such as antibiotic resistance, antibiotic production, degradation of complex organic compounds and production of endotoxins, colicins and restriction or modification enzymes (Maniatis *et al.*, 1982). In nature, genetic material is commonly transferred between bacteria by means of bacterial conjugation or viral transduction (Campbell, 1993). This type of gene transfer is normally limited to bacteria because the mechanism by which it occurs requires compatibility between the host and the transferring organism. However, the bacterium, *Agrobacterium tumefaciens,* can transfer a plasmid known as the Ti-plasmid (Tumour inducing), into plant cells (Armitage *et al.*, 1988) demonstrating that plasmid transfer can occur across species and even genera. In addition plasmids can be altered by the excision of undesirable genetic information and the splicing in of desirable genes. For example the *onc* genes, contained in the Ti-plasmid of *A. tumefaciens,* are responsible for causing the disease phenotype in infected host cells but are not necessary for plasmid replication and are therefore excised from the plasmid before it is used for transformation (Maniatis *et al.*, 1982). Plasmids can also be devised for delivery without the use of *Agrobacterium.* In fact plasmids are often transferred biolisitically or by electroporation. These methods will be discussed in more detail in the following section (Section 2.4.2 c)

Desirable genes possess desirable traits and include reporter genes such as bacterial or firefly luciferase (*lux)* and the β-glucuronidase (*gus*) genes and selectable marker genes such as the neomycin phosphotransferase II (*nptII*) gene (Webb and Morris, 1992). Reporter genes are

used to convey information about the efficacy of a particular transformation experiment since their products are easily assayed (Webb and Morris, 1992). The *lux* and *gus* genes for example are commonly used because their products can be assayed visually to determine whether transformation has been successful. Selectable marker genes are useful because they enable the selection of transformed cells. The *nptII* gene for example encodes the enzyme neomycin phosphotransferase which detoxifies the antibiotics neomycin, kanamycin and G418 (Webb and Morris 1992). Thus cells transformed with the *nptII* gene are resistant to and can be selected by growth on a medium containing one of the above-mentioned antibiotics. Genes of interest are another class of desirable genes and include genes such as the phosphinothricin acetyltransferase (*bar*) gene and mutant 5-enolpyruvyl shikimate-3 phosphate synthase which confer resistance to the herbicides Bialaphos/Basta (Hoechst) and Roundup (Monsanto) respectively (Webb and Morris, 1992).

Apart from desirable genes certain genes and regulatory elements must be present in an effective transformation plasmid vector (Webb and Morris, 1992). Multiple cloning sites or polylinkers incorporate recognition sites for several restriction enzymes into a region of the plasmid that is not essential for plasmid replication. These sites are used for the insertion of foreign DNA (Maniatis *et al.*, 1982).Transcriptional start regions, the 'CAAT' or 'TATA' boxes which are necessary for RNA polymerase binding, translation start (ATG) and stop (TAA, TAG or TGA) codons and transcription termination signals (usually G/AATAA(A)n) are all necessary (Webb and Morris, 1992). Selectable markers for selection of transformed cells have been discussed but marker genes for selection of transformed bacterial hosts are also important (Armitage *et al.*, 1988). An example of such a gene is the β-lactamase gene which confers resistance to the antibiotic ampicillin. Finally, the expression of the genes in the plasmid must be driven by a suitable promoter (Webb and Morris, 1992).

Promoters which have been used successfully in transformation include the bacterial nopaline synthase (*nos*), octopine synthase (*ocs*) and mannopine synthase (*man*) promoters (Webb and Morris, 1992). The most commonly used promoter for plant transformation has been the promoter of the 35S RNA gene from the cauliflower mosaic virus (CaMV) (Webb and Morris, 1992). The CaMV 35S promoter has been particular effective in the transformation of dicotyledonous plants but is less effective in monocotyledonous plants (Webb and Morris, 1992). In fact it has been recognised that many promoters suitable for expression in dicotyledonous plants are less active in monocotyledons (Naik, 2001). A promoter (*Ubi-1*)

consisting of the maize ubiquitin first intron and exon was found to increase expression levels of transgenes in wheat, maize, sugarcane and 3 species of grasses from 5-30 fold (Taylor *et al.*, 1993) compared with expression driven by the maize promoter *Adh1*. *Ubi-1* is a stress responsive promoter which has been found to drive sustained transgene expression unlike the rice, *Act* and artificial, *Osa* promoters which tend to cause transgene silencing when plants are regenerated (Hansom *et al.*, 1998). Gallo-Meagher and Irvine (1993) also found that *Ubi-1* was more effective than a synthetic *Emu* promoter, rice actin 1 promoter (*Act1*) and the CaMV 35S promoter for expression of the *β*-glucuronidase gene in sugarcane. The expression vector, pUBI-510 (ECACC deposit reference number 00042603) containing the *Ubi-1*-CaMV 35S promoter combination in particular has been shown to be very effective in driving expression in monocotyledons (Groenewald *et al*, 2000).

c) Transformation Technologies

The most commonly used methodologies for the generation of transgenic plants are *Agrobacterium*-mediated transformation and biolistic transformation. Less commonly used is protoplast transformation (Hansen and Wright, 1999).

Agrobacterium-Mediated Transformation

Agrobacterium-mediated transformation has been the most widely used (Hansen and Wright, 1999) and efficient (Moore, 1999) system for the production of transformed plants to date. The ability of the soil bacterium, *Agrobacterium*, to inject its DNA into and effectively transform plants cells is the basis of the method (Hansen and Wright, 1999). The technical procedures involved are simple and the cost of the required equipment is minimal compared with other methods (Hansen and Wright, 1999). The most important advantage of the method though is that foreign DNA is most often delivered as a single or low number of copies (Armstrong, 1999) and is not rearranged in any way (Tyagi *et al.*, 1999). Also the genetic material is inherited in a Mendelian manner (Nadolska-Orczyk *et al.*, 2000).

Methods for *Agrobacterium*-mediated transformation of dicotyledonous plants are better developed than those for monocotyledonous plants since it was originally thought that the

latter could not serve as hosts to the bacterium and therefore could not be transformed by this method (Nadolska-Orczyk *et al.*, 2000; Christou *et al.*, 1988). In addition, evidence to suggest that this was not true (Graves and Goldman, 1986; Gould *et al.*, 1991) was overlooked by most groups seeking to transform monocotyledons (Armstrong, 1999). In recent years however *Agrobacterium* has been used to generate, among others, transgenic rice (Tyagi *et al.*, 1999) maize (Armstrong, 1999) sorghum (Zhao *et al.*, 2000) wheat and barley (Nadolska-Orczyk *et al.*, 2000) and sugarcane (Arencibia *et al.*, 2001).

Biolistic Transformation

A popular method for the transformation of monocotyledons in particular has been the biolistic method (Tyagi *et al.*, 1999) also known as particle bombardment (Vaine *et al.*, 1993a). The method was first reported in 1987 (Klein *et al.*, 1987) and stably transformed plants were regenerated the following year (Christou *et al.*, 1988). In this method DNA is delivered into plant cells by means of high density particles onto which the DNA has been precipitated. The particles or microprojectiles are accelerated at high velocity into the plant tissue, most commonly by a burst/stream of pressurised Helium gas (Vain *et al.*, 1993b).

Biolistic transformation is advantageous since transformed plants can be obtained by the bombardment of any regenerable tissue (Tyagi *et al.*, 1999; McCabe and Christou, 1993). For example in maize, type 1 callus, pre-cultured immature embryos, proliferating shoot meristems and young apical meristems have all been used as explants (Armstrong, 1999). Similarly in rice, immature embryos, embryogenic callus and embryogenic cell suspensions are used (Fauquet *et al.*, 1996). Some plants can be regenerated by direct embryogenesis or with a minimal callus stage, reducing the length of time explants are kept in culture and therefore reducing the occurrence of somaclonal variation (Tyagi *et al*, 1999). Plants transformed using the biolistic method are often reported to contain many copies of the transgene (Tyagi *et al.*, 1999; Kohli *et al.*, 1998; Fauquet *et al.*, 1996). In rice for example 80% of biolistically transformed plants contain between 2 and 10 copies of the transgene (Fauquet *et al.*, 1996). Multiple copies of transgenes are also reported to integrate regularly at the same locus (Kohli *et al.*, 1998; Fauquet *et al.*, 1996). The size of the transgene may be limited since the physical nature of the method can cause the DNA to fragment (Kohli *et al.*, 1998) with the result that random portions of the transgene vector are integrated (Fauquet *et*

al., 1996). Both Mendelian and non-Mendelian inheritance of transgenes have been observed (Fauquet *et al.*, 1996) and chimaeric plants are common (Christou *et al.*, 1998; Gambley *et al.*, 1993). The cost of the equipment used in the method has been reported as being a disadvantage to the technique (Nadolska-Orczyk *et al.*, 2000; Tyagi *et al.*, 1999) and commercially available 'gene guns' used in biolistic transformation are expensive (Vaine *et al*,. 1993b). However alternative guns such as 'flowing helium gun' and the subsequently developed 'particle inflow gun' (PIG) (Vain *et al.*, 1993b) have reduced drastically both the initial cost of purchasing or constructing a 'gene gun' and the cost of consumables used in the bombardment procedure. Biolistic transformation is in fact simple enough in principle that a paint sprayer was adapted as a 'gene gun' by one group (Irvine *et al.*, 1999)

Protoplast Transformation

Protoplasts are generated by the removal of the plant cell wall through mechanical or enzymatic action on the cells (Hansen and Wright, 1999). Pre-existing suspensions of embryogenic cell lines or embryogenic callus are used as sources for the initiation of protoplast suspension cultures (Tyagi *et al.*, 1999). Protoplasts have commonly been transformed through direct uptake of foreign DNA (Fromm *et al.*, 1986; Hauptmann *et al.*, 1986; Ozias Akins *et al.*, 1987). In such cases the permeability of the protoplast plasmalemma is temporarily altered by an osmotic potential in the case of polyethylene glycol (PEG) (Bur'yanov, 1999) treatment or electric discharge in the case of electroporation (Fromm *et al.*, 1986). However protoplasts may also be used as explant material in biolistic (Christou *et al.*, 1988; Klein *et al.*, 1989; Russell *et al.*, 1992; Taylor *et al.*, 1993; reviewed in Armstrong, 1999) and *Agrobacterium*-mediated transformation (Hansen and Wright, 1999). Although protoplast transformation can be very efficient, protoplasts are cultured for extended periods of time, increasing somaclonal variation and regeneration of infertile plants (Tyagi *et al.*, 1999). In addition protoplast regeneration can be difficult to optimise (Bur'yanov, 1999) and is very genotype specific (Tyagi *et al.*, 1999).

d) Genetic Transformation of Sugarcane

Sugarcane is a genetically complex plant with estimations of variation in chromosome number being as much as 36-170 (Irvine, 1999). Commercial sugarcane cultivars display polyploidy, and aneuploidy (Butterfield *et al.*, 2001) and little is known about the structure and organisation of this complex genome. In addition, manipulations such as backcrossing and selfing are nearly impossible in sugarcane (Butterfield *et al.*, 2001; Smith *et al.*, 1999). Breeding for specific traits of interest while maintaining elite agronomic traits is therefore very difficult in sugarcane (Smith *et al.*, 1999). In South Africa new cultivars intended for commercialisation must therefore be characterised and selected for agronomic traits and specific traits of interest (Huckett, 1998). This selection process can take up to 14 years (Huckett, 1998) and represents a cost in time, labour and loss of germplasm. However, genetic engineering offers a means of introducing specific traits of interest into sugarcane varieties without altering the pre-existing complement of varietal traits.

One of the most important factors determining the success of sugarcane transformation is the ability of the target tissue to form new plants embryogenically (McCallum *et al.*, 1998). The best target material for sugarcane transformation is type 3 embryogenic callus which is composed of 'small, round, densely cytoplasmic cells forming yellow, hard, compact, smooth-surfaced, globular callus which turns white as it develops' (Snyman *et al.*, 1996). In sugarcane only the basal portion of the apical meristem is able to produce embryogenic callus and only 50% of the callus formed from this region is embryogenic (Arencibia *et al.*, 2001). However, varietal differences affect the potential for embryogenic callus formation (McCallum *et al.*, 1998; Snyman *et al.*, 1996). Those studies show that South African varieties N12, N19, NCo376 and NCo310 are suitable for transformation since they produce high quantities of embryogenic callus. Transformed sugarcane has also been regenerated via direct embryogenesis from meristem tissues, without callus production (Enríquez *et al.*, 1997; Gambley *et al.*, 1993; Snyman, 2001). However, Gambley *et al* (1993) reported that the plants produced were chimaeric.

Sugarcane has been the target of several transformation studies (reviewed in Naik, 2001; Arencibia *et al.*, 2001). The first stably transformed sugarcane plants were recovered following microprojectile bombardment (Bower and Birch, 1992) and have since been

recovered following electroporation, *Agrobacterium*-mediated transfer and microprojectile bombardment (Arencibia *et al.*, 2001). Smith *et al.* (1999) produced sugarcane with resistance to cane grub using a potato proteinase inhibitor II gene or the *Galanthus nivalis* lectin gene as transgenes and resistance to SCMV using the viral coat protein gene. Herbicide resistant cane has also been produced (Snyman *et al.*, 1998; Falco *et al.*, 2000)

CHAPTER 3: ASSESSMENT OF POTENTIAL TRANSGENES AND DEVELOPMENT OF TECHNIQUES FOR THEIR ASSAY IN TRANSGENIC PLANTS

3.1 INTRODUCTION

Sugarcane yield is negatively affected by numerous bacterial and viral pathogens (Rott *et al.*, 2000). Production of resistant varieties through the insertion of single genes is an attractive strategy. Papaya cystatin and the antimicrobial peptide pleurocidin were selected as candidate transgenes for their antiviral and antibacterial activity respectively.

Cystatin, a cysteine protease inhibitor, is recognised as an antifeedant (Brown and Dziegielewska, 1997) due to its ability to inhibit gut proteases of insects (Edmonds *et al.*, 1996). Inhibition of gut proteases by cystatin is lethal to certain insects (Kuroda *et al.*, 1996) and transgenic plants expressing cystatin are reported to be more resistant to insects (Delledone *et al.*, 2001) and nematodes (Urwin *et al.*, 1995; Vain *et al.*, 1998; Urwin *et al.*, 2000). Cystatins isolated from pearl millet were first shown to be powerful inhibitors of phytopathogenic fungi of the genera *Claviceps, Helminthosporium, Curvularia, Alternaria* and *Fusarium* by Joshi *et al.* (1998). Later it was shown that chestnut cystatins also inhibited the phytopathogenic fungi *Botrytis cinerea, Colletotrichum graminicola* and *Septoria nodorum* but had no effect on non-pathogenic *Trichoderma viride* (Pernas *et al.*, 1999). An interesting result of the protease inhibiting activity of cystatins is that they are potential inhibitors of the reproduction of certain viruses (Arai, 1993). Gutierrez-Campos *et al.* (1999) produced transgenic tobacco expressing oryzacystatin I. High levels of expression correlated with increased resistance to two species of Potyvirus, which require cysteine protease activity for replication. Sugarcane mosaic virus (SCMV) is a Potyvirus capable of causing devastating yield losses in sugarcane in South Africa (Goodman *et al.*, 1998). Since cystatin has been used successfully as a transgene in other species, including rice (Vain *et al.*, 1998) a monocotyledonous grass similar to sugarcane, and due to the possibility that cystatin may

prevent the reproduction of SCMV if expressed in sugarcane, it appeared to be a good transgene candidate. For this project papaya cystatin, isolated from the latex of *Carica papaya* (pers. comm., Karl Kunert, 2000) was used.

Pleurocidin is a 25 residue amphipathic α-helical peptide with antibacterial properties, isolated from winter flounder (Cole *et al.*, 1997). Its isolation is much more recent than that of cystatin and pleurocidin and has not yet been used as a transgene. Pleurocidin was shown to inhibit the growth of a spectrum of 11 different species of Gram-negative and Gram-positive bacteria (Cole *et al.*, 1997). This is consistent with similar antibacterial peptides which have been shown to possess biocidal activity against a broad spectrum of bacteria (Boman and Hultmark, 1987; Boman, 1991; Kjuul *et al.*, 1999). Due to the broad range of bacteria against which pleurocidin has been shown to be inhibitory or biocidal it was proposed that it is capable of inhibiting the growth of or killing bacterial pathogens of sugarcane. One of the aims of the current project was, therefore, to assess the potential of pleurocidin as a transgene in sugarcane. To determine its bacteriocidal effects on sugarcane pathogens, bacteriostatic assays were designed and performed by Stuart Rutherford (Pathology, SASEX) and are reported here due to their integral nature to the project as a whole. Four common bacterial pathogens, *Xanthomonas, campestris* pv. *vasculorum, Xanthomonas albilineans, Leifsonia xyli* ssp. *xyli* and *Leuconostoc mesenteroides* were chosen since they represent a broad range of pathogen families and due to the potentially serious economic implications for the sugar industry of the diseases they cause.

Transgene products may or may not be present in transgenic plants. They may be broken down post-translationally or the gene itself may be silenced inactivated/silenced (reviewed in Jorgenson, 1992; Jorgenson, 1993;.Matzke and Matzke, 1993). Alternatively the gene may be expressed transiently in which case the functional gene product is usually present for a period of 24 – 72 hours only (Webb and Morris, 1992). According to Webb and Morris (1992) successful genetic transformation requires the distinct stages of insertion, integration, expression and inheritance of the introduced transgene. To ensure that all of these steps have occurred it is important that candidate transgene products are detectable in transgenic plants.

In order to detect the presence of pleurocidin in transgenic sugarcane plants it was necessary to develop a method for the extraction of leaf exudate. The method was envisaged as a simple procedure for use, in conjunction with bacteriostatic assays, in determining whether the

transgene product was present and functional in the intercellular spaces of the leaves of transgenic plants.

Cystatin can be detected using fluorogenic peptide substrates which it hydrolyses (Solomon, 1999). Further, as the fluorescence of the hydrolysed product greatly exceeds that of the non-hydrolysed substrate in a quantitative manner, the concentration of cystatin can be measured using an appropriate fluorogenic amino acid substrate (Solomon *et al.*, 1999). Assays were carried out to determine whether endogenous sugarcane cystatins could be detected in crude protein extracts of senescent leaf tissue and young leaf roll and in extracts of leaf exudate. These were seen as important preliminary steps towards the application of the assay in transgenic plants.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Culture

Leuconostoc medium (Atlas, 1997) containing: 50 g/l calcium carbonate, 50 g/l malt extract, 2.5 g/l sodium chloride, 1 g/l beef extract, 1 g/l polypeptone™ and 15 g/l agar, was used for the isolation and maintenance of *Leuconostoc mesenteroides*. Wilbrink's agar (Atlas, 1997) containing: 10 g/l sucrose, 5 g/l peptone, 0.5 g/l potassium phosphate, 0.25 g/l magnesium sulphate, 0.05 g/l sodium sulphite, 5 g/l yeast extract and 18 g/l Bacto/Bitek agar adjusted to pH 7, was used for the isolation and maintenance of *Xanthomonas albilineans* and *Xanthomonas campestris* pv. *vasculorum*. Freshly isolated cultures of *X. albilineans* and *X. campestris* pv. *vasculorum* were provided by Professor John Hastings (then in the Genetics Department, University of Natal, Pietermaritzberg) and were stored on Wilbrink's agar at 4°C. Luria Bertani (LB) media containing 10 g/l bacto tryptone, 5 g/l yeast extract, 5 g/l sodium chloride, 1 g/l glucose and 15 g/l agar, adjusted to pH 7.5, was used for the maintenance of cultures of *Escherichia coli* cells, strain JM 109. *Leifsonia xyli* ssp. *xyli* cultures were grown in SC medium (Davis *et al.*, 1980) containing; 17 g/l cornmeal agar, 8 g/l bacto-soytone, 1 g/l potassium dihydrogen orthophosphate, 1 g/l potassium hydrogen orthophosphate, 0.2 g/l magnesium sulphate and 0.015 g/l hemin dissolved in 15 ml 0.05N

sodium hydroxide, pH 6.6. After autoclaving and allowing to cool, filter-sterilised preparations of 200 g/l bovine serum albumin (BSA), 500 g/l glucose and 100 g/l cysteine free base were filtered (0.22 μm pore size) into the SC medium. *Leifsonia xyli* ssp. *xyli* was isolated from infected stalks that had been washed in soapy water, surface sterilised using 70% (v/v) alcohol and flame, peeled and cut into small pieces which were soaked in sterile water for 1-2 hours to allow diffusion of the bacteria into the water. The water (3 ml) was used to inoculate S8 broth (10 ml; same as SC media with no cornmeal agar, 1.5 g/l potassium dihydrogen orthophosphate and 0.5 g/l potassium hydrogen orthophosphate). 5-6 drops of S8 was used to inoculate SC plates and allowed to soak into the medium before the plates were sealed with parafilm and incubated at 30°C for 14 days.

All media were autoclaved at 121°C for 20 min. and allowed to cool below 60°C before pouring. Medium was poured into sterile disposable Petri dishes in a sterile laminar flow hood.

3.2.2 Bacteriostatic Assays

A microdilution assay for minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) was modified from that used by Steinberg and Lehrer (1997). Bacteria were grown to a concentration of 5 X 10^5 colony forming units (CFU)/ml in a final volume of 55 μl of a nutrient broth (1/2 strength) which varied according to the species of bacterium (see 3.2.1) at 37°C overnight. The cultures were grown in serial dilutions of pleurocidin (obtained from Prof. Gill Diamond, New Jersey Medical School, Newark, NJ, USA) in 0.01% (v/v) acetic acid and 2 g/l BSA, ranging from 1 μM to 500 μM. The lowest concentration to visually inhibit growth of bacterial colonies (compared with control) was deemed the MIC and was confirmed by observation of microbial sediment and absorbance reading at 650 nm. Aliquots (5 μl) of cultures containing pleurocidin concentrations greater than and less than the MIC were streaked onto nutrient-agar plates. The lowest concentration preventing growth of the bacteria was said to be the MBC.

3.2.3 Extraction of Leaf Exudate

Mature leaves of sugarcane variety N12 were harvested by cutting with ethanol-washed scissors and used immediately to prevent curling of the leaf blades. The leaves of plants grown in the glasshouse and in the field were used. Midribs were cut away from the leaf blades with ethanol-washed scissors and discarded to avoid extraction of vessel contents. Approximately 3 g leaf blade tissue was cut into rectangular pieces roughly 1.5 cm in length and infiltrated with 20 ml ice-cold infiltration buffer (2 mM phenylmethylsulfonyl fluoride (PMSF) and 2mM iodoacetamide in water) for 3 – 30 min. using 40 ml glass bottles sealed with Mininert® screw-cap valves (VICI Precision Sampling, Inc.). Leaf pieces were removed from the infiltration buffer, blotted dry and loaded into microcentrifuge filtration units (Millipore Ultrafree®-MC, 0.65 µm filter units with low-binding Durapore® membrane). Leaf exudate was collected in 1.5 ml microcentrifuge tubes by centrifugation and was used immediately for microbicidal activity assay, cysteine proteinase activity assay or protein precipitation.

3.2.4 Protein Precipitation from Leaf Exudate

Leaf exudate was made up to 1 ml with infiltration buffer and protein was precipitated by the addition of 180 µl 100% (w/v) tetrachloroacetic acid (TCA), incubated at –80°C for 30 min. and centrifuged at 14 000 rpm for 30 min. at 4°C. The supernatant was discarded and protein pellets washed 3 times by resuspension in ice-cold acetone and centrifugation at 14 000 rpm for 30 min.. Pellets were dried under vacuum to produce acetone powder and were resuspended in 10 µl sterile water for use in bacteriocidal activity assays.

3.2.5 Crude Total Protein Extraction

Mature leaf tissue and young leaf roll tissue was harvested and stored in polythene bags in liquid nitrogen for transport from the field. Tissue was cut into small squares (mature leaf tissue) or sliced into thin discs and macerated (leaf roll). The tissue was ground to a fine powder in liquid nitrogen and homogenised in 20 ml cold extraction buffer (20 mM Tris, 20

mM sodium chloride, 1mM ethylene diamine tetra-acetic acid (EDTA), 1mM dithiothreitol (DTT), 0.6% (w/v) polyvinylpyrrolidone (PVP), pH 7.8) in 50 ml Corning tubes for 4 min. using an Ultraturrax. The homogenised mixture was centrifuged twice at 16000g for 15 min. to remove cellular debris and protein concentration was determined using the Bradford assay (Bradford, 1976).

3.2.6 Bradford Assay

A modified Bradford assay (Bradford, 1976) was used to determine protein concentration of crude protein extracts and resuspended leaf exudate proteins. Standard protein dilutions ranging from 0 – 10 μg/ml increasing at 2 μg/ml intervals were prepared from a solution of BSA in infiltration/extraction buffer (see 3.2.3.1 and 3.2.3.2 for buffers). Absorbance readings (595 nm) were used to construct a standard curve of protein concentration. Samples of unknown protein concentration (10 μl) were added to 100 μl Bradford reagent (Sigma) + 40 μl buffer in a glass cuvette and mixed by pipetting. Absorbance readings were used to calculate protein concentration from the standard curve (0 μg/ml – 10 μg/ml).

3.2.7 Cysteine Proteinase Activity Assay

Fluorogenic peptides, N-*t*-butyl-oxycarbonyl (Boc)-Gly-Lys-Arg-7-amino-4-methylcoumarin (AMC), N-benzyloxycarbonyl (CBZ)-Gly-Gly-Arg-AMC and N-CBZ-Phe-Arg-AMC (maximum absorption at 345 nm and emission peaking broadly at 445 nm) were used to detect cysteine proteinase activity in crude protein extracts (0.025 – 250 μg/ml protein) (Solomon *et al.*, 1999). Fluorogenic peptide stock (1 μl at a concentration of 10mM) was added to a 2 ml reaction cocktail containing 1 ml 2X Mes buffer (40 mM Mes, 300mM sodium chloride, 1 mM EDTA, 0.5mM DTT, pH 6.5) and 1 ml crude protein extract dilution in a glass cuvette. The cocktail was mixed by gently pipetting and the reaction allowed to continue for 20 min.. Fluorescence was measured using a Hoefer DyNA Quant 200 fluorometer with maximum excitation at 365 nm and detection at 455-465 nm.

3.2.8 Chlorophyll Quantification

Chlorophyll concentration in leaf exudate extract was measured spectrophotometrically. Leaf exudate was diluted in 80 % acetone and absorbances at 663 nm and 646 nm were used to calculate the total chlorophyll concentration with the equation; Total chlorophyll (mg/l) = 17.3 x A_{646} + 7.18 x A_{663} (Rai, 1973).

3.3 RESULTS

3.3.1 Effect of Pleurocidin on Pathogens of Sugarcane

The minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) of pleurocidin were determined for four common bacterial infectious agents of sugarcane (Table 3.1). The MIC was the lowest concentration of pleurocidin which visibly inhibited bacterial growth (Fig. 3.1) on solid medium containing pleurocidin (compared with a control containing no pleurocidin) and was confirmed by spectrophotometric cell counts of liquid cultures containing the same pleurocidin concentrations. The MBC was the lowest concentration of pleurocidin that prevented bacterial growth.

Table 3.1: Minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) of pleurocidin for four common bacterial pathogens of sugarcane. Bacteria were incubated for 18 hours in the presence of increasing concentrations of pleurocidin at which time absorbance at 650 nm was read.

Opportunistic Infectious Agent	Disease /Problem Caused	MIC (μM)	MBC (μM)
Xanthomonas campestris* pv. *vasculorum	Gumming	1	2.5
Xanthomonas albilineans	Leaf Scald	1	1
Leifsonia xyli* ssp. *xyli	RSD	<10	not determined
Leuconostoc mesenteroides	Post-Harvest Sucrose Loss	2.5	5

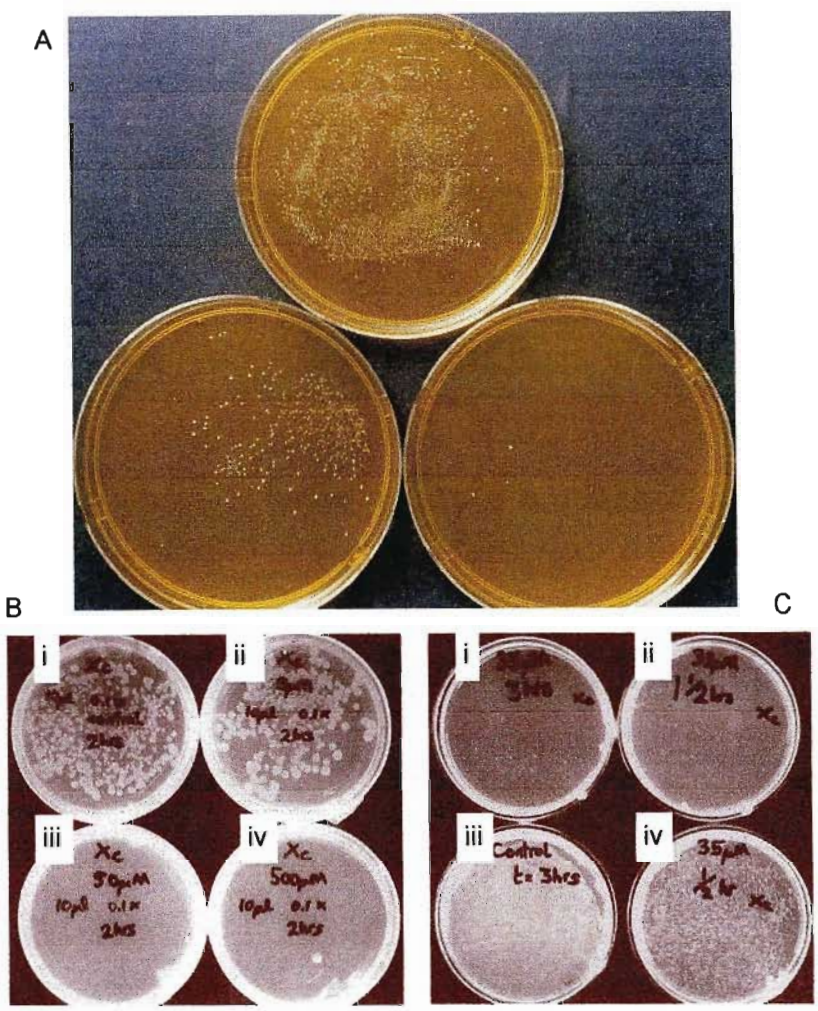

Figure 3.1: Inhibition of growth of two bacterial species by pleurocidin. A: Growth of *Leuconostoc mesenteroides* on nutritive media following incubation with pleurocidin for 3 h at 37°C. Anticlockwise from top, pleurocidin concentrations were 0 μM, 5 μM and 10 μM. **B:** Inhibition of *Xanthomonas campestris* pv. *vasculorum* following incubation for 2 h at 37°C with pleurocidin at; 0 μM (i), 5 μM (ii), 50 M (iii) and 500 μM (iv). concentrations. **C:** Effect of exposure time on growth of *Xanthomonas campestris* pv. *vasculorum* in the presence of pleurocidin. Exposure times of 180 min. (i), 90 min. (ii) and 30 min. (iv) were tested at a pleurocidin concentration of 35 μM against a control of 180 min. (iii) at a pleurocidin concentration of 0 μM.

Absorbance of liquid bacterial cultures at 650 nm confirmed that bacterial cell counts decreased following incubation with increasing concentrations of pleurocidin (results not shown). All bacteria tested were inhibited by pleurocidin at concentrations lower than 10 μM,

X. albilineans being the most sensitive and *L. xyli* ssp. *xyli* being the least sensitive. The MBC of *L. xyli* ssp. *xyli* was high and not determined but was 5 μM or less for the other bacteria (Table 3.1). Inhibition of bacterial growth visibly increased with concentration of pleurocidin (Fig. 3.1 A and B) and with the length of the treatment (Fig. 3.1 C). *X. campestris* pv. *vasculorum* grew well following 3 h exposure to pleurocidin at 20 μM but growth was strongly inhibited after 0.5 h at 35 μM. Growth was not evident following 1.5 h exposure at 35 μM and 0.5 h at 50 μM (Fig. 3.1).

3.3.2 Optimisation of Leaf Exudate Extraction

Sugarcane leaf pieces (3 g) that had been vacuum infiltrated for 1-20 min. in cold extraction buffer were loaded into microcentrifuge filtration units and centrifuged to collect leaf exudate. Preliminary experimentation suggested that the delivery of adequate volumes of leaf exudate required centrifugation speeds higher than 8000 g (Table 3.2). The volume of exudates delivered for centrifugation speeds ≤8000 g was 0 μl/filtration unit (n = 9/centrifugation speed; Table 3.2). Therefore subsequent experimentation was performed at the maximum centrifugation speed (14000 g) and only the infiltration times and centrifugation times were varied.

When a centrifugation time of 10 min. was used the volume of leaf exudate delivered increased as the infiltration time was increased (correlation coefficient = 0.87). When an infiltration time of 1 min. was used the volume of exudates delivered increased from 2.8 μl/filtration (no replicates) unit following centrifugation for 5 min. to 8.95 ± 0.7 (n = 6) μl/filtration following centrifugation for ≥10 min. When an infiltration time of 3 min. was used the volume of exudates delivered was 17.25 ± 1.93 (n = 8) μl/filtration unit for all centrifugation times tested. The correlation between the volume of exudates delivered and centrifugation time at infiltration times of 1 min. and 3 min. was weak (correlation coefficient = 0.76 and 0.59 respectively) although it was stronger at an infiltration time of 1 min.

Table 3.2: Effect of leaf exudate extraction conditions on volume of exudate collected and contamination of exudate by chlorophyll. Sample size = 2 – 4 replicates

Infiltration time (min.)	Centrifuge Speed (x g)	Centrifuge time (min.)	Exudate volume (µl)	Chlorophyll conc. (mg/l)
1	14000	5	2.8 nr	0
1	14000	10	9.13 ± 1.32	0
1	14000	10	nd	0.0197 nr*
1	14000	>10	8.77 ± 0.82	0
3	6000	10	0	-
3	8000	10	0	-
3	14000	5	19.95 ± 1.45	2.43 ± 1.79
3	14000	10	13.03 ± 3.85	1.4 ± 1
3	14000	>10	23 ± 2.83	7.14 ± 2.26
5	14000	10	9.1 ± 1.4	41.98 p
10	5000	10	0	-
10	14000	10	15.1 ± 3.31	41.98p
20	14000	10	17.8 ± 5.22	41.98p

Key: p = concentration determined from pooled samples of leaf exudates, nr = no replicates, nd = not determined, * = leaf pieces intentionally damaged

The chlorophyll content of the leaf exudate was determined as a measure of cellular damage caused by the infiltration and centrifugation of leaf pieces. For chlorophyll content determination 20 µl leaf exudate was added to 80 µl acetone and the absorbtion read at 646 nm and 663 nm. All samples of leaf exudates tested that had been extracted following infiltration for 3 minutes or more (n = 12) contained chlorophyll while those that had been extracted following infiltration for 1 minute (n = 6) did not except one sample which was extracted from leaf pieces that were intentionally damaged (Table 3.2).

Although the sample sizes were small overall results indicated that an infiltration time of 1 minute followed by centrifugation at 14 000 g for 10 min. was suitable for extraction of leaf exudate containing no chlorophyll. This was adequate for the purposes of the present work therefore no further experimentation was performed.

3.3.3 Cysteine Proteinase Activity Assay

In order to test for the presence of cysteine proteinases, the fluorogenic substrate N-CBZ-Phe-Arg-AMC was incubated in a crude protein extract from mature leaf tissue (total protein concentration = 25 µg/ml). Accumulation of hydrolysed substrate was measured fluorometrically and used as a measure of cysteine proteinase activity. Cysteine proteinase, present in the crude protein extracts, was confirmed to be responsible for the observed hydrolysis since leupeptin, a known cysteine proteinase inhibitor (Solomon *et al.*, 1999) completely inhibited hydrolysis (Fig. 3.2) whereas phenylmethylsulfonyl fluoride (PMSF), a serine proteinase inhibitor also inhibited hydrolysis but to a lesser extent (Fig. 3.2). Denaturation of the protein extract by boiling also resulted in maximum inhibition of hydrolysis.

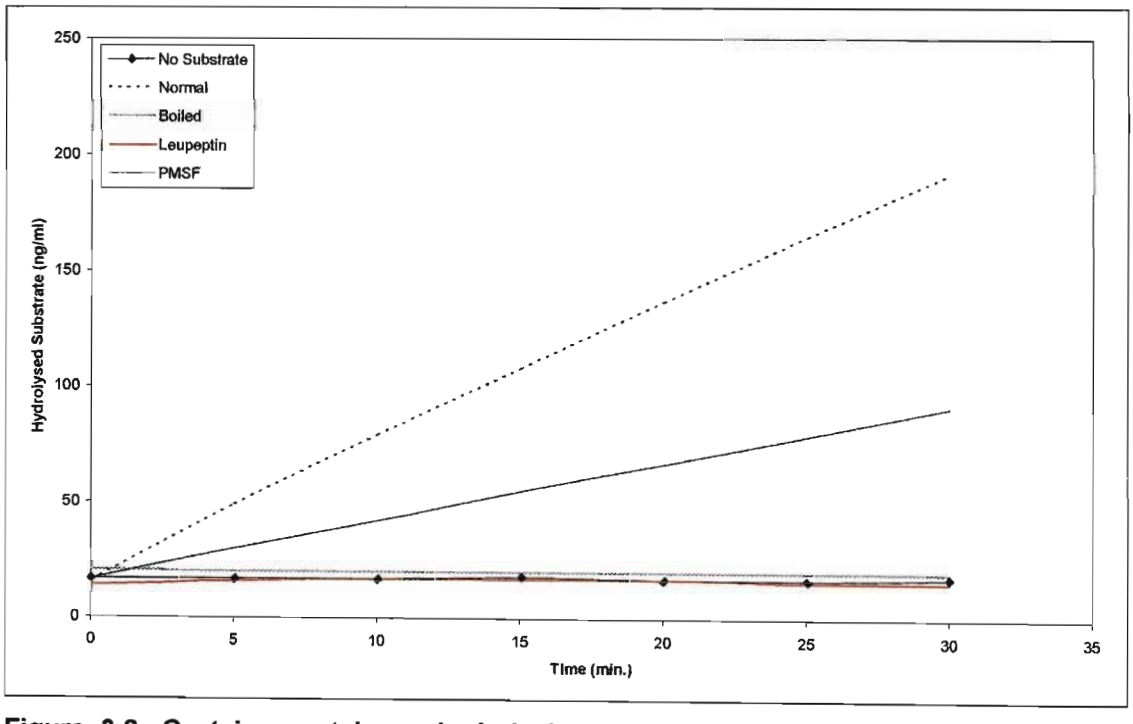

Figure 3.2: Cysteine proteinase hydrolysis of fluorogenic substrate, N-CBZ-Phe-Arg-AMC. Fluorescence of reactions was measured at five minute intervals and used to calculate the amount of hydrolysed substrate present. The proteinase inhibitors leupeptin and phenylmethylsulfonyl fluoride (Leupeptin and PMSF respectively) were added to confirm that activity was due to cysteine proteinase activity. Additional controls contained boiled crude protein extract (Boiled) or no substrate (No Substrate). Controls were compared with the activity observed in the reaction containing crude protein extract and N-CBZ-Phe-Arg-AMC only. All reactions were performed using crude protein extract diluted to a total protein concentration of 25 µg/ml.

To determine the lowest total protein concentration at which cysteine proteinase activity was measurable, the crude protein extracts from immature leaf roll and mature leaf tissue were diluted to five different total protein concentrations ranging from 250 μg/ml to 0.25 μg/ml and incubated in the presence of the fluorogenic substrates N-*t*-Boc-Gly-Lys-Arg-7-AMC, N-CBZ-Gly-Gly-Arg-AMC and N-CBZ-Phe-Arg-AMC. Crude protein extracts were used from immature and mature tissue to determine whether different levels of proteinase activity could be detected in the tissues. The level of cysteine proteinase activity was expected to be higher in the mature leaf tissue since cysteine proteinases are involved in programmed cell death (Solomon, 1999) which was expected to occur more in mature leaf tissue than in immature leaf tissue. Three different fluorogenic substrates were tested to determine which of the three was the most suitable for future use in similar assays. Reactions were performed for each total protein concentration, leaf tissue and fluorogenic substrate. The reactions were performed in triplicate, the average reaction rates of which are shown in Table 3.3.

Table 3.3: Rates of production of hydrolysed fluorogenic substrate. Reactions were allowed to continue for 20 min. at which time the fluorescence of the reaction mixture was measured. Background fluorescence of the unhydrolysed substrates and from the crude protein extracts were measured in control reactions and used to calculate the increase in hydrolysed substrate.

Leaf Tissue	[Protein] (μg/ml)	Hydrolysed Substrate (ng/ml/min.) n = 3		
Mature		Gly-Gly	N-t-Boc	Phe-Arg
	0.25	-0.31 ± 0.03	0.82 ± 0.71	0.07 ± 0.12
	1.25	1.16 ± 0.8	1.67 ± 0.75	0.07 ± 0.13
	2.5	0.43 ± 0.46	0.18 ± 0.38	0.35 ± 0.19
	25	2.29 ± 0.1	3 ± 0.25	0.135 ± 0.03
	250	3.73 ± 0.08	4.5 ± 1.63	-0.05 ± 0.07
Immature				
	0.25	-0.16 ± 0.04	-0.4 ± 0.03	-2.9 ± 0.09
	1.25	0.11 ± 0.02	-0.23 ± 0.19	-2.8 ± 0.07
	2.5	0.03 ± 0.08	1.2 ± 1.11	-2.94 ± 0.03
	25	1.36 ± 0.12	1.93 ± 0.03	-2.95 ± 0.02
	250	2.73 ± 0.14	6.13 ± 0.27	-3.02 ± 0.04

Proteinase activity was detected at all concentrations of protein extract tested. At the lowest concentration (0.125 μg/ml) proteinase activity was measurable only when the substrate N-*t*-

Boc-Gly-Lys-Arg-7-AMC was used and was near the limit of detectability (Table 3.3, N-t-Boc). Proteinase activity was consistently lower in extracts from leaf roll than in extracts from mature leaf at the same concentration for all concentrations tested (Table 3.3). Reaction rates were negative or close to zero when the substrate N-CBZ-Phe-Arg-AMC was used (Table 3.3, Phe-Arg). It is likely that this was due to the fact that the batch of the substrate was old at the time of use since it had been used for experiments prior to the one shown here. The highest reaction rates were obtained using the substrate N-*t*-Boc-Gly-Lys-Arg-7-AMC and this substrate was therefore used in subsequent experiments.

3.3.4 Cysteine Proteinase Activity Measured in Leaf Exudate

To determine whether the cysteine proteinase assay could be used to test for the presence of cystatin in the intercellular spaces, samples of leaf exudate were tested for the presence of cysteine proteinase activity by incubation in the presence of the fluorogenic substrate N-*t*-Boc-Gly-Lys-Arg-7-AMC. Leaf pieces were infiltrated with Tris buffer for either 1 or 3 min. and centrifuged at 14000 g for 10 min. To test for cysteine proteinase activity, 60 - 100 µl leaf exudate was collected made to a final volume of 2 ml with Tris buffer. The fluorogenic substrate was added and the fluorescence of the reaction was read at 5 minute intervals. Cysteine proteinase was detected in leaf exudate containing chlorophyll and in leaf exudate that contained no chlorophyll (Table 3.4). The average protein concentration of leaf exudates was determined to be 144.3 ± 7.33 µg/ml (n=5) by the Bradford assay and the proteinase activities in Table 3.4 are expressed in terms of the activity per µg total protein present.

Table 3.4: Rates of cysteine proteinase activity (ng/ml/min./µg protein) detected in extracts of leaf exudate. The chlorophyll concentration of the leaf exudates samples tested are shown.

Proteinase Activity (ng/ml/min./µg protein)	Chlorophyll Conc. (µg/ml)
0.14 nr	0 nr
0.34 ± 0.06*	0.97 ± 0.42*

Key: nr = no replicates, * = average for 3 replicates

3.4 DISCUSSION

3.4.1 Suitability of Pleurocidin as an Antibacterial Transgene

Results (Table 3.1) indicate that pleurocidin is an effective bacteriocidal agent against sugarcane pathogens, particularly *X. albilineans* and *X. campestris* pv. *vasculorum* which cause leaf scald disease (LSD; Rott and Davis, 2000) and gumming disease (Saumtally and Dookun, 2000) respectively. *Leifsonia xyli* ssp. *xyli*, which causes ratoon stunting disease (RSD; Davis *et al.*, 1980) and *L. mesenteroides* which causes post-harvest sucrose loss, were less sensitive to pleurocidin but still strongly inhibited (Fig. 3.1) by it at concentrations lower than 10 μM. This is consistent with the fact that amphipathic α-helical peptides, such as pleurocidin, are known to possess bacteriocidal activity against a broad spectrum of bacteria (Cole *et al.*, 2000). However the results also indicate that certain genera of bacteria are more susceptible to the effects of antimicrobial peptides than others. This was demonstrated by the fact that the growth of both *Xanthomonas campestris* pv. *vasculorum* and *Xanthomonas albilineans* was inhibited by lower concentrations of pleurocidin than that of *Leifsonia xyli* ssp. *xyli* or *Leuconostoc mesenteroides* (Table 3.1).

The concentration of pleurocidin and the duration of exposure to pleurocidin were important factors influencing the survival of bacteria. Higher levels of bacterial inhibition were observed when higher concentrations of pleurocidin were used (Fig. 3.1 A and B) and when the length of exposure to pleurocidin was increased (Fig. 3.1 C). This is compatible with the known mechanism of action of antibacterial peptides (Hancock and Scott, 2000). Expressed as a transgene in the intercellular spaces of sugarcane, pleurocidin could be expected to act as a first line of defense against the above mentioned and potentially other opportunistic infectious agents. According to Hancock and Scott, 2000 antimicrobial peptides are known to be highly effective in killing bacteria and are likely to kill invading bacteria before they are able to multiply. Thus it is likely that the concentration of pleurocidin in the plant need not be high in order for it to be effective to at least some degree. According to the results shown here (Table 3.1 and Fig. 3.1) at high concentrations pleurocidin could be expected to kill certain bacterial pathogens, in particular the highly susceptible *Xanthomonas* species and *Leuconostoc mesenteroides*. At low concentrations pleurocidin could be expected to inhibit the growth of the bacterial pathogens and therefore reduce the occurrence systemic bacterial infections

resulting in disease. A reduction in the population of pathogens residing on each sugarcane stalk would potentially reduce the size of the population residing in the field and this may reduce the occurrence of varietal decline by preventing a buildup of the pathogen population to critical levels.

3.4.2 Phenotypically Effective Levels of Expression of Pleurocidin and Cystatin

Table 3.5 provides information from several studies on expression levels of transgene products, including cystatins, in several plant species. The table is a compilation of reports from various authors and gives an indication of the range of effective expression levels that can be achieved in transgenic crops. According to the various authors, all transgenes in Table 3.5 produced the desired phenotypic effect in transgenic plants at the levels of expression reported. Urwin *et al.* (2000) however reported that for a range of 0.1 – 0.4 % total soluble protein, the upper level of expression was required for nematode resistance in *Arabidopsis thaliana*. Cao *et al.* (1999) indicate that higher levels of Cry 1C expression correlated well with higher levels of resistance to three species of insect larvae while moderate resistance occurred in plants with lower expression levels. Similarly, Maqbool *et al.* (2001) report that transformed plants expressing all three of the genes, Cry 1Ac, Cry 2A and GNA were much more resistant to three types of insect pests than transformants expressing only one of the genes. Conversely, GNA protein expressed at levels as low as 0.25 % total soluble protein in rice conferred significantly higher resistance to brown planthopper (Tinjuangjun *et al.*, 2000) and, expression of Oc-IΔD86 at 0.2 % total soluble protein reduced egg production of the nematode *Meloidogyne incognita* by 55 % in transgenic rice (Vain *et al.*, 1998).

Natural levels of cystatin are reported to be 2-3 mg/kg seeds in rice for oryzacystatin-I (Michaud, 2000) and 0.6 mg/g fresh weight in potato leaves for potato multicystatin (Walsh and Strickland, 1993). This is equivalent to a range of natural expression levels from 0.00375 % total soluble protein in rice seeds to 3 % in potato leaves, assuming a total soluble protein content of 8 % for rice and 2 % for potato leaves (Boulter and Berbyshire, 1977). The range of phenotypically effective transgene expression reported in Table 3.5 (0.1 % - 2 %) falls within this range. In addition a recombinant cystatin expressed to 2 % total soluble protein in rice was driven by the CaMV 35S promoter (Vain *et al.*, 1998) which is not the most suitable

promoter for expression in monocotyledonous plants (Naik, 2001; Gallo-Meagher and Irvine, 1993). It was therefore reasonable to assume that, using the *Ubi-1*-CaMV 35S promoter which has been shown to be very effective in driving expression in monocotyledons (Groenewald *et al*, 2000) cystatin could be expressed in sugarcane at levels similar to those reported in Table 3.5. At such levels cystatin would likely have an effect on the endogenous levels of cysteine proteinase activity.

Table 3.5: Levels of inhibitory transgene products expressed in transgenic plants. Transgenes reported include synthetic *Bacillus thuringiensis*-toxin genes (Cry 1Ac, Cry 1B, Cry 2A), snowdrop lectin gene (GNA) , maize ribosome inactivating gene (b-32) and natural and synthetic cystatin genes (Oc-I, Oc-IΔD86, CC-I)

Gene	**Expression Level (% total soluble protein)**	**Plant**	**Reference**
Cry 1Ac	0.03 – 1		
Cry 2A	0.01 – 0.5	Rice	Maqbool *et al.*, 2001
GNA	0.01 – 2.5		
Cry 1B	0.01 – 0.4	Mediterranean rice	Breitler *et al.*, 2000
GNA	- 0.25	Thai rice	Tinjuangjun *et al.*, 2000
Oc-IΔD86	0.1 - 0.4	*Arabidopsis thaliana*	Urwin *et al.*, 2000
Cry 1C	- 0.4	Broccoli	Cao *et al.*, 1999
b-32	0.5 - 1	Rice	Kim *et al.*, 1999
Oc-I	0.2	Rice	Vain *et al.*, 1998
Oc-I and Oc-IΔD86	- 0.5	Tomato hairy root	Urwin *et al.*, 1995
CC-I	- 2	Rice	Irie *et al.*, 1989

There is currently no literature available concerning the effective concentrations of pleurocidin as a transgene. However several authors have reported on the effective *in vitro* concentrations of antimicrobial peptides on a range of bacterial species. The average minimum inhibitory concentration (MIC) of pleurocidin for clinical isolates of *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Staphylococcus aureus* was 43.02 µg/ml, 23.9 µg/ml and 69.3 µg/ml respectively (Cole *et al.*, 2000). In addition the MIC of pleurocidin for *E. coli* was determined to be 3.5 µM (Cole *et al.*, 1997). Cole *et al.*, (1997) showed also that factors such as pleurocidin concentration, time of exposure and bacterial species affected the MIC. The MIC of cecropins A, B and P1 were determined for *Salmonella typhimurium, Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Bacillus megaterium, Streptococcus*

pyogenes and two strains of *Escherichia coli* (Boman, 1995). The MICs for cecropins A and B ranged from 0.2 μM to 1.4 μM, and those of the synthetic cecropin P1 ranged from 0.3 μM to 44 μM. The MICs of the natural cecropins for *E. coli* were between 0.2 μM and 0.3 μM which is a similar result to that obtained by Cole *et al.* (1997). In general those results were reflective of similar experiments and results reported by Boman and Hultmark (1987). The dermaseptins are a group of peptides similar to both cecropins and pleurocidin in that they are linear helical peptides (Nicolas and Mor, 1995). The MICs of five dermaseptins from the skin of the frogs *Phyllomedusa sauvagei* and *Phyllomedusa bicolor* (Hylidae family) were determined for *Aeromonas caviae, Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Nocardia brasiliensis, Saccaromyces cerevisiae, Candida albicans* and *Cryptococcus neoformans* (Nicolas and Mor, 1995). Those authors found that the MICs ranged from 0.5 μM to 40 μM although the majority were between 0.5 μM and 10 μM. Buforin II is another amphipathic antimicrobial peptide which was shown to kill both gram negative and gram positive bacteria at concentrations in the range of 1-4 μg/ml (Park *et al.*, 2000).

Cary *et al.* (2000) reported a significant reduction of infection of transgenic tobacco by the pathogenic fungi *Colletotrichum destructivum* expressing the synthetic antimicrobial peptide, D4E1. Extracts of the transgenic plants were also shown to inhibit the growth of germinating conidia of *Aspergillus flavus* and *Verticillium dahliae*. Although the level of expression of D4E1 was not reported by Cary *et al.* (2000) the authors had previously determined that the MIC of purified D4E1 was 7,75 μM, 0.6 μM and 13.02 μM for *A. flavus, V. dahliae* and *C. destructivum* respectively. Those MICs are in the same range as those calculated for pleurocidin in the present work (Table 3.1) against *X. campestris* pv. *Vasculorum* (1 μM), *X. albilineans* (1 μM), *L. xyli* ssp. *xyli* (<10 μM) and *L. mesenteroides* (2.5 μM). It is likely therefore that transgenic plants could express pleurocidin at effective levels.

3.4.3 Application of the Cysteine Proteinase Activity Assay in Transgenic Plant Analysis

Cysteine proteinase activity has been shown to be affected by the presence of cystatin (Solomon *et al.*, 1999). This property was thought to be potentially useful in determining the presence or absence of cystatin in a putative transgenic plant. An assay was therefore

developed to measure the level of cysteine proteinase activity in sugarcane total protein extracts and leaf exudate.

Cysteine proteinase activity was detected in total protein extracts (Fig. 3.2, Table 3.3) and in chlorophyll contaminated and uncontaminated extracts of leaf exudate (Table 3.4). The activity was confirmed to be cysteine proteinase activity through the use of leupeptin and PMSF. Both substances are known to inhibit cysteine proteinases (Solomon *et al.*, 1999) but PMSF also inhibits serine proteases (Minami and Fukuda, 1995) and is less effective at inhibiting cysteine proteases than leupeptin (Solomon *et al.*, 1999; Michaud *et al.*, 1994). Results here were consistent in that PMSF caused partial inhibition and leupeptin total inhibition of cysteine proteinase activity in total protein extracts (Fig 3.2).

It was shown in the present work (Table 3.4) that cysteine proteinase activity could be detected in leaf exudates that contained no chlorophyll. The absence of chlorophyll was taken to indicate that the cells of the leaf pieces were undamaged by the extraction process and that the exudates was intercellular fluid. In addition cysteine proteinase activity was detected in concentrations of crude total protein extract as low as 0.125 µg/ml and different levels of cysteine proteinase activity were detected in crude protein extracts from different tissue types (Table 3.3). Based on these results and the expectation that the expression of cystatin transgenes in sugarcane would lower the endogenous levels of cysteine proteinase activity, it is suggested that cystatin transgenes expressed in the intercellular spaces of sugarcane could be detected the cysteine proteinase assay using leaf exudate extracted by the method described.

In the experiments performed here the fluorogenic peptide N-*t*-Boc-Gly-Lys-Arg-7-AMC, gave the best results over the range of crude total protein extract concentrations tested (Table 3.3). In addition cysteine proteinase activity was detected in the lowest crude total protein extract concentration (0.125 µg/ml) only when N-*t*-Boc-Gly-Lys-Arg-7-AMC was used. Therefore it is recommended that this peptide is used as the substrate in future applications of the cysteine proteinase assay.

3.4.4 Potential Methods for Pleurocidin Assay in Putative Transgenic Plants

The assay of pleurocidin in putatively transformed sugarcane could potentially be performed by a method similar to that used to determine the MIC and MBC for pleurocidin. Since pleurocidin was targeted to the apoplast it would be present in leaf exudate extracted as was described in this chapter (see Section 3.2.3). The leaf exudate could be placed onto solidified LB-medium in a Petri plate and allowed to absorb into the medium. The plate could then be inoculated with a volume of a liquid bacterial culture that is known to result in the growth of a lawn of bacteria following overnight incubation under controlled conditions. If pleurocidin was present in the leaf exudate a zone of inhibition should be visible at the point on the medium where the exudate was placed. Leaf exudate extracted from a non-transformed control plant could be used in a parallel experiment as a negative control and a known concentration of pleurocidin or other antibacterial agent as a positive control.

3.5 CONCLUSIONS

- A method was developed for the extraction of intercellular fluid from leaves of sugarcane, providing a means of extracting cystatin and pleurocidin proteins, targeted for transport to the cell exterior in transgenic lines. For the extraction of leaf exudate that is uncontaminated with chlorophyll, an infiltration time of 1 min. combined with a centrifugation time of 10 min. at a centrifuge speed of 14000 g, is recommended.
- A fluorometric assay was tested, and found to be adequate, which is highly sensitive to differential levels of cysteine proteinase activity and which could be used for the detection of cystatin in putative transgenic plants.
- The presence of cysteine proteinases in the intercellular spaces of leaves of the sugarcane variety N12 was confirmed.
- Minimum inhibitory concentrations (MICs) of pleurocidin were determined for the sugarcane pathogens *Xanthomonas campestris* pv. *vasculorum, Xanthomonas albilineans, Leifsonia xyli* ssp. *xyli* and *Leuconostoc mesenteroides* to be less than 10 μM.
- It was reasoned using supportive literature that at the levels of transgene expression that can potentially be expected in sugarcane, cystatin could be expected to affect endogenous

levels of cysteine proteinase activity and pleurocidin could be expected to inhibit the growth of bacterial species important in sugarcane pathology.

CHAPTER 4: GENERATION AND ANALYSIS OF TRANSFORMATION CONSTRUCTS

4.1 INTRODUCTION

Plasmid vectors are the conventional means of delivering transgenes into target tissue. The choice of vector is important since different vectors are used for *Agrobacterium*-mediated and direct delivery methods. Choice also depends to a large extent on the target species since the promoters, which will drive expression of the transgene and which do not function with the same efficiency in all species, are contained in the vector. Apart from consideration of the vector, the transgene itself may not be ideally suited for expression in a host plant and may require modification by the addition of host-consensus start and stop sequences. It may also be desirable to direct the transgene product to a particular subcellular location, in which case the signal sequence of the transgene may need to be modified. In this project cystatin and pleurocidin genes were modified and cloned into a suitable plasmid vector, pUBI510, for biolistic insertion and expression in sugarcane.

Extracellular localization of transgene products decreases the possibility of their being processed by endogenous host enzymes. In addition this location ensures a greater possibility that sugarcane pathogens will come into contact with the transgene products. Cystatin and pleurocidin transgene products were therefore targeted for this region. In Chapter 3 it was shown that intercellular fluid could be extracted. Thus transgene products targeted to this region could be easily extracted. Assays for both pleurocidin and cystatin were also tested and found to be potentially useful in the analysis of transgenic plants.

The reasoning algorithm called PSORT assigns probabilities of sub-cellular locale to amino acid sequences based on a database of known signal sequence-locale combinations (Nakai and Kanehisa, 1992; Nakai and Horton, 1999; Nakai, 2000). Analysis of pleurocidin and cystatin transgenes with PSORT by Dr Stuart Rutherford (pathology SASEX) showed that cystatin was likely to be transported out of the cell (55%) while pleurocidin required a signal sequence

substitution for extracellular localisation. Alterations to the pleurocidin transgene was therefore performed by Dr Stuart Rutherford: The pleurocidin signal sequence from winter flounder was replaced with a signal sequence derived from a maize peroxidase gene. PSORT analysis of the modified transgene sequence indicated that it was likely to transported out of the cytoplasm into the ER lumen (85%) or intercellular space (76%). For both transgenes it was considered likely that at least a portion of the transgene product would be harboured in the intercellular spaces. This was considered acceptable since low concentrations of both of the gene products are potentially efficacious as was argued in chapter 3.

During the process of transgene manipulation, cloning and amplification, alterations may occur in the sequences of the transgene insert or the vector. Small variations in the sequence of either may have deleterious effects on transgene expression or functioning of the transgene product. It was therefore necessary to perform a series of checks to ensure the transgene-plasmid vector constructs were intact and true to the original design before the constructs could be used for delivery via microprojectile bombardment. These tests, involving PCR analyses, restriction enzyme analyses and sequencing of the constructs, are reported in this chapter.

4.2 MATERIALS AND METHODS

4.2.1 Pre-Constructed Plasmids

The commercially available pBluescript® II SK Phagemid (Stratagene) and pGem-T® Easy Vector (Promega) are plasmids used for cloning and replication of genes (Fig. 4.1; top left and top right respectively). Both plasmids contain a multiple cloning site (MCS) with several restriction endonuclease sites, the ß-galactosidase alpha-fragment (*lac Z*) for blue-white colour selection of recombinant phagemids/vectors, an f1 filamentous phage origin of replication (fi (+) ori) for recovery of the sense strand of the *lac Z* gene plus insert, and an ampicillin resistance gene (Amp) for antibiotic selection of transformed bacterial cells. The MCS is flanked by T7 and T3 RNA polymerase promoters in the case of the pBluescript® II SK Phagemid and by T7 and SP6 promoters in the case of the pGem-T® Easy Vector.

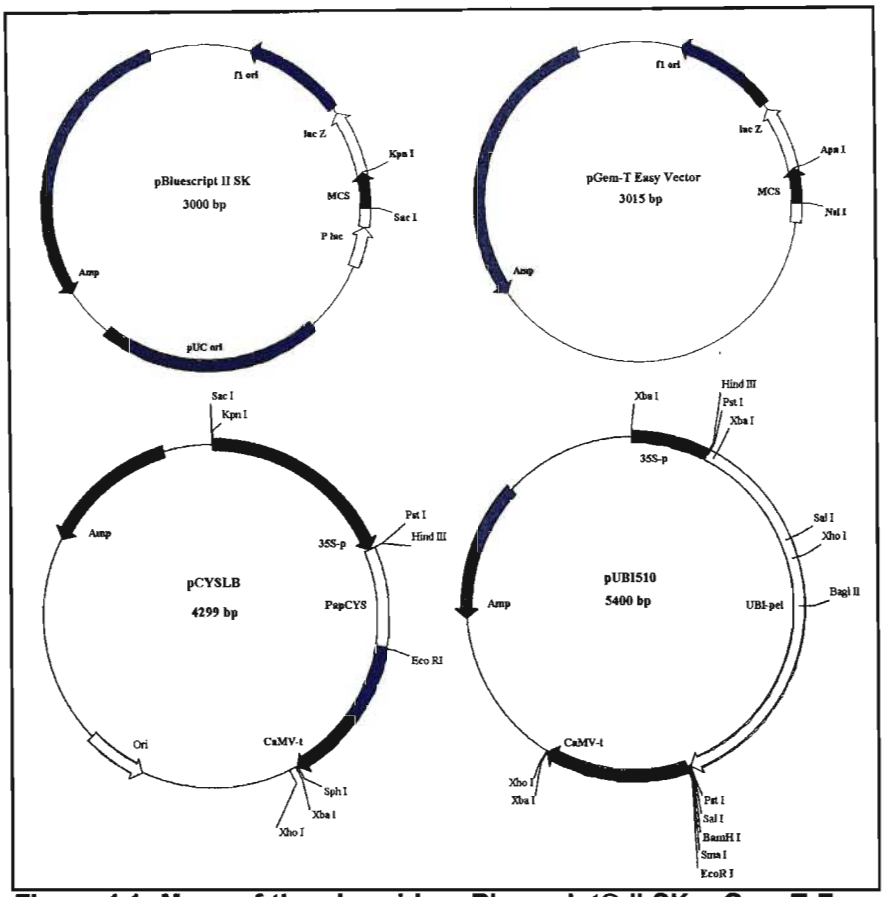

Figure 4.1: Maps of the plasmids; pBluescript® II SK, pGem-T Easy Vector, pCYSLB and pUBI510. Shown are: f1 filamentous phage origin of replication (f1 ori), ß-gaclactosidase α-fragment (*lac Z*) and promoter (p *lac*), ampicillin resistance gene (Amp), plasmid origin of replication (pUC ori and Ori), cauliflower mosaic virus promoter (35S-p) and terminator (CaMV-t), a promoter derived from the maize polyubiquitin gene (UBI-pei) and the papaya cystatin gene (PapCYS). PBluescript® II SK, containing the pleurocidin gene in the multicloning site (MCS), was donated by Dr Gill Diamond (UMDNJ – New Jersey Medical School).

The pleurocidin gene was donated to SASEX by Dr Gill Diamond (UMDNJ – New Jersey Medical School) having been cloned into the pBluescript® II SK Phagemid. The plasmid pCYSLB (Fig 4.1; bottom left) was donated as a gift from Professor Karl Kunert (FABI, University of Pretoria). It contains the papaya cystatin coding sequence, cloned between the CaMV promoter and terminator sequences, and the ampicillin resistance gene (Amp). The plasmid, pUBI510 (Fig. 4.1; bottom right; Groenewald, *et al.*, 2000) contains the maize polyubiquitin-derived promoter including the first exon and intron (UBI-pei), the 35S cauliflower mosaic virus (CaMV) promoter and derived transcription termination

(Terminator) sequences, and the ß-lactamase coding region (Amp) for ampicillin resistance, which is necessary for bacterial selection. Cystatin and pleurocidin transgenes were cloned into pUBI510 between the Bam HI and Eco RI sites highlighted in bold (Fig. 4.1; bottom right) to yield two separate constructs; pUBI510-cys and pUBI510-pleuro.

4.2.2 Primers

Primers used for transgene manipulation and cloning (Table 4.1) were designed by Dr Stuart Rutherford (SASEX). Primer stocks were stored at -80°C at a concentration of 100 µM but were diluted to a working concentration of 6 µM stored at –20°C. The primer pairs PleuroA-for/PleurA-rev and Bamsignalperox/Hindsignalperox were used in the construction of the plasmid pUBI510–pleuro8 and for manipulation of the pleurocidin transgene insert (see section 4.3.1) but were not used for the subsequent amplification of transgene inserts. The primers Pleuro-consensus and Pleuro-antisense were used for the amplification of the pleurocidin transgene insert from the purified plasmids, from transformed bacterial cells or from putative transgenic plants (see Chapter 5). Similarly the primers Papaya 3' start and Papaya 5' start were used for the amplification of the cystatin transgene insert.

Table 4.1: Primers used for transgene manipulation and cloning. Restriction endonuclease cleavage sites, GGATCC (Bam HI) GAATTC (Eco RI) and AAGCTT (Hind III) within each primer are shown in bold. The start codon and consensus adjacent codons for monocotyledons are shown in green and red respectively. All primers are written in the 5' to 3' direction. For each primer pair the sense primer is followed by the antisense primer.

Primer Name	Primer Sequence (5'-3')
Papaya 3' Start	**GGATCC**ACCATGGCGCCCGGAATTGTG
Papaya 5' Start	**GAATTC**TAATGAGCATCACCA
PleuroA-for	T**GGATCC**GCACAAAGCCCACTTTG
PleuroA-rev	GC**GAATTC**AGGTAAAAGTCTG
Pleuro-consensus	**GGATCC**GCAACCATGGCGTTCACTG
Pleuro-antisense	**GAATTC**TGATTTATTTCATTAAAAGCAAGAAG
Bamsignalperox	AGGCACA**GGATCC**AGGCTGCCATGGCGTC
Hindsignalperox	**AAGCTT**CCCCAGCCTGCGTGGGCGACACAGG

4.2.3 PCR Amplification Conditions

PCR reactions were performed in PCR tubes designed for use in either a Gene Amp® PCR System 9700 or a Hybaid Omnigene PCR Thermal Cycler. The thermal cycling regime generally consisted of an initial denaturing step of 95°C for 1 min, followed by an amplification step of 30 cycles of 95°C for 30 sec., 55°C for 30 sec. and 72°C for 1 min, ending with 1 cycle of 72°C for 5 min However, slight variations of this regime were used depending on the specific requirements of the experiment. Reagent concentrations of PCR were always 1X Taq DNA polymerase buffer (Promega), 1.5mM $MgCl_2$, 0.2 μM primers, 2mM dNTPs and 1.25 U/reaction Taq DNA polymerase (Promega). Final reaction volumes and template DNA concentrations varied depending on the PCR machine and the specific requirements of the experiment.

4.2.4 Manipulation and Cloning of Transgenes

a) Pleurocidin Vector, pUBI510–pleuro8

The pleurocidin gene was amplified from pBluescript® II SK with the primers PleuroA-for and PleuroA-rev, incorporating BamHI and EcoRI sites respectively (Table 4.1) and cloned into the pGEM® T-Easy Vector (Promega). The start codon was altered by amplification using the primers Pleuro-consensus and Pleuro-antisense (Table 4.1) which incorporated the consensus monocotyledon start sequence just downstream of the Bam HI cleavage site. The consensus start sequence was altered slightly (compare primers Pleuro-consensus and Bamsignalperox in Table 4.1) with the addition of a signal sequence derived from the maize peroxidase signal sequence using the primers Bamsignalperox and Hindsignalperox (Table 4.1) which incorporated Bam HI and Hind III sites at the 5' and 3' ends of the signal sequence respectively. The Hind III site was used to splice the 3' end of the signal sequence with a Hind III at the 5' end of the pleurocidin sequence. The pleurocidin sequence plus the added signal sequence was cloned into the plasmid vector pUBI510.

b) Cystatin Vector, pUBI510-cys3

The cystatin gene (Fig. 4.2) isolated by PCR from papaya cDNA was donated to SASEX by Dr Karl Kunert, having been cloned into the vector pCYSLB. Cystatin was transferred from pCYSLB to pGEM-T® Easy and subsequently to pUBI510 by amplification using the primers Papaya 3' Start and Papaya 5' Start (Table 4.1), followed by restriction and ligation. Further manipulation of the cystatin transgene was not required.

Cystatin

GGATCCACCATGGCGCCCGGAATTGTGATCGGTGGTTTGCAGGAC
GTCGAGGGAGATGCTAACAATCTTGAATACCAGGAACTTGCTCGGT
TCGCCGTCGATGAGCACAACAAGAAAACTAATGCGATGCTCCAGTT
CAAGAGGGTTGTGAATGTAAAGCAGGCAGTGGTTGAAGGCTTAAAG
TACTGCATCACTTTGGAGGCTGTAGATGGTCACAAGACGAAGGTAT
ATGAGGCCGAGATCTGGGTGAAGCTCTGGGAGAATTCCAGGAGCT
TGGAGGGATTC**AAGCTT**CTTGGTGATGCTCAT**TAGAATTC**

Pleurocidin

GGATCCAGGCTGCCATGGCGTCTCCCACCTTGATGCAATGCCTGG
TCGCCGTTTCCCTCCTCTCCTGTGTCGCCCACGCAGGCTGGGG**AA**
GCTTTTTTAAAAAGGCTGCTCACGTTGGCAAGCATGTTGGCAAGGC
GGCCCTTACTCATTACCTTGGCGATAAGCAGGAGCTCAACAAGCGT
GCAGTCGATGAAGACCCAAATGTTATTGTTTTTGAA**TGA**AGAAATCG
CCTTGAAGGAGCCTTCAGATGATATATAATCCTTCTTGCTTTTAATG
AAATAAATCA**GAATTC**

Figure 4.2: Cystatin and pleurocidin transgene sequences. Start codons (bold, green) consensus sequences (bold, blue) and stop codons (bold, red) are shown. Priming sites are underlined. Bam HI (GGATCC) Hind III (AAGCTT) and Eco RI (GAATTC) are shown in bold black letters. The coding region of the pleurocidin signal sequence is shown in magenta letters, the 3' untranslated region is shown in red letters and the region coding for the mature peptide in black letters.

4.2.5 Bacterial Transformation

E. coli, strain JM109 HEC (Promega) competent cells were thawed, from storage at –80°C, on ice, mixed by gently flicking the tube and transferred (50 µl aliquot) to 1.5 ml microcentrifuge tubes containing 2 µl of plasmid (between 200 ng and 500 ng DNA). The tubes were again

mixed gently, incubated on ice for 20 min. and transferred to a water bath at exactly 42°C for 45 – 50 sec. Carefully the tubes were returned to ice for 2 min. and 950 µl of room temperature SOC medium (2 g/ml Bacto tryptone, 0.5 g/ml Bacto yeast extract, 0.05 g/ml NaCl, 0.2 M glucose, 0.1 M $MgCl_2$, 0.1 M Mg SO_4, pH 7 with KOH) was added. The tubes were then incubated for 1.5 hours at 37°C with shaking at 150 rpm. 100 µl of each transformation reaction was then spread onto Luria Bertani plates (1.0% m/v bactotryptone, 0.5% m/v yeast extract, 0.55 m/v NaCl and 0.1% m/v glucose, pH 7.5) containing 50 µg/ml ampicillin in a sterile laminar flow hood. The plates were incubated at 37°C overnight. Colonies were picked off of the plates, transferred to gridded, numbered plates, which were incubated at 37°C overnight.

4.2.6 Maintenance and Storage of Transformed Bacterial Cultures

Colonies of transformed cells were grown on antibiotic plates (see 3.2.1 for details) at 37°C overnight. The plates were sealed with parafilm, stored at 4°C and colonies were transferred to fresh plates every three weeks.

4.2.7 Colony PCR

Colonies of putatively transformed cells were subject to PCR using primers specific for the cystatin and pleurocidin transgene inserts. Cells were transferred, using sterile toothpicks, to PCR tubes containing 29 µl PCR cocktail and subject to amplification as described in section 4.2.3. PCR products were analysed by gel electrophoresis (see section 4.2.12)

4.2.8 Plasmid Isolation

Single colonies of transformed *E. coli* JM109 HEC were used to initiate 50 ml cultures in LB broth containing 100 µg/ml ampicillin in 250 ml Ehrlenmeyer flasks. Flasks and LB broth were prepared and autoclaved in advance and ampicillin was added from stock (5 mg/ml

ampicillin in 50% ethanol stored at –20 °C) after the broth had cooled sufficiently. The flasks were incubated at 37 °C overnight with shaking at 150 rpm. The plasmids were isolated by alkaline lysis from the cell cultures the following day using the Nucleobond AX100 plasmid purification kit and manufacturers protocol. Following elution of the plasmid DNA from the Nucleobond anion exchange columns it was precipitated in 1.5 ml microcentrifuge tubes with 0.7 volumes of isopropanol at room temperature and centrifuged at 12000 g for 15 min. at 4 °C to pellet the DNA. The pellet was washed with 70% (v/v) ethanol and allowed to air dry, either upside down on the bench top or in the vacuum centrifuge, and re-dissolved in 50-100 µl sterile water.

4.2.9 Quantification of Plasmids

Plasmid concentrations were measured fluorometrically. The Hoefer® DyNA Quant® 200 fluorometer was used for all fluorometric assays, which were carried out according to the supplied instructions manual. The fluorometer was calibrated with 2µl of a 1000 µg/ml dilution of calf thymus DNA (Sigma) added to 2 ml of assay buffer (1 µg/ml Hoechst dye, H33258 + 1X TNE; 0.2 M NaCl, 10 mM Tris-Cl, 1mM EDTA, pH 7.4). DNA concentrations were determined against this standard.

4.2.10 Restriction Digestion of Plasmid DNA

Constructs were linearised by digesting 1 µg of plasmid DNA with 10 U restriction endonuclease Bam HI in a final volume of 20 µl of 1X concentrated SuRE/Cut™ Buffer B recommended by the manufacturer (Roche). Reactions were allowed to continue overnight in an incubator at 37°C in 1.5 ml microcentrifuge tubes sealed with parafilm (American National Can.) and LidLock safety clips (Sorenson™ Bioscience, Inc.). If the reactions could not be processed immediately the following morning they were stored at 4°C until later in the day.

4.2.11 Purification of Digestion Products

One volume of a phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added and the tubes were vortexed briefly before being centrifuged for 10 min. at 12 000 g at room temperature. The aqueous layer was drawn off using a pipette and transferred to a clean labeled 1.5 ml microcentrifuge tube. The old tube and remaining contents were discarded. The DNA was precipitated in 2.5 volumes of 100% (v/v) ethanol and 0.3 M NaOAc. Incubation at -20°C for a few hours or at -80°C for 30 min. increased the precipitation efficiency. Tubes were centrifuged for 30 min. at 14 000 g at 4°C to pellet the DNA. The supernatant was poured off, the DNA rinsed with 70% (v/v) ethanol and the open tubes were placed upside down on paper toweling on the bench top to dry. Once completely dry the DNA was redissolved in 30 µl water.

4.2.12 Gel Electrophoresis of Linearised Plasmid DNA

Agarose gels (0.8%, w/v) were made up in 0.5X TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0) and ethidium bromide (10mg/ml) was added to a final concentration of 500 µg/l prior to electrophoresis. Gel-loading buffer (10% w/v sucrose, 0.25% w/v xylene cyanol, 0.25% w/v bromophenol blue, 1 M NaCl, 20 mM Tris-Cl, pH 7.5, 5 mM EDTA) was added to DNA samples at a ratio of 1:6 (v/v) to achieve a final sample volume of 30 µl. The DNA samples were loaded under buffer and run at 50 V until the dye front had migrated ¾ of the length of the gel. The DNA fragments were viewed by placing the gel onto a UV transilluminator and images of the gels were captured using polaroid film. No destaining was required.

4.2.13 DNA Sequencing

Construct sequences containing the transgene insert were analysed by capillary electrophoresis to check the correctness of the sequence. Sequences were primed using the UBI-exp primer which is specific to the polyubiquitin-derived promoter (UBI-pei; see Fig 4.1) contained in the pUBI510 plasmid. The ABI Prism™ sequence analyser, which

determines DNA sequence by dye termination cycle sequencing, together with a Perkin Elmer ready reaction kit with AmpliTaq® DNA polymerase, FS, was used for all sequencing reactions.

Good quality plasmid DNA (500 ng), 2 µl primer (6 µM) and 6 µl Big Dye cocktail were added to ultrapure, autoclaved water to make final reaction volumes of 20 µl in autoclaved PCR tubes. A thermal cycling regime consisting of an initial denaturing step of 95°C for 1 min. followed by 30 cycles of 95°C for 30 sec, 40°C for 30 sec and 60°C for 3 min. with a ramp of 2 sec/°C, was used for amplification. The contents of the PCR tubes were transferred to 1.5 ml microcentrifuge tubes the DNA precipitated in 80 µl of 60 ± 3% non-denatured ethanol by briefly vortexing and incubating at room temperature for 15 min.. The DNA was pelleted by centrifugation at maximum speed for 15 min.. The supernatants were carefully aspirated with a pipette and the pellets washed, with brief vortexing and centrifugation for 10 min., in 100 µl of 70% (v/v) ethanol. The supernatants were aspirated with a pipette and the residual liquid removed by blotting with a twisted piece of tissue paper. The ethanol wash and following steps were repeated and the pellets were then dried in a vacuum centrifuge for 10-15 min. 18 µl of Template Suppression Reagent (Perkin Elmer) was added and the samples denatured for two min. in a boiling water bath. The tubes were incubated on ice for 1 minute and centrifuged before being transferred to autosampler tubes. The samples could be stored at –4 °C for up to a week before being loaded into the ABI Prism Genetic Analyser for analysis by capillary electrophoresis.

4.3 RESULTS AND DISCUSSION

4.3.1 Verification of Plasmid Integrity

a) Confirmation of the Presence of Transgene Inserts

Since the plasmids pUBI510-pleuro8 and pUBI510-cys3 were newly designed (Dr Stuart Rutherford, SASEX) and constructed it was necessary to analyse them to determine their integrity. The plasmids were subject to a PCR test to ensure the presence of transgenes (Fig. 4.3). Transgene specific primers were used to amplify cystatin and pleurocidin transgenes from the plasmids. PCR products were analysed by electrophoresis (section 4.2.12) to check for the expected 314 bp and 292 bp amplification products from pUBI510-pleuro8 and pUBI510-cys3 respectively. Plasmids yielding positive PCR results were analysed by restriction digestion and sequencing.

b) Restriction Analysis

Plasmid integrity was checked by restriction digestion and electrophoresis of the restriction products on 0.8% agarose gels. The sizes of restriction products were compared with expected fragment sizes (Table 4.2) which were determined from the location of restriction enzyme cleavage sites in the plasmid vectors. Restriction product sizes were estimated using molecular weight markers III and V (Boehringer Mannheim) (Fig. 4.4). The plasmids pUBI510-cys3 and pUBI510-pleuro8 were digested using Hind III. Since the transgene inserts had been cloned into the vectors using the restriction enzymes Bam HI and Eco RI, plasmids were digested with both of these enzymes to excise the transgenes from the constructs. This type of analysis was suitable for checking that no major re-arrangements or deletions had occurred in the plasmids. Had such alterations occurred the restriction products would have been substantially different from what was expected. However this type of analysis was not suitable for checking for relatively minor alterations in the plasmids such as single base pair substitutions or deletions. For more thorough analysis the plasmids were therefore sequenced

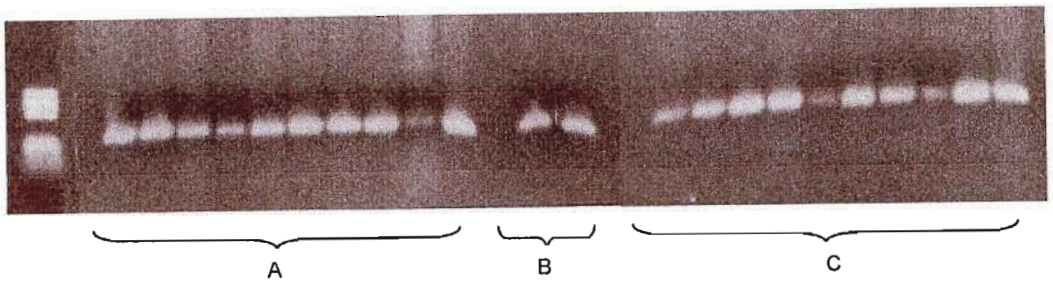

Figure 4.3: Electrophoresis of PCR products: A check for the presence of transgene inserts. Pleurocidin (**A**) and cystatin (**C**) transgenes were amplified from colonies of *E. coli* transformed with pUBI510-pleuro8 and pUBI510-cys3. Transgenes were also amplified from the plasmids pUBI510-pleuro8 and pUBI510-cys3 (**B**, left to right as written). Molecular weight marker V, far left of figure, indicates the size of the amplified products is in the expected range, i.e., pleurocidin = 314 bp, cystatin = 292 bp.

Table 4.2: Expected fragment sizes resulting from digestion of pUBI510-pleuro8 and pUBI510-cys3. Expected fragment sizes were calculated from the position of the restriction sites in the plasmids.

Enzyme/s	Plasmid	Expected Fragment Sizes (base pairs)
Hind III	pUBI510-pleuro8	2716 + 3002
	pUBI510-cys3	2076 + 3616
Bam HI + Eco RI	pUBI510-pleuro8	314 + 5400
	pUBI510-cys3	292 + 5400

c) Sequence Analysis

For comprehensive assessment of plasmid integrity, plasmids were sequenced (Fig. 4.5). Electropherograms generated from the analysis of sequences were checked visually (Fig. 4.6), base by base and corrected in instances where overlapping dye peaks led to incorrect base assignment. In all cases sequences were found to match the original sequences. The expected sequences were compared with the actual sequences to ensure that the two matched (Fig. 4.5).

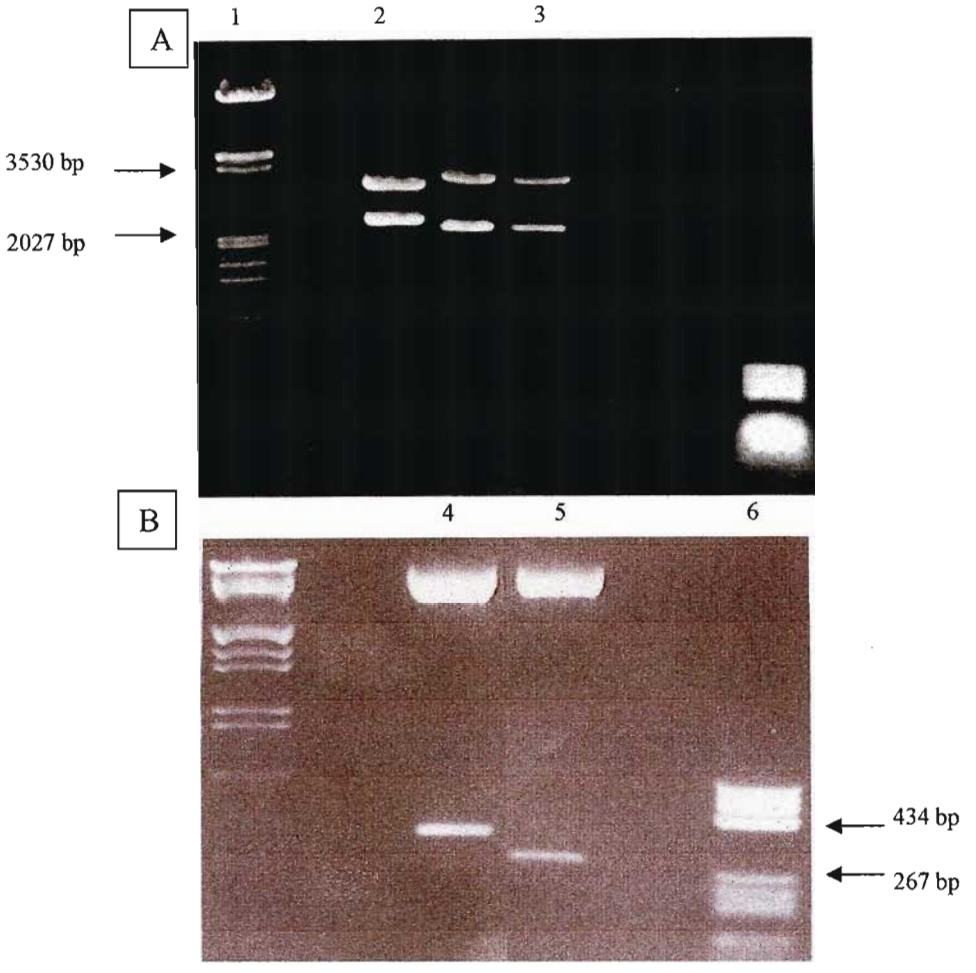

Figure 4.4: Electrophoresis of digested plasmid DNA. A: Products of plasmid digestion using Hind III. Relevant lanes shown are: molecular weight marker III (lane 1), pUBI510-pleuro8 (lane 2) pUBI510-cys3 (lane 3). **B:** Products of plasmid digestion using Bam HI and Eco RI. Lanes shown are: pUBI510-pleuro (lane 4), pUBI510-cys (lane 5)and mkr V (lane 6). Sizes, in base pairs (bp), of the relevant bands (arrows) from Mkr III and Mkr V are given.

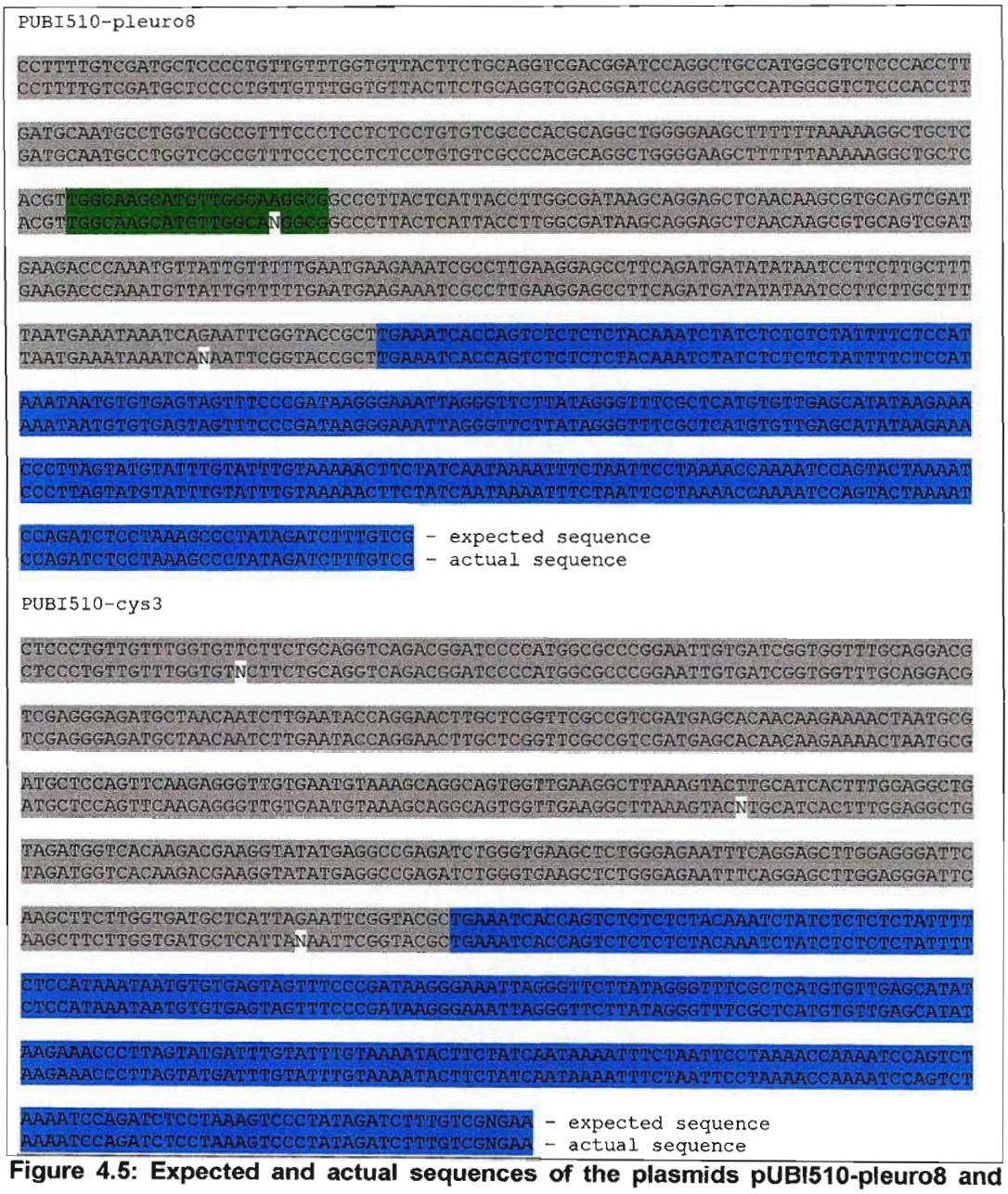

Figure 4.5: Expected and actual sequences of the plasmids pUBI510-pleuro8 and pUBI510-cys3. The plasmids pUBI510-pleuro8 and pUBI510-cys3 were sequenced by dye terminator cycle sequencing using an ABI Prism™ sequence analyser. The expected sequences are shown with the actual sequence in the line directly below. The transgene inserts are highlighted in grey and a portion of the plasmid vector highlighted in blue. The letter 'N' (un-highlighted) indicates a base that was undecipherable by the sequence analyser. These bases were determined by visual examination of the electropherogram corresponding to the sequence (Fig. 4.6). The area highlighted in bright green corresponds to the electropherogram shown in Fig. 4.6.

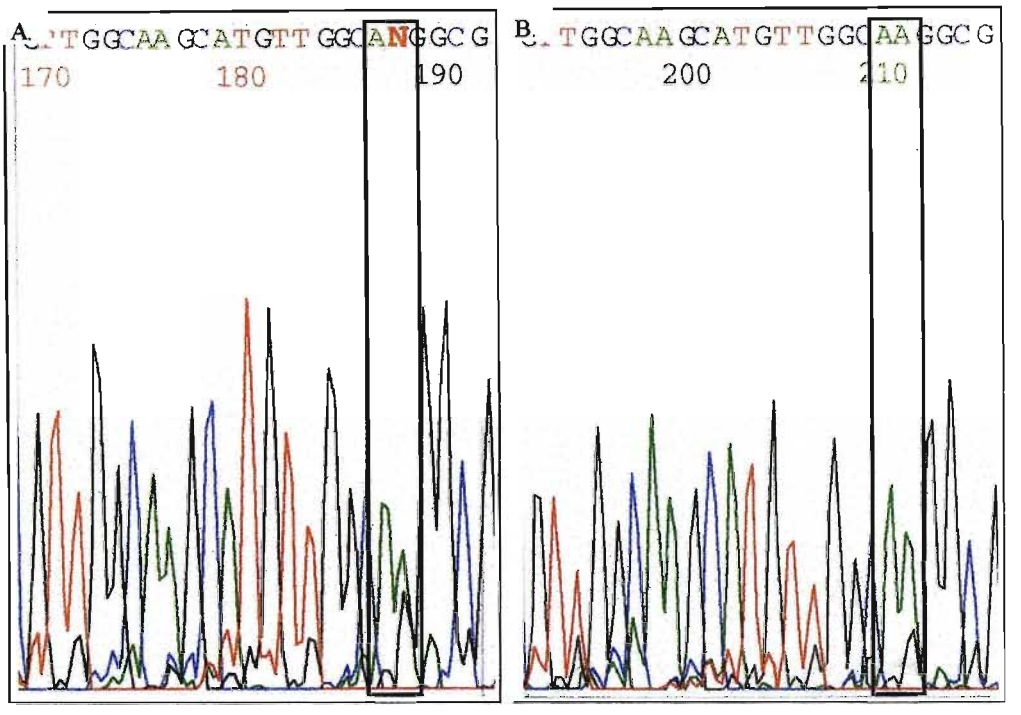

Figure 4.6: Electropherograms of identical portions of the plasmid pUBI510-pleuro8. The electropherograms were produced from the sequencing of the plasmid on two separate occasions. The area of interest is surrounded by a bold rectangle in both A. and B.. The double peak in the green line indicates that the proper sequence should read AA as it does in B. however in A. interference can be seen in the form of a peak in the black line quite close to the second peak in the green line. Visual examinations shows that the green peak is the dominant one and the proper base here is A.

4.4 CONCLUSIONS

- The modification of pleurocidin and cystatin transgenes and the cloning of the transgenes into vectors suitable for expression in sugarcane was described.
- The untested plasmids pUBI510–pleuro8 and pUBI510–cys3 were analysed by PCR, agarose gel electrophoresis and capillary electrophoresis to ensure that undesirable alterations had not occurred during the transgene modification and plasmid construction phases.

- In both cases the plasmids tested gave the expected results indicating that they could be used in the transformation process.
- The methods described in this chapter used for the initial testing of the newly constructed plasmids were subsequently used throughout the project for testing freshly isolated plasmids before they were used for delivery into sugarcane cells via biolistic transformation as described in Chapter 5.

CHAPTER 5: SUGARCANE TISSUE CULTURE, GENE DELIVERY AND MOLECULAR ANALYSIS OF PUTATIVE TRANSGENIC PLANTS

5.1 INTRODUCTION

The most accessible technology, financially and logistically, for the implementation of genetic transformation in sugarcane has been microparticle bombardment or biolistic transformation. This technology has been used successfully for sugarcane transformation in foreign and local research laboratories (Bower and Birch, 1992; Gambley *et al.*, 1993; Bower *et al.*, 1996; Snyman *et al.*, 1996; Hansom *et al.*, 1998). Both embryogenic callus (Bower and Birch, 1992; Bower *et al.*, 1996; Hansom *et al.*, 1998) and meristematic leaf discs (Gambley *et al.*, 1993; Snyman *et al.*, 2001) have been used as target material for bombardment. One aim of the work in this chapter was to compare three different protocols for *in vitro* culture, regeneration and transformation of sugarcane. The protocols involved the use of either leaf discs or callus as target tissue for transgene delivery followed by plant regeneration by either direct or indirect embryogenesis respectively, for the production of transgenic sugarcane. Regeneration by direct embryogenesis from leaf discs was either in the presence of low (0.6 mg/l) or high (3 mg/l) concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), whereas regeneration from callus by indirect embryogenesis was necessarily in the presence of a high concentration (3 mg/l) of 2,4-D. The constructs pUBIKN, pUBI510-pleuro8 and pUBI510-cys3 were delivered biolistically to target material and putative transgenic plants were regenerated for molecular analyses.

Production of transgenic plants via the direct route of embryogenesis is attractive because it requires that plant material is *in vitro* 10-12 weeks less than in the indirect route (Snyman *et al.*, 2000; Snyman *et al.*, 2001) and involves exposure to lower concentrations of auxin (Grisham and Bourg, 1989; Snyman *et al.*, 2000; Snyman *et al.*, 2001). The incidence of somaclonal variation and infertility in regenerated plants is increased when plant tissue is

maintained in the undifferentiated state (callus) for long periods in culture at high auxin concentrations (Moore, 1999; Tyagi *et al*, 1999). Therefore it is advantageous to avoid the culturing of callus for extended periods and the use of high auxin concentrations when possible. However, the use of biolistic transformation in conjunction with direct embryogenesis in sugarcane has not been studied as extensively as it has with indirect embryogenesis. When direct embryogenesis has been used the efficiency has been lower (Snyman *et al.*, 2001) than that reported for the indirect route, i.e. 1 (Falco *et al.*, 2000) - 19.8 ± 3.7 (Bower *et al.*, 1996) independent genotypes regenerated per bombardment event.

The major role of naturally produced auxins is to promote cell elongation in young shoots (Campbell, 1992). Therefore plant tissue cultured in the presence of high concentrations of the synthetic auxin 2,4-D may be more metabolically active than those cultured in the presence low concentrations of 2,4-D and therefore may be better able to repair cellular damage caused by gene delivery by microprojectile bombardment. It was therefore proposed that the higher transformation efficiency of the indirect route may be partially attributable to the high concentration of 2,4-D used. An attempt was made to combine the benefit of increased transformation efficiency potentially caused by high 2,4-D concentration with the advantages of transformation using direct embryogenesis as a means of regenerating transformed plants. Leaf discs intended for use as target material for gene delivery were therefore cultured on medium containing 3 mg/l or 0.6 mg/l 2,4-D and in both cases were cultured according to a protocol known to induce regeneration by direct embryogenesis in sugarcane. A third protocol was used in which leaf discs cultured in the presence of 3 mg/l 2,4-D according to conditions known to induce indirect embryogenesis in sugarcane.

A further aim of the work was to produce plants for preliminary analysis of the pleurocidin and cystatin transgenes. In Chapter 3 it was shown that pleurocidin and cystatin were suitable candidates for use as transgenes in sugarcane for increasing sugarcane resistance to bacterial and viral, opportunistic infectious agents. In preparation for their transfer to sugarcane these genes were cloned into plasmid vectors suitable for expression in sugarcane (Chapter 4). Expression in plasmid vectors was driven by the Cauliflower Mosaic Virus 35 S/maize polyubiquitin-derived promoter combination (CaMV/*Ubi*-1). The plasmids pUBI510-cys3 and pUBI510-pleuro8 containing cystatin and pleurocidin respectively were co-delivered along with pUBIKN, containing the *nptII* gene for kanamycin resistance, by microprojectile bombardment into embryogenic callus and meristematic leaf discs of sugarcane.

Sugarcane varieties NCo376 and N12 were chosen for transformation. Despite yielding high sucrose levels, NCo376 is highly susceptible to diseases such as SCMV and common rust while N12, a commonly grown commercial variety, is very susceptible to RSD and rated as intermediate with regard to SCMV and rust. Since these varieties already possess very good agronomic traits and since their specific deficiencies could be addressed through the expression of either pleurocidin or cystatin transgenes or both, these varieties were considered suitable candidates for gene transfer. Ideally the expression of the transgenes would be required to increase the resistance of NCo376 and N12 to bacterial and viral infectious agents without altering any other genetic traits.

In all cases the kanamycin antibiotic variant, geneticin (G418) was used as a selective agent for the identification of transformed plants through its inclusion at a concentration of 45 mg/l in all media used for the culture of plant material following bombardment.

The molecular analysis of the putative transgenic plants was an important aspect of the present work. Putative transformed plants were analysed using PCR to test for the presence of integrated transgenes and Southern hybridization to determine integration pattern and transgene copy number. Transgene copy number constructions were employed in conjunction with the PCR and Southern hybridization analyses to gain further information about the number of transgene copies present in each line. In addition RT-PCR analysis was used to test for the presence of mRNA (transcription) in PCR positive plants.

5.2 MATERIALS AND METHODS

5.2.1 Plasmids

Plasmids pUBI510-cys3, pUBI510-pleuro8 and pUBIKN (Chapter 4) containing the cystatin, pleurocidin and *npt-II* genes respectively were maintained in *E. coli*, extracted as described in chapter 4 and stored at –20°C.

5.2.2 Primers

The primer pairs, 'Papaya 3' start' and 'Papaya 5' start', 'Pleuro-consensus' and 'Pleuro-antisense' (Table 4.1) and 'NPT-for' and 'NPT-rev' were used to amplify the cystatin, pleurocidin and *npt-II* genes respectively. The same primers were used for the production of probes for Southern blot hybridization analysis and RT-PCR. Primer stocks were stored at a temperature of -80°C and at a concentration of 100 µM but were diluted to a working concentration of 6 µM and stored at –20°C.

5.2.3 Sugarcane Varieties

Sugarcane varieties NCo376 and N12 were used.

5.2.4 Harvest of Sugarcane and Preparation of Explants

The stalks of mature (> 6 months) sugarcane, that had been field grown from setts on site at SASEX, were cut using a cane knife that had been washed in a 10% Jeyes fluid solution. The upper section of the stalk (first 4 or 5 internodes) was separated from the lower section of the stalk which was discarded. The upper sections were placed in black plastic bags, transported to the laboratory and were stored overnight (26 ± 1°C).

Sugarcane tops were stripped down to the first 3 or 4 internodes and the leaf-roll trimmed to within 10 cm of the first internode. The tops were then placed apical-side down in a beaker of 70% (v/v) ethanol in the laminar flow hood. In a sterile atmosphere in the laminar flow hood, the fibrous, outer layers of each leaf roll were stripped away and the inner, softer portion was sliced into discs (2 mm thick) using a sterile scalpel blade and a glass plate. The discs (14 from each stalk) were taken from the portion of the leaf roll nearest the first internode.

5.2.5 Three Protocols for *In Vitro* Culture and Regeneration of Sugarcane

For *in vitro* culture and regeneration of sugarcane three protocols were used which involved regeneration by direct embryogenesis from leaf discs or regeneration via a callus stage by indirect embryogenesis. The three protocols are described below and shown in Fig. 5.1.

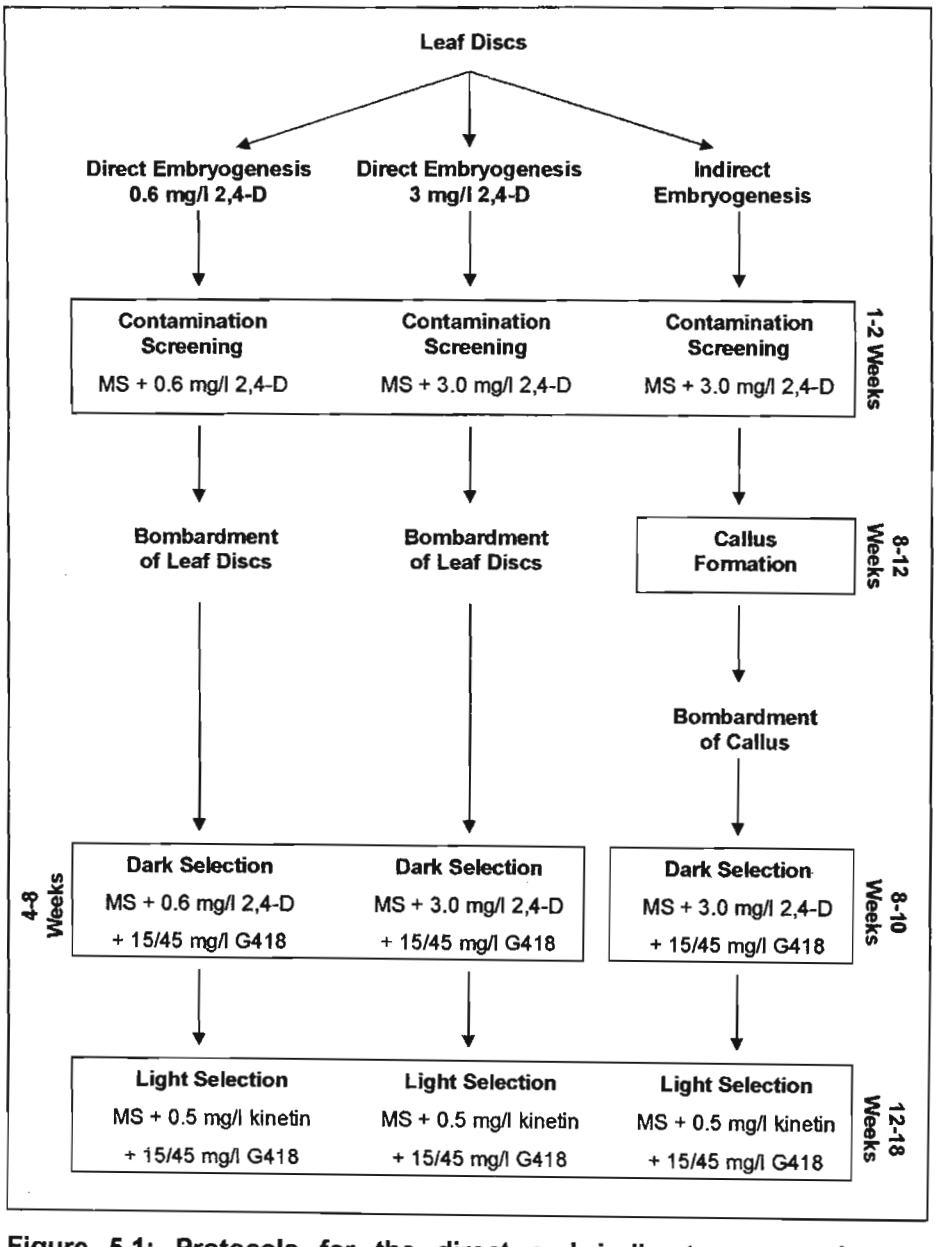

Figure 5.1: Protocols for the direct and indirect regeneration of sugarcane. The tissue culture media and the number of weeks required for each stage (far right and far left) are shown. For non-bombarded controls the bombardment stage was omitted. The bombardment stage is shown to indicate its position in the pathway relative to other events.

a) Protocols 1 and 2: Regeneration by Direct Embryogenesis

The protocol below describes the culture of leaf discs intended for use as target material for gene delivery by microprojectile bombardment and regeneration by direct embryogenesis on medium containing 0.6 mg/l or 3 mg/l of the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D). The protocols were the same except for the concentration of 2,4-D used. The culture of leaf discs to be used as non-bombarded controls was the same as that described below with the exception that geneticin was excluded from the media.

Leaf discs (section 5.2.4) were placed onto medium containing full strength MS formulation (Murashige and Skoog, 1962), casein hydrolysate (CH; 1 g/l), sucrose (30 g/l), agar gel (Sigma; 5 g/l) and 2,4-D (0.6 mg/l or 3mg/l) to promote cellular metabolism. The discs were cultured for 1–2 weeks at which time discs with visible bacterial or fungal contamination were discarded. Discs that were not contaminated were used as target material for gene delivery by microprojectile bombardment. For specific experiments some discs were cultured for an additional 1-2 weeks before being used for bombardment. Cultures were kept in the dark at 26°C.

Leaf discs were transferred to osmoticum (MS containing 0.2 M sorbitol and 0.2 M mannitol; Vain *et al.*, 1993) for four hours before and four hours following bombardment. The surface of the medium was scored with sterile forceps before placing the discs onto the medium to increase the contact of the medium with the cells. The side of the leaf discs to be bombarded was placed face down on the medium and the same orientation maintained following bombardment. During the treatment, plates were wrapped in aluminium foil to prevent light entry and left in the laminar flow hood.

Following the post-bombardment osmoticum treatment leaf discs were cultured for 4 days on a medium identical to that used for the initial culture period (full strength MS, 1 g/l CH, 30 g/l sucrose, 5g/l agargel and 0.6 mg/l or 3.mg/l 2,4-D). Leaf discs were then transferred to a medium of the same formulation but also containing geneticin (G418; Roche South Africa) at a concentration of either 15 mg/l or 45 mg/l. Leaf discs were cultured on this medium

(selection medium) in the dark at 26°C for 4-8 weeks. Somatic embryos were transferred to regeneration medium (MS, 1 g/l CH, 30 g/l sucrose, 5 g/l agargel, 15 or 45 mg/l G418 and 0.5 g/l kinetin) and cultured with a 16 h/ 8 h (light/dark) photoperiod (35 $\mu m/m^2/s$) at 26°C. All cultures were subcultured every 2 weeks. Plantlets were transferred to half strength MS, 5 g/l sucrose and 15 or 45 mg/l in Magenta jars (Sigma) when they were approximately 2 cm high with visible roots to allow room for growth. Plantlets were subcultured once a month and transferred to small pots containing a fertilized soil mixture for hardening off in the glasshouse when they were approximately 10 cm high.

b) Protocol 3: Regeneration by Indirect Embryogenesis

Leaf discs were cultured on MS, 1 g/l CH, 30 g/l sucrose, 5 g/l agargel and 3 mg/l 2,4-D to induce callus formation. Discs were cultured on this medium in the dark at 26°C for 8-12 weeks and subcultured every two weeks. Callus was not removed from the leaf discs on which it grew until it was harvested for use as target material for gene delivery. Hard yellow callus consisting of small densely packed cells was subcultured 1 day prior to bombardment and transferred to osmoticum the following morning. Osmotic treatment of callus was the same as for leaf discs with the exception that callus was buried in the osmoticum so that roughly ¾ of it was submerged in the medium and ¼ protruded from the surface. Treatment of callus following the post-bombardment osmoticum treatment was the same as that described for leaf discs with the exceptions that the culture medium contained 3 mg/l 2,4-D instead of 0.6 mg/l and that callus was cultured for 8-10 weeks in the dark on selection medium before being transferred to regeneration medium and a 16 h/8 h (light/dark) photoperiod. Callus used for non-bombarded controls was cultured using the same conditions but geneticin was excluded from the media.

5.2.6 Callus Vitality Check

Callus exhibiting no growth on selection medium containing 45 mg/l Geneticin was tested using vital staining in 0.8% 2,3,5, triphenyltetrazolium chloride – tetrazolium red (TTC) in 0.05 M phosphate buffer (0.05 M NaHPO, 0.05 M NaH_2PO). The callus was placed into a 1.5

ml microcentrifuge tube containing 500 µl TTC and left at room temperature overnight. The callus was examined the following day.

5.2.7 Transformation of Sugarcane

a) Plasmid Purification and Integrity Check

All procedures described were performed as in Chapter 4. For full details refer to sections 4.2.5-4.2.13

Colonies of *E. coli*, strain JM109 HEC (Promega) cells were transformed with the plasmids pUBI510-cys3 and pUBI510-pleuro8. These colonies were stored at 4°C on Luria Bertani medium (10 g/l bacto tryptone, 5 g/l yeast extract, 5 g/l sodium chloride, 1 g/l glucose and 15 g/l agar, adjusted to pH 7.5) containing 50 µg/ml ampicillin and transferred to fresh medium every three weeks. Plasmids were grown up from colonies and isolated for use in bombardment. Plasmid concentration was determined fluorometrically and plasmids were checked electrophoretically following digestion with restriction endonucleases and phenol purification. Plasmids were sequenced periodically to check for sequence aberrations.

b) Precipitation of Plasmid DNA onto Micro-carriers

Protocol 1

A protocol modified from Sanford *et al.*, (1993); Chen *et al.* (1998); Dekalb, (1998); and Klein *et al.*, (1988) was used for the preparation of microparticles for bombardment and the precipitation of DNA onto the particles. Particles, either tungsten (0.2-3 µm in diameter) or gold (0.6 µm or 1.0 µm), were washed by vortexing in TE, pelleted by centrifugation and resuspended in 70 % (v/v) ethanol. The procedure was repeated and the particles resuspended in 100 % (v/v) ethanol (100 mg/ml).The particles were stored at – 20 °C.

For precipitation of plasmid DNA onto the particles, 30 µl (3 mg) of the particle suspension was transferred to a sterile, non-stick Eppendorf tube. The ethanol was taken off after centrifuging the tube. The particles were washed in 1 ml of sterile water and the water was then drawn off. While continuously vortexing the tube, 20 µl water, 20 µl plasmid DNA suspension (containing a maximum of 25 µg DNA), 40 µl $CaCl_2$ (2.5 M), and 16 µl fresh spermidine (100 mM) were added at room temperature in the order stated. The mixture was vortexed for a further 1-2 min. and then placed on ice for two min.. The tube was pulse centrifuged briefly and the liquid drawn off. The particles were washed with 70 % and then 100 % (v/v) ethanol and then resuspended in 30 µl 100 % (v/v) ethanol. The suspension was used immediately for bombardment of leaf discs. Two or four µl were used per bombardment.

Protocol 2

Particles were washed in absolute ethanol and rinsed in water three times before being resuspended in water at a concentration of 100 mg/ml. To coat the particles, 10 µg DNA (5 µg pUBIKN + 5 µg pUBI510-Cys3 or pUBI510-Pleuro8) dissolved in 20 µl water was added to 50 µl of the particle suspension, followed by 50 µl $CaCl_2$ (2.5 M) and 20 µl spermidine (100 mM) while continuously vortexing. After allowing the particles to settle 110 µl of the supernatant was drawn off. The particle suspension was used for bombardment in 4 µl aliquots.

c) Microprojectile Bombardment

All bombarding was done using the particle inflow gun (PIG, Finer *et al.*, 1992) to accelerate tungsten (M10, 0.73 median diameter size) and gold (0.6 µm and 1.0 µm median diameter sizes) micro-carriers (BioRad) in a stream of helium (1000 kPa for 0.5 sec) into sugarcane leaf discs or callus contained in an evacuated chamber (10 kPa). Two to four µl of a suspension of tungsten or gold micro-carriers, onto which appropriate constructs had been precipitated, was pipetted onto a 0.2 mm^2 mesh supported by a 13 mm Swinny filter holder (Millipore). The filter holder was screwed into a needle adapter in the PIG chamber attached to the helium line. Plant material to be bombarded was placed onto the center of a metal grid 6

cm below the filter unit and covered with a Jik-sterilised, plastic-mesh baffle. The door was placed over the chamber, the chamber evacuated and the bombardment carried out. Atmospheric pressure was returned to the chamber immediately after bombarding.

5.2.8 Molecular Analyses of Putative Transgenic Plants

a) DNA Extraction

DNA was extracted either from sugarcane tillers, leaf roll or leaf material from plants at various stages of growth. Whenever possible, whole tillers, ranging in size from 5-20 cm in height, were used for DNA extraction. When no small tillers were present a combination of leaf roll and leaf material were used. Both the cetyltrimethylammonium bromide (CTAB) method (Draper and Scott, 1988) and a method modified from Dellaporta *et al.* (1983) were used. The Dellaporta method was used for large-scale (6 g plant tissue) extractions only while the CTAB method was used in both large-scale and small-scale (100 mg plant tissue) extractions. DNA concentrations were determined spectrophotometrically. For highly concentration-dependant applications, concentrations were also determined using the fluorometer (Section 4.2.9.). DNA preparations were diluted to a concentration of 500 ng/µl to facilitate their use in PCR and Southern hybridization analyses.

CTAB Method for DNA Extraction

Freshly harvested plant leaf material (small scale: 100 mg; large scale: 6g) was frozen using liquid nitrogen and ground to a fine powder in liquid nitrogen and transferred to 1.5 ml Eppendorf tubes for small scale extractions or 50 ml Corning tubes for large scale extractions. CTAB buffer (100 mM Tris pH 8.0, 20 mM EDTA, 2% CTAB, 1.4 M NaCl) was added (500 µl; 20 ml) and the tubes were incubated at 55°C for 45 min. Chloroform (300 µl; 15 ml) was added and the contents mixed. Tubes were vortexed in small-scale extractions but gently rocked for 5-10 min for large-scale extractions. The tubes were then centrifuged at maximum speed for 5 min. and the aqueous layer transferred to a fresh tube. One volume of room temperature isopropanol was added and the tubes mixed well and centrifuged at maximum

speed for 10 min. The supernatant was then poured off and the pellets were washed in 75% (v/v) ethanol and then in 100% (v/v) ethanol. The tubes were placed upside down on the benchtop overnight to allow the pellet to dry and resuspended the following morning in 0.5X TE (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0).

Dellaporta Method for DNA Extraction

Freshly harvested leaf and leaf roll material (6 g) was cut into small pieces with alcohol-washed scissors and frozen in liquid nitrogen. The material was ground to a fine powder in a mortar and pestle and transferred to 50 ml Corning tubes on ice to which 35 ml cold extraction buffer (100 mM Tris-HCl, 500 mM NaCl, 10 mM mercaptoethanol, 50 mM EDTA, pH 8.0) had been added. The tubes were mixed vigorously and 3.5 ml 20% (w/v) SDS was added. The tubes were incubated at 65°C for 45 min and then 7 ml 5M potassium acetate was added. The tubes were mixed and incubated on ice for 20 min. The tubes were centrifuged at 8000 rpm for 30 min. and the supernatant was then filtered through Whatmann paper or pre-sterilised mutton cloth into sterile Corning tubes (the supernatant from each sample was divided between two tubes at this stage). One volume of isopropanol was added and the tubes were mixed and incubated at 4°C overnight or at –80°C for 30 min to increase the yield. The DNA was spooled out using glass hooks and dissolved in 0.5 X TE at 37°C overnight.

b) RNA Extraction

For the preparation of a small scale RNA extractions all working surfaces were cleaned using RNase Away and all utensils and glassware was pre-washed with RNase Away, wrapped in foil and autoclaved before use. Freshly harvested leaf material (100 mg) was placed into a 1.5 ml Eppendorf tube and frozen in liquid nitrogen. The leaf material was ground as finely as possible using a plastic pestle. 500 µl of a 1:1 mixture of saturated phenol (pH 7.9) and extraction buffer (100 mM LiCl, 100 mM Tris, pH 8.0, 10 mM EDTA, 1% w/v SDS) preheated to 80°C was added to the ground plant material and the tubes vortexed for 30 seconds. The samples were centrifuged at maximum speed for 10 min and the aqueous layer was transferred to a fresh tube. One volume of acid phenol (phenol:chloroform = 5:1, pH 4.7) was added and the tubes vortexed for 30 seconds and centrifuged again at maximum speed for

10 min.. The aqueous layer was again transferred to a fresh tube to which 1 volume of 5M LiCl was added. The tubes were mixed and stored at – 20°C overnight. The following morning the tubes were centrifuged at maximum speed for 20 min. to pellet the RNA. Excess liquid was aspirated using a pipette and the pellet was allowed to dry before being resuspended in 5 µl nuclease free water. RNase inhibitor and DNase were added (1U per µg RNA based on an estimated delivery of 200 µg RNA per gram fresh weight of leaf tissue) and the tubes incubated at 37°C for 30 min. Enzymes were then denatured by incubation at 75°C for 5 min. The volume was increased to 50 µl using nuclease free water and again 1 volume of 5M LiCl was added. Incubation at –20°C, centrifugation and pellet drying steps were repeated and the pellet resuspended in 5 µl nuclease free water. RNA preps were stored at –20°C for use in RT-PCR.

c) PCR

Polymerase chain reactions were performed using the Applied Biosystems Gene Amp® PCR System 9700 and the Hybaid Omnigene thermal cycler. The final concentration of reagents in all PCR reactions was; 1X Taq DNA polymerase buffer (Promega) 1.5mM $MgCl_2$, 0.2 µM primers, 2mM dNTPs and 1.25 U/reaction Taq DNA polymerase (Promega). In all cases 500 ng sugarcane genomic DNA was used as template. Final reaction volumes were 25 µl or 50 µl depending on which of two PCR thermal cycling machines were used. Reactions were performed using standard thermal cycling regimes comprising Denaturation, annealing and elongation phases (specific protocol information is provided in section 5.3.4).

d) RT-PCR

The Titan One Tube RT-PCR System (Roche) was used for RT-PCR according to the manufacturer's instructions for one-step RT-PCR. Mastermix 1 (0.2 mM dNTPs, 0.4 µM upstream and down stream primers, 5 mM DTT, 5 U RNase inhibitor, 1 µg total RNA) was prepared to a final volume of 25 µl with nuclease free water and added to mastermix 2 which contained 1X RT-PCR buffer for a final $MgCl_2$ concentration of 1.5 mM and 1 µl supplied Expand High Fidelity enzyme mix (reverse transcriptase, AMV in: 20 mM Tris-HCl, 100mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% (v/v) Tween 20, 0.5% (v/v) Nonident P40, 50% (v/v)

glycerol, pH 7.5) prepared in 25 µl nuclease free water for a final reaction volume of 50 µl. The tubes were incubated in a heating block at 95°C for 30 min and then subject to PCR thermal cycling regime 1 for amplification of pleurocidin.

e) Southern Hybridization Analysis

DNA was digested using the restriction enzymes Bam HI and Eco RI either separately or together. Ten µg DNA (20 µl of 500 µg/µl stock) was added to a reaction mix (4 mM spermidine, 1X SureCut Enzyme Buffer B, 30 U each of restriction enzymes) prepared with ultrapure water to a final volume of 400 µl and were incubated at 37°C overnight. 10 µl aliquots of the digests were separated electrophoretically and viewed using ethidium bromide staining and a UV trans-illuminator to ensure complete digestion had occurred. If digestion appeared incomplete 10 U restriction enzyme were added and the sample was again incubated at 37°C overnight. In extreme cases when digestion was still incomplete following the second incubation, the DNA was phenol cleaned and the digestion process repeated. Substituting Buffer A for Buffer B was also helpful in these cases and generally facilitated digestion more efficiently than Buffer B despite the manufacturer's recommendations for Bam HI and EcoRI.

One volume (400 µl) of a phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) mixture was added to the DNA digestion mixture. The samples were vortexed and centrifuged at maximum speed for 10 min and the aqueous layer was transferred to a fresh tube. The DNA was precipitated by the addition of 1/10 of the volume 3M sodium acetate and 2.5 volumes of 100% (v/v) room temperature ethanol. Samples were incubated at –80°C for 30 min before being centrifuged at maximum speed for 10 min.. The ethanol was poured off and the pellets washed with 100% and then 70% ethanol. The tubes were left inverted on paper toweling on the bench-top overnight to dry the pellet. The DNA was redissolved in 20 µl nuclease free water. It was necessary to leave samples overnight at 4°C and incubate for at least 30 min before loading to ensure DNA had fully dissolved. A small amount of DNA was expected to be lost during the procedures described above but it was assumed that the amount of DNA in the tubes at this stage was close to 10 µg.

Loading dye was added to the restricted DNA samples and the DNA was separated by electrophoresis on a 1% agarose gel in 0.5X TBE. The separation was allowed to continue at 50 V overnight until the bromophenol blue front had migrated roughly 20 cm. Gels were stained for 20 min in ethidium bromide (100 μg/l) and viewed with a UV trans-illuminator to ensure even migration of the DNA. The gels were then incubated in 125 mM HCl for 30 min (or until the bromophenol blue turned yellow) with shaking at room temperature. The gels were rinsed in distilled water, transferred to 1.5 M NaCl, 500 mM NaOH and incubated with shaking for 30 min at room temperature.

The DNA was transferred to HyBond XL membrane by an upward capillary blotting procedure (Fig. 5.2). The membrane was then UV crosslinked (70 000 $\mu J/cm^2$) DNA side up to bind the DNA permanently to the membrane. The membrane was then rinsed in 2X SSC (0.3M sodium chloride and 0.03M sodium citrate) and drained. The membranes were then rolled and placed into hybridization bottles. The bottles were placed into the hybridization oven at 65 °C and rinsed in 5X SSC for a few min.. The SSC was poured off and 25 ml pre-heated pre-hybridization buffer which contained 5X SSC (final concentration), 5X Denhardt's solution (2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone (PVP), 0.5% (w/v) SDS and 200 μg/ml denatured, fragmented salmon sperm) was added. The membranes were incubated in the pre-hybridization for no less than 4 hours before fresh hybridization buffer was added (same as pre-hybridization buffer but with 100 μg/ml denatured, fragmented salmon sperm). Membranes were incubated in hybridization buffer overnight.

Following hybridization membranes were washed twice for 5 min with 2X SSC and 0.1% SDS (w/v), and twice for 10 min. with 1X SSC and 0.1% SDS (w/v) at 65°C. The membranes were rinsed in distilled water and drained briefly before being placed into plastic bags and placed between 2 sheets of x-ray film in x-ray cassettes. The cassettes were stored in black bags at –80°C and the x-ray film developed after 5 and 10 days.

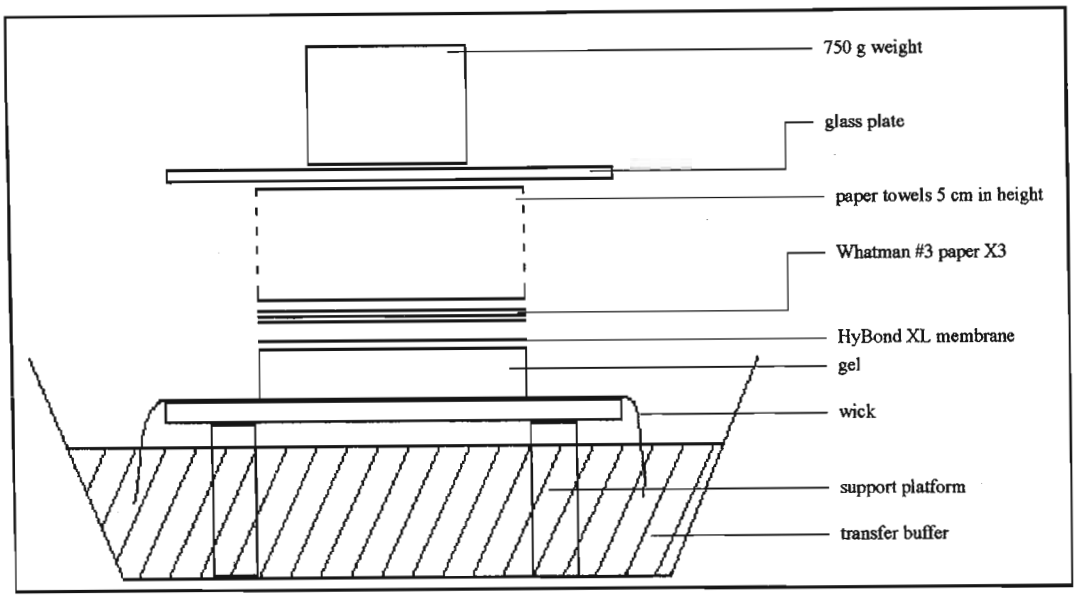

Figure 5.2: Schematic diagram of upward capillary blotting apparatus. Dry paper towel and Whatmann #3 filter paper, cut to fit the exact shape of the gel were used to draw transfer buffer from the basin via the wick (filter paper) through the gel and thus transfer the DNA in the gel onto the membrane above. The weight at the top ensured the construction was packed tightly to facilitate capillary action and the glass plate ensured even distribution of the weight.

f) Dot Blot Analysis

Putative transgenic plant genomic DNA (5 µg) was transferred to sterile microcentrifuge tubes and 3 volumes 99% (v/v) ethanol and NaOAc to a final concentration of 0.3 M was added. The tubes were incubated for 30 min. at -80°C and the DNA pelleted by microcentrifugation at maximum speed for 30 min at 4°C. The supernatant was poured off and the pellets dried. When completely dry the DNA was dissolved in 6 µl 0.4M NaOH (overnight at 4°C). The tubes were incubated for 30 min at 37°C the following morning to ensure the DNA was completely dissolved. The DNA was pipetted in successive 2 µl aliquots onto a precut piece of HyBond XL membrane using a Perkin Elmer PCR plate as a grid to ensure that each 2 µl aliquot from a single sample was placed onto the same spot on the membrane. Successive 2 µl aliquots were pipetted only after the previous one had fully dried. The membrane was allowed to dry fully before the DNA was bound to it using a UV crosslinker set at 70 000 $\mu J/cm^2$.

Hybridization solutions and procedures were the same as those described for Southern hybridization analysis.

g) Probe Preparation and Labeling.

Probes for use in Southern hybridization and dot blot analysis were generated by amplification of cystatin, pleurocidin and npt-II inserts from the plasmid vectors; pUBI510-cys3, pUBI510-pleuro8 and pUBIKN respectively. PCR products were purified using Quiagen PCR Purification Kit according to the manufacturer's instructions. Purified product was quantified using a fluorometer and checked electrophoretically on a 0.8% (w/v) agarose gel to ensure the correct fragment had been obtained.

To label, 25 ng insert DNA was added to a final volume of 25 µl nuclease free water containing; 0.3 µM upstream and downstream insert specific primers and the mixture was boiled for 5 min and snap-cooled in a slush-ice bath. The contents of the tube were then spun down and added to a second reaction mixture (25 µl) containing; 10 µl labeling buffer (Amersham), 2 µl PCR grade BSA, 5 µl α-^{32}P labeled dCTP (1 mCi/100 µl; 3000 Ci/mmol) and 5 U (1µl) Klenow enzyme. The reaction mixture was gently mixed by pipetting and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 20 µl 50 mM EDTA (pH 8.0).

To ensure that the labeling reaction had proceeded efficiently, 1 µl of the reaction was placed onto a narrow piece of chromatographic paper and placed into a 500 ml beaker containing 100 ml Na_2PO_4 (750 mM) at the bottom. The solution was allowed to migrate up the paper for 15 – 30 min at which time the paper was covered in plastic and placed into an X-ray cassette on top of a piece of X-ray film. The film was developed after 20 min. In addition the reaction mixture was passed through a NucTrap Column to remove unincorporated radionucleotides and short fragments which decrease the signal to noise ration of the probe. Successfully labeled probe was boiled for 1 minute to denature and snap-cooled in a slush-ice bath before being added to the hybridization buffer.

h) Transgene Copy Number Reconstructions

Pleurocidin, cystatin and npt-II transgene inserts were amplified from their respective pUBI510 based vectors and were purified using a Quiagen PCR Purification Kit according to the manufacturers instructions and resuspended in nuclease free water and stored at –20°C. Purified product was quantified using a fluorometer and checked electrophoretically on a 0.8% (w/v) agarose gel. Stock dilutions were maintained at concentrations of < 50 ng/µl.

Dilutions for transgene copy number reconstructions were calculated (see example in Appendix 1) based on an inferred haploid sugarcane genome size of 1.1×10^{10} base pairs (bp; Bower and Birch, 1992). Transgene inserts were diluted and spiked into genomic DNA from a non-transformed sugarcane plant such that a 1 transgene copy number reconstruction represented a single copy of the sugarcane genome containing a single transgene copy. Similarly a 10 transgene copy number reconstruction represented a single copy of the sugarcane genome containing 10 transgene copies. For PCR analyses copy number reconstructions were prepared in 500 ng genomic DNA. For Southern hybridization they were prepared in either 10 or 20 µg genomic DNA. Copy number reconstructions degraded quickly at low concentrations and were therefore prepared fresh for use in PCR or Southern hybridization analyses. Serial dilutions of the inserts, ranging from 1 copy/µl to 100 000 copies/µl increasing at 10-fold intervals, were prepared from stock on the day of use.

5.3 RESULTS AND DISCUSSION

5.3.1 Comparison of Sugarcane Regeneration using Three Protocols

a) Regeneration by Direct Embryogenesis in the Presence of 0.6 mg/l 2,4-D

Leaf discs were cultured on MS, 1 g/l CH, 5 g/l agargel and 0.6 mg/l 2,4-D in the dark at 26°C and were subcultured every two weeks. Two to four weeks after culture initiation pin-head sized, white embryos could usually be seen developing on the exposed surfaces of the discs,

particularly around the edges. Embryos developed singly or in clusters. New embryos continually developed and became visible while those already visible grew larger. At six to eight weeks after culture initiation clusters of embryos were visible which were white and roughly the size of a peppercorn. Also visible at this stage were small white shoots with small but clearly visible leaves present. At this stage the embryos were transferred to regeneration medium without geneticin and a 16 h/8 h (light/dark) photoperiod. Discs from which embryos had been removed and transferred to regeneration medium were retained in the dark for a further two to four weeks to allow the continued development of new/younger embryos. Within a week of transfer to regeneration medium, the white shoots became green although albino shoots were occasionally observed which remained white/translucent permanently. Two weeks after being placed onto regeneration medium, small, green, leafy shoots could be seen emerging from the embryo clusters. It was common to observe the shoots of 10-20 plantlets emerging from the same area on a single cluster. Probably due to competition for resources between the plantlets, usually less than five of these plantlets grew larger while the others remained small. The larger plantlets were therefore separated from the smaller plantlets during routine subculturing to allow the smaller plantlets to grow. Six to eight weeks after being placed onto regeneration medium some plantlets were roughly 1-2 cm tall and possessed clearly visible roots. Such plantlets were transferred to magenta jars to allow for further growth. From a single non-bombarded control (4-6 leaf discs) ten to fifty plantlets were usually transferred. Shoots were maintained in magenta jars for a further six to eight weeks before being transferred to small pots or speedling trays for hardening off in the glasshouse.

b) Regeneration by Direct Embryogenesis in the Presence of 3 mg/l 2,4-D

Leaf discs were cultured in the presence of 3.0 mg/l 2,4-D in the dark and subcultured every two weeks. Two weeks following culture initiation the surface of the discs appeared rough and dry. Four to eight weeks following culture initiation small yellow clusters of dense callus were visible and peppercorn-size clusters of embryos were visible on the surface of some discs. At this stage discs on which embryos could be seen were transferred to regeneration medium without geneticin and a 16 h/8 h (light/dark) photoperiod. Due to the high concentration of 2,4-D far fewer direct somatic embryos were formed using this protocol than when leaf discs were cultured in the presence of 0.6 mg/l 2,4-D. Regeneration of plantlets

from those embryos that did form however was similar to that described for leaf discs cultured in the presence of 0.6 mg/l 2,4-D. Due to the fact that fewer numbers of embryos were produced, fewer plants were regenerated by direct embryogenesis from leaf discs cultured in the presence of 3 mg/l 2,4-D than from those cultured in the presence of 0.6 mg/l 2,4-D.

c) Regeneration from Callus by Indirect Embryogenesis

Leaf discs were initiated onto MS containing 3.0 mg/l 2,4-D, maintained in darkness and transferred to fresh medium every two weeks. Two weeks following initiation, the surface of the leaf discs appeared rough and dry. Six weeks after culture initiation small yellow clusters of dense callus were visible growing on the surface of the discs and a ring of loosely-packed cells which appeared slimy had formed around the edges of some discs. Also present at this stage were dry, 'fluffy' agglomerations of cells that were either yellow or grayish in appearance. Six weeks following culture initiation clusters of callus were larger and the discs from a single plate were often split between two or three plates. At eight to twelve weeks, clusters of pea-sized or larger, hard, yellow callus were separated from the leaf discs from which they had been produced and placed onto fresh medium. Four to eight weeks later callus was placed on regeneration medium without geneticin and a 16 h/8 h (light/dark) photoperiod. Green shoots were visible within the first two weeks after being placed onto regeneration medium. Plantlets grew and it was necessary to divide the material from a single plate into two or more plates every two weeks. Thus the material from a single non-bombarded control was gradually divided into several plates. Plantlets were transferred to magenta jars when they were approximately 2 cm tall to allow room for growth. The callus continued to produce new plantlets and more than 100 plants could be regenerated from a single non-bombarded control.

d) Comparison of Regeneration by Direct and Indirect Embryogenesis

Six non-bombarded controls were observed in each of the three *in vitro* culture protocols. Regeneration by direct embryogenesis in the presence of low (0.6 mg/l) and high (3 mg/l) concentrations of 2,4-D required 17-28 weeks for completion while regeneration by indirect embryogenesis required 25-40 weeks for completion. The number of plants regenerated from

each of the protocols was not counted but more plants were regenerated by indirect embryogenesis than direct embryogenesis. The least number of plants were regenerated by direct embryogenesis in the presence of a high (3 mg/l) concentration of 2,4-D with plants being regenerated from only one of six non-bombarded controls. Plants were regenerated from each of the six non-bombarded controls by direct embryogenesis in the presence of a low (0.6 mg/l) concentration of 2,4-D and by indirect embryogenesis.

5.3.2 Effect of Regeneration Route on Transformation

To determine the effect of the tissue culture pathway on transformation, transgenes were delivered to leaf discs and callus produced. Leaf discs were bombarded six days after culture initiation and plantlets regenerated by direct embryogenesis in the presence of either 0.6 mg/l or 3 mg/l 2,4-D. Callus was bombarded 8-12 weeks following culture initiation and plantlets were regenerated by indirect embryogenesis. Only hard yellow callus was used for bombardment. Both leaf discs and callus were from the sugarcane variety NCo376. The plasmids pUBIKN and either pUBI510-pleuro8 or pUBI510-cys3 were co-delivered biolistically using tungsten (M10) microcarriers accelerated using a particle inflow gun (PIG). Plant material was subject to pre- and post-bombardment osmotic treatment. Deep Petri dishes sealed with microporous tape were used for pre-and post-bombardment tissue culture of the plant material, including selection and regeneration.

Following bombardment plant material was placed on medium containing 45 mg/l synthetic kanamycin homologue, G418. Leaf discs (Fig. 5.3) were cultured in the dark for 4-6 weeks, at which time white embryos could be seen growing on dead or dying leaf discs (Fig. 5.3 A). The death of the leaf discs was evident by the fact that they became dark brown to black in colour. Embryos were cut off of the leaf discs on which they grew and transferred to regeneration medium containing 45 mg/l G418 and a 16 h/8 h (light/dark) photoperiod. Small embryos were left on the discs for support until they had grown larger but were moved into the light at this stage as well. In most cases green, leafy shoots could be seen within two weeks of being transferred to regeneration medium (Fig 5.3 B). Plantlets (Fig. 5.3 C) were kept in Petri dishes until roots were well developed and were then transferred to magenta jars

in which they were maintained until they were 5-7cm tall at which time they were transferred to small pots to be hardened off in the glasshouse.

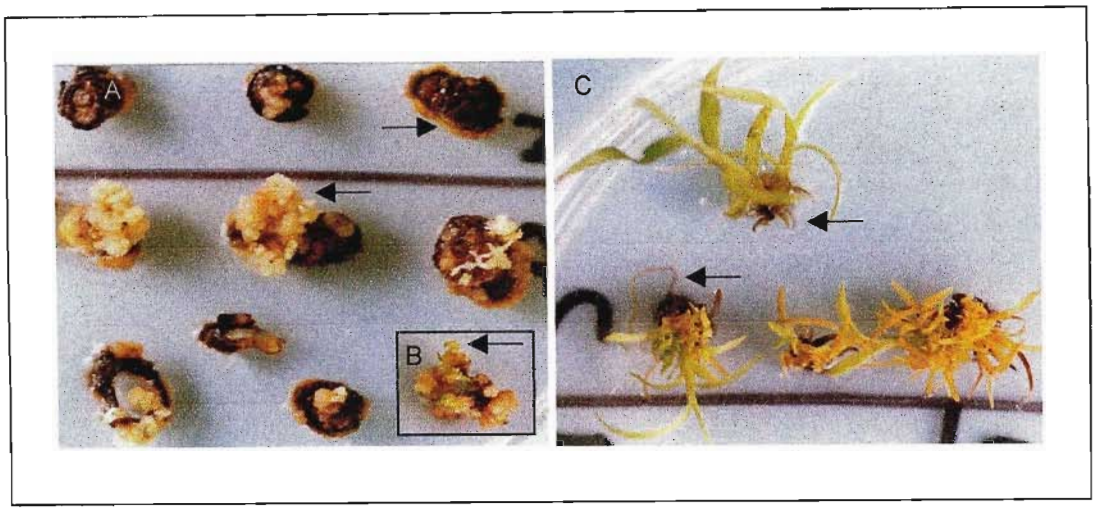

Figure 5.3: Regeneration of sugarcane plants from leaf discs on selection medium. **A:** Appearance of leaf discs at 4-6 weeks after bombardment. Predominantly brown (arrow, top right) leaf discs with actively growing white pro-embryos protruding from the tops and sides (arrow, center). **B:** 6-8 weeks post-bombardment. Following transfer to regeneration medium, green shoots could be seen growing from the plant material (arrow indicates shoot). **C:** 8-12 weeks post bombardment. Shoots 1-2 cm in height (arrow, top) some with visible roots (arrow, bottom left).

A total of 7 and 2 independent, putative transgenic lines were regenerated by direct embryogenesis in the presence of low (0.6 mg/l) and high (3 mg/l) concentrations of 2,4-D respectively (Table 5.1). The overall regeneration efficiency of these routes was 0.11 plants per bombardment and 0.014 plants per bombardment respectively. No plants were regenerated from bombarded callus. In the first two to three weeks following bombardment, small, white, growths were visible on the callus however these remained small and no further growth was observed. Following a 10-12 week period on selection medium in the dark, during which time no growth was observed, vital staining with tetrazolium red (TTC) confirmed that bombarded callus was dead and that non-bombarded callus was still alive (Fig 5.4). That callus was regenerable at the time of bombardment was confirmed by the fact that many plantlets were regenerated from non-bombarded callus. An additional, 29 and 12 bombardments were performed on callus produced from sugarcane varieties N12 and N19 respectively. The bombardment and tissue culture conditions were the same for these bombardments as those

described for plant material from NCo376 above. No plants were regenerated from this material.

Table 5.1: Putative transgenic plants regenerated by direct and indirect embryogenesis. For each group of bombardments (Grp.) the regeneration protocol (Prot) number of uncontaminated bombardments (No. Bombs) plasmids used (Plasmids) and number of putative transgenic plants produced (Putative Transgenics) are given. The plasmids pUBIKN (K) and either pUBI510-cys3 (C) or pUBI510-pleuro8 (P) were co-delivered. The transformation efficiency (Efficiency) is the number of putative transgenic plants produced per number of uncontaminated bombardments. The transformation efficiency is given for both the group and the experimental pathway.

Grp.	Prot	No. Bombs	Plasmids	Putative Transgenics	Efficiency	
					Grp.	EP
A	1	26	K, P	4	0.154	0.106
B	1	40	K, C	3	0.075	
C	2	72	K, P	0	0	0.014
D	2	66	K, C	2	0.030	
E	3	46	K, P	0	0	0
F	3	47	K, C	0	0	

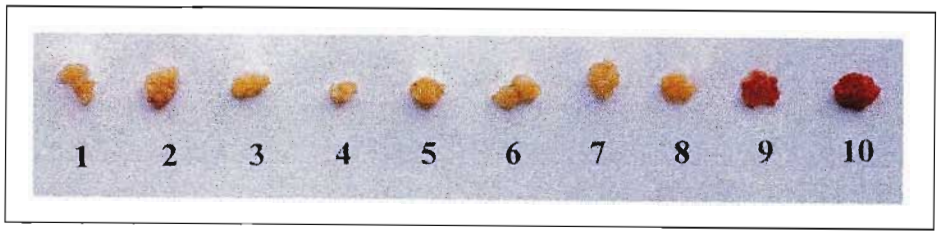

Figure 5.4: Callus following tetrazolium staining. Callus at 12 weeks after bombardment was placed overnight in a 0.8% TTC solution. Non-bombarded callus (9 and 10) that had been growing on medium with no selective agents was stained red indicating the material was metabolically active. Bombarded callus (1-8) which had been growing on selection media was not stained, indicating the material was dead.

More non-bombarded control plants were regenerated by indirect embryogenesis than by direct embryogenesis in the presence of either low or high 2,4-D concentrations. It was therefore expected that the same trend would be observed for bombarded plant material. The

lack of regeneration by indirect embryogenesis from bombarded callus observed in this work is inconsistent with previous studies using this method. Falco *et al.* (2000) report the regeneration of 1 transformed plant per bombardment event while Bower *et al.* (1996) report the regeneration of up to 19.8 ± 3.7 transformed plants per bombardment. However, in the laboratory where the current project was undertaken, Snyman *et al.* (2001) report that 0.1 transformed plants were regenerated by indirect embryogenesis per bombardment. Since plants were regenerated from bombarded leaf discs it is unlikely that the lack of regeneration from bombarded callus can be attributed to the bombardment procedure as this was the same for both callus and leaf discs.

The experience of the technician is reported to be an important factor in biolistic transformation. More experienced technicians have been shown to generate more plants per bombardment than inexperienced technicians (Fauquet *et al.*, 1995). The level of experience required when using callus as a target material for bombardment is possibly greater since only certain kinds of callus have a high probability of regeneration and since selection of transformed callus requires visual interpretation (pers. comm. Dr. Bernard Potier, 2001) as well as the selective agent G418. This is probably the most likely explanation for the lack of plants regenerated from bombarded callus in this project, given the inexperience of the bombarder with the culture of callus.

5.3.3 Effect of Other Variables on Transformation

a) Age of Leaf Discs at the Time of Bombardment

To allow for the bombardment of leaf discs in varying states of metabolic activity transgenes were delivered to leaf discs at varying numbers of days following initiation. The plasmids pUBIKN, pUBI510-pleuro8 and pUBI510-cys3 were co-delivered to leaf discs from sugarcane variety N12. The discs were not subject to pre- and post-bombardment osmotic treatment, and thin Petri dishes, sealed with parafilm were used for tissue culture. For the selection of transformed cells, bombarded leaf discs were cultivated on medium containing 15mg/l G418 and plantlets were regenerated by direct embryogenesis in the presence of 0.6 mg/l 2,4-D. The plasmids were precipitated onto tungsten or gold microcarriers by protocol 1

(section 5.2.2). The number of putative transgenic plants produced from such bombardments is shown in Table 5.2.

Table 5.2: Number of putative transgenic plants produced from leaf discs bombarded at varying numbers of days following initiation. For each experiment (Exp.) the number of bombardments (No. Bombs) plasmids and microcarrier used are given. The age of the discs is given in days. The number of putative transgenic plants regenerated and the regeneration efficiency (average number of putative transgenic plants regenerated per bombardment) is shown.

Exp.		No. Bombs	Plasmids	Age of Discs	Microcarrier	Putative Transgenics	Efficiency
1	A	56	K,C,P	6	T	3	0.054
	B	53	K,C,P	13	T	6	0.113
	C	10	K,C,P	17	T	6	0.600
2	A	8	K,P	6	T	1	0.125
	B	50	K,P	13	T	2	0.040
3	A	25	K,C,P	6	G	32	1.280
	B	22	K,C,P	20	G	4	0.182

Key: K = pUBIKN, C = pUBI510-cys3, P = pUBI510-pleuro8, T= tungsten, G = Gold

In the first experiment (Exp. 1) the regeneration efficiency increased as the age of the discs increased from 6 to 17 days. In experiments 2 and 3 the regeneration efficiency decreased as the age of the discs increased. The number of bombardments performed in experiments 1C and 2A were few and the regeneration efficiencies may therefore not represent the treatment accurately. The regeneration efficiencies for leaf discs bombarded 6 and 13 days after culture initiation varied between experiments. A general effect of the age of discs on regeneration efficiency could not be identified and further investigation using larger sample sizes would be required in order to elucidate this.

b) Effect of the Microcarrier on Regeneration Efficiency

Experiments 1 and 3 in Table 5.2 were performed using the same protocols for tissue culture and bombardment with the exception that in experiment 1 tungsten microcarriers were used for transgene delivery and in experiment 3, gold microcarriers were used. The regeneration efficiency in experiment 1A was lower (0.054 plants per bombardment) than that in

experiment 3A (1.28 plants per bombardment). This is consistent with reports in the literature that gold microprojectiles result in higher transformation efficiencies than tungsten microprojectiles (Russel, 1992; Randolph-Anderson *et al.*, 1998).

c) Overall Regeneration Efficiency of Direct Embryogenesis-Based Transformation

The overall efficiency of regeneration of putative transgenic plants from bombarded leaf discs by direct embryogenesis was 0.21 plants per bombardment when 0.6 mg/l 2,4-D (calculated from results shown in Table 5.1 and Table 5.2) was used and 0.014 plants per bombardment when 3 mg/l 2,4-D was used (Table 5.2). Snyman *et al.* (2001) working in the same laboratory in which the present work was performed, report a higher figure of 1.8 plants per bombardment regenerated from bombarded leaf discs by direct embryogenesis using 0.3 mg/l 2,4-D concentration. The culture conditions and bombardment protocols used by Snyman *et al.* (2001) were the same as those used for this project and it is proposed that the lower regeneration efficiency reported here was due to the inexperience of the technician as was proposed for the lack of regeneration from bombarded callus.

5.4.4 Molecular Analyses of Putative Transgenic Plants: Optimisation of Techniques

a) Inefficiency of Dot Blots for Screening Putative Transgenic Sugarcane

Dot blot hybridization was tested as a potential method of screening putative transgenic plants. Five micrograms of genomic DNA was extracted from 30 putative transgenic plants and dotted onto a HyBond XL membrane. The membrane was probed for the presence of the nptII, pleurocidin and cystatin transgenes (Fig. 5.5) using ^{32}P-labelled probes generated by amplification from the plasmids pUBIKN, pUBI510-pleuro8 and pUBI510-cys3, respectively. In each case the transgene insert was amplified from the plasmid using insert specific forward and reverse primers. Hybridization of the probes to the membrane was visualized on an autoradiograph. All of the probes hybridized to both positive and negative controls as well as to putative transgenic plant genomic DNA. However hybridization to positive controls was always much greater than that to negative controls and differences in intensity were clearly

visible among the putative transgenic plant samples. Thus it was thought that both specific and non-specific hybridization was occurring. Due to non-specific hybridization the dot blot was abandoned as a method of screening putative transgenic plants. However, those individuals for which the intensity of hybridization appeared strong were noted and subject to PCR analysis.

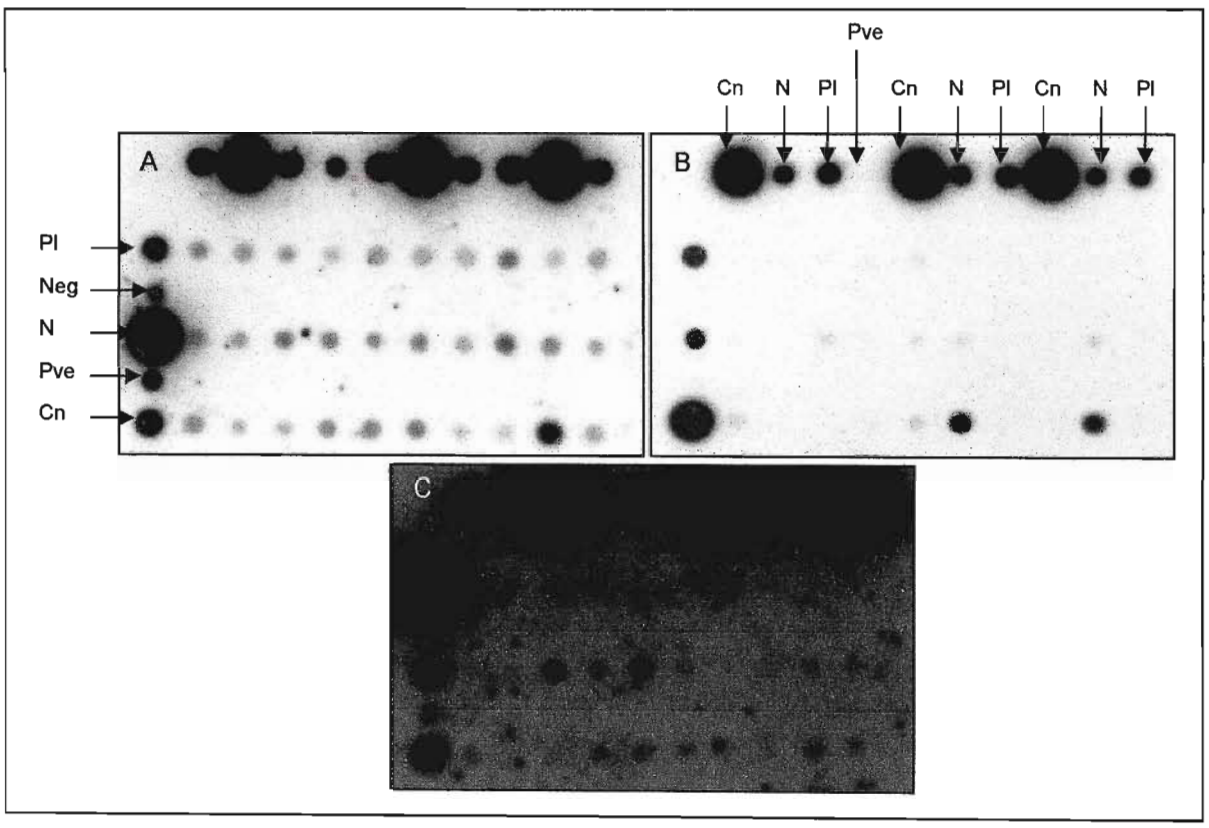

Figure 5.5: Autoradiographs of a dot blot of genomic DNA from 28 putative transgenic plants. The dot blot was probed with ^{32}P-labelled nptII (A) cystatin (B) and pleurocidin (C) transgene inserts. Positive controls for nptII (N) cystatin (Cn) and pleurocidin (Pl) were prepared from the plasmids pUBIKN, pUBI510-cys3 and pUBI510-pleuro8 respectively. A positive control (Pve) consisting of genomic DNA from a known nptII-positive sugarcane plant and a negative, non-bombarded control (Neg) were also included.

b) Optimisation of PCR Protocols

Prevention of Contamination of Negative Controls

Initially PCR analysis was problematic due recurring contamination of the negative controls with the gene of interest. Negative controls consisted of a water/no template control which contained PCR reagents and water but no template DNA and the non-bombarded control which contained PCR reagents, water and genomic DNA from a non-bombarded (non-transformed) sugarcane plant. In order to eradicate the contamination of the negative controls fresh working stocks of primers (6 µM) were prepared from the concentrated stocks (100 µM) when there was any suspicion that they may have been contaminated. Reagents such as $CaCl_2$ (Promega) and PCR reaction buffer (Promega) were divided into 20 µl and 50 µl aliquots respectively and stored in UV irradiated, sterile microcentrifuge tubes at -20°C. In addition, deionised H_2O was autoclaved and filtered through sterile micropore filters before use. Pipettes, reaction tubes, pipette tips and reagents that could withstand the treatment were exposed to UV irradiation in the laminar flow hood overnight before reactions were set up the following morning. Positive controls were kept in the freezer until it was used and was replaced immediately to minimize the risk of contamination. In addition the positive control was the last reaction to be set up and a separate pipette was used for aspirating the plasmid DNA. Latex gloves were worn during the entire process of preparing PCR reactions. The measures described here dramatically reduced the occurrence of contamination of the negative controls.

Quantity of Template DNA in PCR Reactions

It is known that excess template DNA is not beneficial to PCR reactions. For amplification of transgenes from putative transgenic plants it was desirable to use a quantity of genomic DNA that was large enough to adequately represent the entire sugarcane genome but not so large as to interfere with amplification. To determine the quantity of DNA to be used in this project, five, fifty, one hundred and five hundred nanograms of genomic DNA from putative transgenic sugarcane was tested for the presence of the cystatin transgene (Fig. 5.6). The individuals 12 02 and 12 O3d were selected since hybridization to genomic DNA from these

individuals had been strong for nptII, cystatin and pleurocidin probes in a dot blot analysis. Amplification was greatest in those in which either 100 ng or 500 ng genomic DNA had been used.

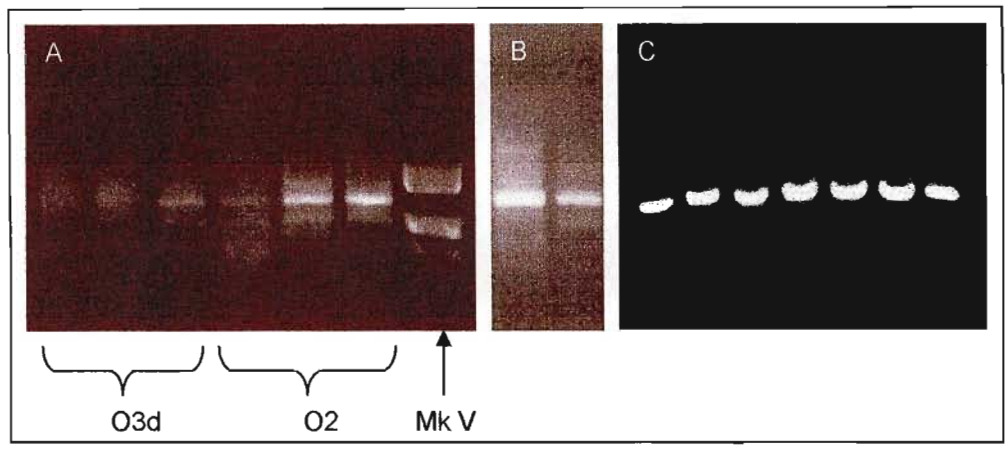

Figure 5.6: Amplification of the cystatin transgene from genomic DNA of putative transgenic plants. A: Five, fifty and one hundred nanograms (left to right) of genomic DNA of the individuals 12 O2 and 12 O3d was used as template for PCR. Five hundred nanograms of the individuals 12 O2 and 12 O3d (**B**) and of seven putative transgenic plants (**C**) was used as template for PCR. Products of PCR reactions were electrophoresed on 0.8% agarose gels, stained with ethidium bromide and visualised using a UV transiluminator. Products were identified by their position relative to molecular weight marker V (MkV).

In a second experiment 500 ng genomic DNA from all potential dot blot positive individuals was tested for the presence of the cystatin transgene by PCR analysis. A high level of amplification occurred in all of the reactions in the second experiment (Fig. 5.6C). Previous attempts to amplify transgene from 100 ng genomic DNA of one of the individuals in this experiment had failed. Since good amplification was obtained using 500 ng genomic DNA as template it was decided to continue using this quantity in subsequent reactions.

c) PCR protocol efficiency

It was necessary to develop a protocol capable of amplifying transgenes from a plant containing a single transgene. Therefore experiments were performed to test the ability of 5 PCR thermal cycling protocols to amplify a transgene from copy number reconstructions

containing 1, 10, 100, 1000, 10 000 or 100 000 transgene copies. Transgene copy number reconstructions were prepared as was described in section 5.2.3 h). The transgene copy number reconstructions were spiked into 500 ng non-bombarded control, genomic DNA for use as template DNA in PCR reactions. For testing of PCR protocols the cystatin transgene and cystatin specific primers were used.

Protocols 1 and 2 (Table 5.3) were standard PCR protocols which had previously been used for the amplification of cystatin, pleurocidin and nptII transgenes from plasmids and from transgenic plants (not shown). Protocols 3, 4 and 5 (Table 5.3) were designed to increase amplification of low copy number transgenes. In protocol 3 the thermal cycling regime began with a very low annealing temperature which increased in a stepwise manner. The rationale for using this protocol was that the low annealing temperature used in the beginning would allow both specific and non-specific amplification to occur. Although the non-specific amplification was not desirable it was proposed that the concentration of the gene of interest would increase during the initial cycles and that amplification of this gene would therefore be greater during subsequent cycles when the annealing temperature was raised. In protocols 4 and 5 the initial cycles were completed with very high annealing temperatures which gradually became lower throughout the reaction. In protocol 5 the annealing temperature decreased by 0.6°C each cycle while in protocol 4 it decreased by 5°C every 5 cycles. The rationale of these protocols was that the high annealing temperature would only allow highly specific annealing in the initial cycles. This was to ensure that the primers remained accessible to the transgene inserts since they were not likely to be involved in non-specific annealing. Thus the occurrence of successful primer-transgene annealing would be increased. The PCR protocols were tested with either standard *Taq* DNA polymerase (Promega) or Amplitaq Gold (Promega) or, in the case of protocols 4 and 5, both.

The standard protocols (protocols 1 and 2; Table 5.3) did not amplify cystatin from transgene copy no. reconstructions containing 1000 transgene copies or less but did amplify cystatin from a positive control containing 2 ng pUBI510-pleuro8 (not shown). Protocol 3 amplified pleurocidin from transgene copy number reconstructions containing 100 transgene copies or more (Fig. 5.7). Protocols 4 and 5 amplified cystatin from transgene copy number reconstructions containing 1 or more transgene copies. Protocol 4 was therefore used in conjunction with the respective transgene specific primers to test for the presence of cystatin, pleurocidin and nptII transgenes in putative transgenic plants.

Table 5.3: Thermal cycling protocols for amplification of transgene inserts from putative transgenic plants. Denaturation (D), annealing (A) and extension (E) phases of thermal cycling protocols 1-5 are shown. Temperature and duration of each phase as well as the no of cycles within each step are given. Protocol 2 is identical to regime 1 except the annealing temperatures are 5°C higher. The symbols 'Δ' indicates that the annealing temperature lowers by 0.6°C per cycle, 'Δ1' that 3 steps follow in which the annealing temperature increases by 5°C per step (5 cycles each) and 'Δ2' that 2 steps follow in which the annealing temperature decreases by 5°C per step (5 cycles each).

Thermal Cycling Protocol	Step	Phase	Temperature	Time	No. Cycles
1 and 2	1	D	95°C	2 min.	1
	2	D	95°C	30 sec.	10
		A	55°C	30 sec.	
		E	72°C	1 min.	
	3	D	95°C	30 sec.	30
		A	50°C	30 sec.	
		E	72°C	1 min.	
	4	E	72°C	5 min.	1
3	1	D	95°C	5 min.	1
	2	D	95°C	30 sec.	5
		A	50°C	30 sec.	
		E	72°C	1 min.	
	3	D	95°C	30 sec.	10
		A	55°C$^{\Delta 1}$	30 sec.	
		E	72°C	1 min.	
	7	E	72°C	7 min.	1
4	1	D	95°C	5 min.	1
	2	D	95°C	30 sec.	15
		A	65°C	45 sec.	
		E	72°C	1 min.	
	3	D	95°C	30 sec.	5
		A	60°C$^{\Delta 2}$	30 sec.	
		E	72°C	1 min.	
	6	E	72°C	7 min.	1
5	1	D	95°C	5 min.	1
	2	D	95°C	30 sec.	20$^{\Delta}$
		A	50°C	45 sec.	
		E	72°C	1 min.	
	3	D	95°C	30 sec.	10
		A	50°C	30 sec.	
		E	72°C	1 min.	
	4	D	95°C	30 sec.	5
		A	50°C	30 sec.	
		E	72°C	1 min.	
	5	E	72°C	7 min.	1

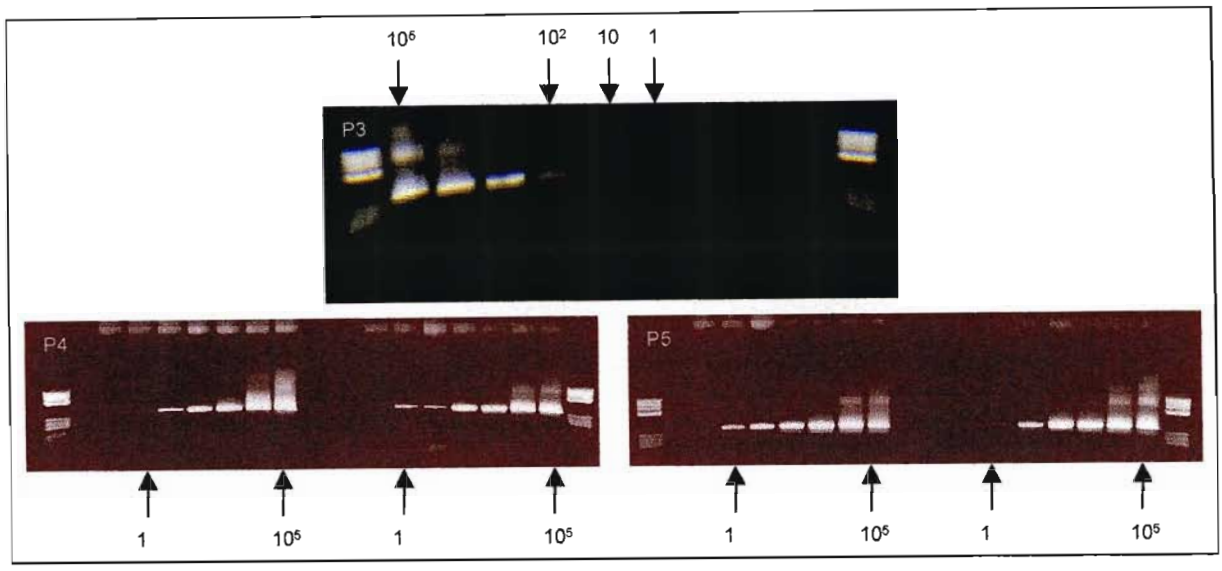

Figure 5.7: Products of PCR using protocols 3, 4 and 5. Cystatin transgene copies (1, 10, 100, 1000, 10000 and 100000 copies) were spiked into 500 ng genomic DNA of non-transformed sugarcane. **P3:** Products of PCR using protocol 3 (P3). **P4 and P5:** Products of PCR reactions using protocols 4 and 5. In each picture two sets of reactions are shown. On the left are reactions using *Taq* DNA polymerase (Promega) and on the right are reactions using Amplitaq Gold (Promega).

5.4.5 Occurrence of Transgenes: Evidence for Chimaeric Plants

a) Evidence from PCR Analysis of Putative Transgenic Plants

Genomic DNA from 48 putative transgenic plants was tested for the presence of the nptII, cystatin and pleurocidin transgenes by PCR analysis using transgene specific primers. A further 13 putative transgenic plants were tested for the presence of the cystatin and pleurocidin transgenes but not for the presence of the nptII gene. Genomic DNA used as template for PCR reactions was extracted from putative transgenic plants less than three months, 6 months, 1 year and again +1 year after they had been regenerated. The results of PCR using transgene specific primers for each of the transgenes are shown in Table 5.4. The results shown for each transgene were pooled from all of the reactions in which the transgene specific primer was used and only those plants which gave positive results for one or more transgenes are included.

Table 5.4: Record of PCR results for PCR-positive putative transgenic plants. Individuals from which the nptII, cystatin and/or pleurocidin transgene/s were amplified are shown. The time periods shown; 3 months, 6 months, 1 year and +1year, indicate the age of the plants at the time of DNA extraction. Positive reactions are indicated by a plus sign (+) and negative reactions by a minus sign (-). Plants for which positive and negative results were obtained are indicated by a plus sign followed by a minus sign (+-). Plants which gave positive results and later died are indicated by the letter 'd'. The letters 'nr' indicate that no result was obtained for a given individual. Data for PCR-negative individuals is not shown (cystatin and pleurocidin columns). The shaded and unshaded areas demark the data sets for each of the transgenes.

Plant	nptII		cystatin				pleurocidin		
	1 year	+1 year	3 months	6 months	1 year	+1 year	3 months	1 year	+1 year
1	+	-							
11 B1b	+	-							
11 F	+	-							
12 A1	+	-	+	+-	-	-	+	-	-
12 Abii	+-	-							
12 Eb	d	d	+-	d	d	d	+	d	d
12 G1	nr	-	+	nr	-	-			
12 G2	nr	-	+	nr	-	-	+	nr	-
12 H	+-	-	+	+-	-	-	+	+	-
12 I2	d	d	+	d	d	d			
12 I3a	d	d	+	d	d	d			
12 I3b	+	-							
12 I4	+	-	+	+-	-	-			
12 I7i	d	d	+	d	d	d			
12 I7ii	d	d	+	d	d	d	+	d	d
12 J	d	d	+-	d	d	d	+	d	d
12 K1c	+	-	+	+-	-	-	+	-	-
12 K3	+	-							
12 K4	+	-							
12 K5a	+	-	+	-	-	-			
12 K5b	+	-							
12 N1c	+-	-	+	-	-	-			
12 N1f	+-	-	+	-	-	-	+	+	+-
12 N3b	+	-	+	+	-	-			
12 O1	+-	-	+	-	-	-	+	-	-
12 O1c	+	-	+	+-	-	-			
12 O2	+-	+	+	+-	-	-	+	+	-
12 O3d	d	d	+	d	d	d			
13 1D	+	-	nr	+-	-	-			
13 1D3	+-	-	nr	+-	-	-			
13 2B3b	+-	+	-	+-	-	-			
13 2E	+-	-	nr	+	-	-			
13 2Eb	+	-	nr	+	-	-			
17 A1	+-	+	nr	+	-	-	nr	+	-
17 A3	+	-					nr	+	-
17 E3	+	-							
17 E4	+-	-					nr	+	-
30 G	+	-							

Thirteen putative transgenic plants (Table 5.4) died less than 3 months after regeneration. Consequently PCR results were only obtained for these plants using DNA extracted less than three months following regeneration. The plants were not visibly infected with bacteria or fungi and were subcultured together with many of the other 48 plants tested (Table 5.4). The cause of the death of these plants is therefore unknown. Although 10 of the individuals were PCR-negative for the presence of pleurocidin and 6 were negative for the presence of cystatin

it is possible that the presence of the cystatin gene in those plants testing positive for cystatin had deleterious effects on the growth and survival of the plants. This is supported by the fact that tobacco plants transformed with cystatin have been reported to display a dwarfed phenotype (pers. comm. Karl Kunert, 2000). The plants were reported to be normal in appearance but were slower growing than non-transformed controls. In it has been shown that cystatin expressed in transformed soy bean cells blocks the advent of programmed cell death (PCD) under conditions known to induce PCD in untransformed cells (Solomon *et al.*, 1999). Programmed cell death is involved in xylogenesis (Fukuda, 1996; Groover *et al.*, 1997) and senescence (Greenberg, 1996) and it is possible that a cystatin transgene could interfere with PCD during the normal growth and development of a transgenic host plant and lead to the death or slow growth of the plant.

Of the remaining plants all of them gave both positive and negative results. Positive results were obtained from samples of genomic DNA extracted earlier than those from which negative results were obtained. The majority of positive results for the cystatin and pleurocidin gene were obtained from DNA extracted from plants 6 months or less after regeneration. Negative results were obtained for DNA from the same plants extracted 1 year or more after regeneration. A similar pattern of positive and negative results was observed for the nptII gene although most of the plants were still positive 1 year following regeneration. These results suggest that regenerated plants were not completely transformed but were chimaeric in nature. Chimaerism has been previously reported to be associated with biolistic transformation (McCabe *et al.*, 1988; Christou, 1990; Christou and Ford, 1995). It has also been associated with regeneration of sugarcane by direct embryogenesis following microprojectile bombardment of sugarcane explants (Gambley *et al*, 1993). Those authors observed expression of the Gus gene in streaks of transformed cells surrounded by streaks of non-transformed cells in 20-40% of regenerated plants. Further support for chimaerism is that at the stage at which many of the leaf discs were bombarded, i.e., 1-2 weeks following culture initiation embryos are already forming (Snyman *et al.*, 2001). Bombardment of such tissue could conceivably leave some cells of a developing embryo transformed and others un-transformed. A plant developing from such an embryo would be chimaeric.

Gambley *et al.* (1993) observed that larger shoots showed Gus activity predominantly at the base while smaller shoots showed more extensive Gus activity up into the leaves. This is visual evidence indicating a reduction in concentration of transformed cells with the age of

the plant. It is proposed that a similar reduction in the concentration of transformed cells occurred in the plants regenerated in this project and that this accounts for the observed PCR results, i.e. that plants giving positive results for one or more transgenes in initial PCR analyses gave negative results for pleurocidin and cystatin more than 6 months after regeneration negative results for the nptII gene more than 1 year after regeneration.

b) Regeneration of Chimaeric Plants Supported by Southern Hybridization Analyses

Southern hybridization analysis of putative transgenic plant genomic DNA was undertaken to determine whether PCR positive plants could be confirmed as positives and to gain information about the number of copies present in the plants. For Southern hybridization analysis, DNA was extracted from 3 or more replicates of the same individual and cleaved using either Bam HI only or Bam HI and Eco RI. Restricted genomic DNA (10 or 20 μg) from each individual was then separated by electrophoresis on a 0.5% agarose gel. Before the DNA was transferred onto a membrane, the gel was stained with ethidium bromide and viewed using a UV transiluminator to ensure that the DNA had separated evenly. The DNA was then transferred to a Hybond XL membrane by upward capillary blot and bound to the membrane using UV irradiation. The membrane was incubated in a buffer containing ^{32}P-labelled transgene-probes generated by amplification of the transgene inserts from the plasmids pUBIKN, pUBI510-cys3 and pUBI510-pleuro8. Following incubation with the probes the membranes were washed and placed onto X-ray film in a light-tight X-ray cassette. The X-rays were exposed to the membrane for 5-10 days at which time they were developed. Table 5.5 shows the results of the Southern analyses performed on putative transgenic plants.

In experiment 1 (Table 5.5) genomic DNA was cleaved using both Bam HI and Eco RI. Because both enzymes were used, the transgenes were cut out of the genomic DNA at both ends. Therefore all copies of the transgene for each individual migrated at the same rate during electrophoresis. Hybridization was observed as distinct bands in lanes containing DNA from the individuals 12 H, 12 O2, 13 1D3 and 17 A1. The bands were identified as being cystatin since hybridization occurred to a positive control containing the plasmid pUBI510-cys3 but not to controls containing the plasmids pUBI510-pleuro8 or pUBIKN. Three of the individuals, 12 H, 12 O2, and 17 A1 were PCR-positive for the nptII, cystatin and pleurocidin

transgenes while the other, 13 1D3 was PCR-positive for the nptII and cystatin transgenes only.

Table 5.5: Southern hybridization experiments performed on genomic DNA extracted from putative transgenic sugarcane plants. For each experiment the number of plants analysed (Number of Plants) age of plants at time of DNA extraction (Age) amount of DNA used per individual (Sample Size) the transgene probe used (probe) and number of positive plants (Number of Positives) is shown. Hybridization to single copy reconstructions is denoted by 'Y' and lack thereof by 'N'. Experiments for which single copy-controls were not included are denoted by '-'.

Experiment	Number of Plants	Age	Sample Size (µg DNA)	Probe	Single Copy Hybridization	Number of Positives
1	28	< 6 Months	10	cystatin	-	4
2	25	> 1 Year	20	cystatin	-	0
3	25	> 1 Year	20	cystatin	-	0
4	8	> 1 Year	20	pleurocidin	Y	0
5	48	> 1 year	20	cystatin	N	0
6	48	> 1 year	20	pleurocidin	Y	0

The DNA used in experiment 1 had been extracted when the plants were less than 6 months old, many being less than 30 cm tall at the time of extraction. All subsequent Southern analyses (experiments 2-6; Table 5.5) were done using DNA that was extracted after the plants were over a year old. In experiments 4 and 6 hybridization of the pleurocidin probe was observed in lanes in which pleurocidin transgene copy no. reconstructions containing 1 and 10 transgene copies had been loaded. In experiment 5 hybridization of the cystatin probe was observed in the lane in which a cystatin transgene copy no. reconstruction containing 10 transgene copies had been loaded. However hybridization of the cystatin and pleurocidin probes was not observed in any of the lanes containing DNA from putative transgenic plants.

Southern hybridization results were similar to those observed for PCR analyses in that positive results were only obtained using DNA that was extracted from plants less than 6 months after they had been regenerated.

c) Production of Transcripts and Transgene Stability in the Individual 12 N1f

c) Production of Transcripts and Transgene Stability in the Individual 12 N1f

To test for the production of mRNA in putative transgenic plants RT-PCR analysis was performed on 7 plants which had been determined to contain the pleurocidin transgene by PCR analysis. Total plant RNA was extracted from the 7 putative transgenic plants and 1 non-bombarded control plant. The Titan One Tube RT-PCR System (Roche) was used for the production of cDNA. Pleurocidin specific primers were used for the amplification step. To ensure that the RNA was not contaminated with DNA it was used as template for a PCR reaction using pleurocidin specific primers. The PCR and RT-PCR products were electrophoresed on a 0.5% agarose gel and visualised by staining with ethidium bromide and viewing on a UV-transiluminator. The pleurocidin transgene was amplified from the cDNA of only one plant, 12 N1f (Fig. 5.8).

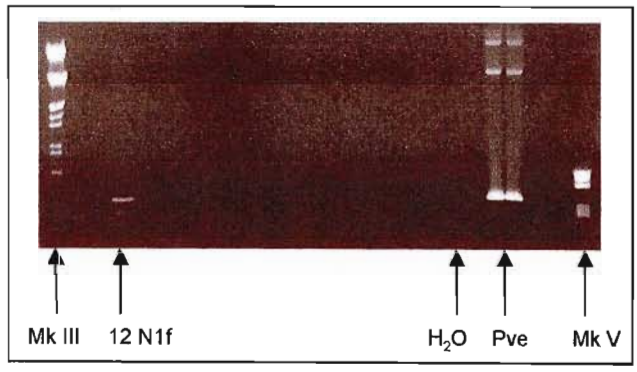

Figure 5.8: Reverse Transcriptase-PCR of seven pleurocidin PCR positives. Products of PCR were run in the lane adjacent to and to the right of those of containing RT-PCR products. The RT-PCR product of 12 N1f cDNA is shown (12 N1f). Note that amplification occurred from the plasmid control (Pve) and not from the negative control (H_2O).

Replicates of the transgenic individual 12 N1f were grown from setts cut from two stalks (tillers) of the originally regenerated plant. The setts were planted in a nutrient rich soil in plastic trays and cultivated in the glasshouse. When the plants had reached a height of roughly 45 cm, DNA was extracted from the leaves and small tillers of each of 12 replicates. Since the individual 12 N1f was determined by PCR and RT-PCR analysis to contain the pleurocidin

cystatin transgene was stably integrated in the individual 12 N1f. It is considered significant that this was the only individual for which transcript production was demonstrated.

5.4.5 Regeneration of True Transgenic Plants from Potentially Chimaeric Plants

To regenerate completely transformed plants from suspected chimaeric plants, leaf discs were cut from 30 putative transgenic plants and cultured in the dark (26°C) on MS, 1 g/l CH, 30 g/l sucrose, 0.6 g/l 2,4-D for one week. The discs were then transferred to medium of the same formulation but also containing 45 mg/l G418 for the selection of transformed cells. The putative transgenic plants were one year in age and were ± 1 m in height at the time of harvest. Two replicates of each putative transgenic individual were used to prepare a total of 36 leaf discs per individual. Leaf discs were sublcultured every two weeks.

After 2-4 weeks on selection embryos were visible on many of the discs. Six to eight weeks following culture initiation the embryos were transferred to regeneration medium and a 16 h/8 h (light/dark) photoperiod at 26°C. Although the embryos looked healthy at the time of the transfer to regeneration medium, no plants were regenerated from the material.

It was thought that the failure of the embryos to regenerate may have been due to the fact that the putative transgenic plants that were used were too old at the time of harvest and that the transgene may have been diluted or lost by this stage due to the proliferation of non-transformed cells. A similar experiment was therefore performed using leaf discs cut from 15 putative transgenic plants that had been regenerated more recently and were less than six months in age. The leaf discs did not respond well to the treatment and all of the discs died during the dark phase of selection. The experiment using younger plants was repeated a second time with the same result.

The failure of the leaf discs cut from plants less than six months after regeneration may have been due to the fact that the plants were so young. The failure of leaf discs cut from 1 year old plants however may be due to the fact that the transgenes were not present, at the time of harvest, in the apical portions of the plants from which leaf discs were cut. Although PCR results indicated that the nptII gene was present in 1 year old plants (Table 5.4) it is possible it

was limited to the basal portions of the plants. This phenomenon was observed in sugarcane transformed with the Gus gene by Gambley *et al.* (1993). Since whole tillers were normally used for DNA extraction the transgene would have been detectable by PCR but would not necessarily be present in the leaf roll, from which leaf discs were cut.

Reports of natural sugarcane resistance to kanamycin-type selective agents are varied. Bower *et al.* (1996) report that 45 mg/l G418 was lethal to non-transformed, control callus and that selection of transformed sugarcane using 45 mg/l was reliable, favored the growth of embryogenic vs. non-embryogenic callus and resulted in only 5% of the regenerate plants being escapes. Escape rates of 4% on 43.3 µM geneticin (Falco *et al.*, 2000) and 0-7% on 144 µM geneticin (Ritata *et al.*, 1993) have also been reported. Arencibia *et al.* (2001) however report that non-transformed control callus grew well on concentrations of Kanamycin as high as 200 mg/l. High natural levels of Kanamycin tolerance were also reported by Ozias *et al.* (1986) and Hauptmann *et al.* (1988). Hauptmann *et al.* (1988) also reported that Kanamycin only inhibited the growth of sugarcane cells if added before protoplasts were five days old. Natural sugarcane resistance to G418 may explain why embryos formed on leaf discs cultured in the presence of 45 mg/l G418 in this project.

5.5 CONCLUSIONS

- The indirect route of morphogenesis was relatively more efficient than the direct route for the regeneration of plants from non-bombarded controls.
- No transgenic plants were regenerated via the indirect route of morphogenesis.
- Based on results of PCR and Southern hybridization analysis it was interpreted that the plants regenerated following bombardment were either non-transformed escapes or incompletely transformed chimaeras.
- The transformation protocol used in this project did not produce transformation efficiencies that have been achieved in other laboratories using the same protocol.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUDING REMARKS

6.1 OVERVIEW

In Chapter 3 the suitability of cystatin and pleurocidin as transgenes for use in sugarcane was established. Both of the genes were chosen for the desirable traits they may potentially confer upon sugarcane in which they are expressed as transgenes. Further, cystatin has been used successfully in other transformation studies and pleurocidin was shown to be an effective inhibitor of four known, and economically serious, bacterial sugarcane pathogens. In addition a method was developed for the extraction of intercellular fluid/exudate from sugarcane leaves. It was proposed that the absence of chlorophyll in exudate extracted using an infiltration time of 1 minute and a centrifugation time of ten minutes was an indication that the cells were not damaged. Further experimentation is needed to ensure that the assumption that cellular damage had not occurred is correct. Although the above mentioned extraction conditions were adequate for the purposes of this project, further experimentation with infiltration times and centrifugation times may increase the volume of exudate extracted.

In this project transgene products were targeted for expression and subsequent transport out of the cell. Expressed in the intercellular spaces, transgene products are less likely to be processed by the enzymes of the host plant and are in a suitable position to come into contact with invading pathogens. The method for extraction of leaf exudate (section 3.3.2) used in this project will potentially be useful in the future to test for the presence of transgene products in putatively transformed plants.

In the current project a cysteine proteinase assay was tested. It was shown that leaf exudate extracted by the method developed here was a suitable substrate for the cysteine proteinase assay (Table 3.4). Since cystatin is known to affect the levels of cysteine proteinase in transformed plants it was envisaged that the cysteine proteinase assay could be used to test for the presence of cystatin in leaf exudate from putatively transformed plants. The leaf exudate could potentially be used directly to test for the presence of pleurocidin by means of a bacterial growth inhibition assay (section 3.3.1).

The modification and cloning of pleurocidin and cystatin transgenes into plasmid vectors suitable for expression in sugarcane was discussed in Chapter 4. Also described in this chapter was the important step of assessing the integrity of the vectors produced from this process. It was necessary to test the plasmid vectors and ensure that the transgenes were in the correct position in the vector, that the priming sites were intact and effective and that no sequence alterations had occurred. This was achieved through restriction enzyme analysis, PCR analysis and sequencing of the constructed plasmid vectors (section 4.3.1). The analysis of plasmid vectors always forms an important part of transformation routines in that each new batch prepared must be tested before use.

In Chapter 5 the use of the transgene constructs for the production of transgenic plants was presented. Cystatin and pleurocidin transgenes were delivered to sugarcane leaf discs and callus biolistically. Plants were regenerated from sugarcane leaf discs by direct embryogenesis in the presence of a low or high concentration of 2,4-D and from callus by indirect embryogenesis. In the current work the indirect route of embryogenesis was relatively more efficient than the direct route in terms of the regeneration of plants from non-bombarded controls (section 5.3.1) however no plants were regenerated by indirect embryogenesis from bombarded material (section 5.3.2). This is unusual considering that the protocol used for this work has been used for the production of transformed sugarcane both at SASEX (Snyman et al., 1996; Snyman et al., 2000; Snyman et al., 2001) and in foreign laboratories (Bower and Birch, 1992; Gambley et al., 1993; Bower et al., 1996; Hansom et al., 1998). It was proposed in Chapter 5 that the failure of this protocol in the current work could be due to practical inexperience.

6.2 INSIGHTS

Outcomes of interest from this project include the leaf exudate extraction method, the cysteine proteinase assay, chimaeric plants and the abandonment of cystatin as a potentially useful transgene for use in sugarcane. The method for the extraction of leaf exudate will be useful in the future for the analysis of transgenic plants. The cysteine proteinase assay which was tested here may also be useful for the analysis of chimaeric plants produced in this project to test for

the presence of cystatin. The production of chimaeric plants and the abandonment of cystatin as a transgene are discussed in more detail below.

Based on the results of PCR and Southern hybridization analysis it was proposed that the putative transgenic plants produced in this work were chimaeric. Chimaerism has been previously reported to be associated with biolistic transformation (McCabe *et al.*, 1988; Christou, 1990; Christou and Ford, 1995). It has also been associated with regeneration of sugarcane by direct embryogenesis following microprojectile bombardment (Gambley *et al*, 1993). Further support for chimaerism is that at the stage at which many of the leaf discs were bombarded, i.e., 1-2 weeks following culture initiation embryos are already forming (Snyman *et al.*, 2001). Bombardment of such tissue could conceivably leave some cells of a developing embryo transformed and others un-transformed. A plant developing from such an embryo would be chimaeric.

If the short culture times associated with the direct route are to be advantageous, the plants produced cannot be chimaeric. Chimaeric plants are essentially useless for commercial production. To solve the chimaera problem will require further investigation to determine the optimum pre- and post-bombardment culture conditions and timing of bombardment needed to produce non-chimaeric plants. Such an investigation will be long-term and labour intensive in nature.

Cystatin was chosen as a transgene mainly due to its potential ability to prevent the establishment of sugarcane mosaic virus (SCMV) infections. Extensive literature review during the course of the work presented here, however, has shown that the expression of cystatin in transgenic plants may interfere with programmed cell death (PCD) in plants. Cystatin is an inhibitor of cysteine proteinases which are key enzymes in the onset of PCD. It has been shown that cystatin expressed in transformed soy bean cells blocks the advent of programmed cell death (PCD) under conditions known to induce PCD in untransformed cells (Solomon *et al.*, 1999). Since PCD has been shown to be an important part of natural plant developmental processes such as xylogenesis (Fukuda, 1996; Groover *et al.*, 1997) and senescence (Greenberg, 1996) the expression of cystatin in transgenic plants may block natural developmental processes and therefore be deleterious in nature. Tobacco plants transformed with cystatin have been reported to display a dwarfed phenotype (pers. comm. Karl Kunert, 2000). The plants were reported to be normal in appearance but were slower

growing than non-transformed controls. Certain of the putative transformed plants produced in this project died for no apparent reason within three months of regeneration. Although only some of these plants were PCR-positive for the presence of the cystatin transgene it is possible that the cause of death in these individuals may have been the presence of cystatin. This casts doubt on the use of the cytatin gene for transformation of sugarcane in the future.

6.3 FUTURE OUTLOOK

The transformation of sugarcane can be performed routinely at present although the transformation efficiency is often lower than is desirable. From studies using sugarcane and other monocotyledonous species there is evidence to suggest that transformation efficiencies may be increased through continued investigation into the factors that affect transformation. In the sugarcane industry, genetic transformation will potentially enable the production of genetically superior crops possessing unique mechanisms of disease resistance, a decreased tendency to deteriorate in vigour, higher levels of sucrose accumulation and improved milling characteristics for higher sucrose recovery. Further attempts at the transformation of sugarcane, and all crops for that matter, must therefore be targeted at the commercial production of crops.

At present it is predicted that the regulations surrounding the production of GM varieties will increase (Smyth *et al.*, 2002). The regulations are said to be a response to societal and political pressures which are not necessarily grounded on scientific evidence to support increased regulation (Smyth *et al.*., 2002). Dale *et al.* (2002) report that there is no compelling scientific evidence to suggest that GM crops are innately different than non-GM crops and that there is no reason to suspect that free DNA originating from transgenic crops is likely to have a significant impact on the environment. Nevertheless, gene flow from GM crops to related non-GM crops, weeds or wild relatives is among the biggest concerns surrounding their cultivation (Smyth *et al.* 2002; Snow, 2002). The majority of other concerns are ethical or socioeconomic in nature. Currently research is being directed towards limiting the flow of transgenes from GM crops into the environment (Daniell, 2002; Hare and Chua, 2002).

The ability to produce transformed crops is the result of advances in several areas of biology and decades of labour intensive research in laboratories around the world. As the current

project has demonstrated the process is time consuming and laborious. This is contrary to the layman's perception of genetic transformation as a recent, somewhat mystical phenomenon. The media and certain extreme environmental awareness groups have done much to aggravate this type of perception and have cast doubt and uncertainty on this field of research. It is up to researchers then to continue making progress and to dispel misconceptions regarding genetic transformation at every opportunity.

APPENDIX 1

Calculation of dilution factor required to prepare a single-transgene copy no. reconstruction of the pleurocidin transgene insert.

IF:

Size of pleurocidin transgene insert: 314 bp

Size of haploid sugarcane genome: 1.1×10^{10} bp (Bower and Birch, 1992)

THEN:

Insert:	haploid sugarcane genome
314 bp:	1.1×10^{10} bp
314 *f*g:	1.1×10^{10} *f*g
314 *f*g:	11 µg
285.45 *f*g:	10 µg

THEREFORE:

A single-transgene copy no. reconstruction using 10 µg genomic DNA requires 285.45 *f*g pleurocidin transgene insert.

IF:

[pleurocidin transgene insert]	= 0.0637 ng/µl
	= 63694.26 *f*g/µl

THEN:

Required dilution	= 63694.26 / 285.45
	= <u>223 X</u>

NOTE: The above calculation is for 10 µg genomic DNA. Southern hybridization analyses were performed using either 10 µg or 20 µg genomic DNA loaded per lane. For PCR analyses 500 ng genomic DNA was used template. In addition the transgene inserts were not equal in size. The calculation was therefore changed depending on the type of analysis to be used, the amount of genomic DNA required, and the specific transgene for which copy no. reconstruction were being prepared.

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