Molecular characterisation of *Eucalyptus grandis* PGIP

by

Raksha Bhoora

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Study Leader

Prof. B.D. Wingfield

Co-study Leaders

Prof. D.K. Berger

Dr. P.M. Chimwamurombe

Prof. M.J. Wingfield

Dedicated to my late father

Vasantrai Bhoora

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List Of Abbreviations

AA	amino acids
Amp^R	ampicillin resistance
ADP1	adaptor primer 1
ADP2	adaptor primer 2
ADA	agarose diffusion assay
ARC	Agricultural Research Council
Avr	avirulence
BAP	6-Benzylaminopurine
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumin
CAT	chloroamphenicol acetyltransferase
CAMV	cauliflower mosaic virus
cDNA	complementary DNA
CWDE	cell wall-degrading enzymes
cv	cultivar
dH_2O	distilled water
ddH_2O	double distilled water
DEB	DNA extraction buffer
DIG	digoxygenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
endo-PGs	endopolygalacturonase
EtOH	ethanol
GUS	β-glucuronidase
hpt	hygromycin phosphotransferase
IAA	indole-3-Acetic Acid
IEF	isoelectric focusing
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan^R	kanamycin resistance

kb	kilobasepair
kDa	kilodalton
Km	kanamycin
LB	Luria Bertani
LRR	leucine-rich repeat
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog
NAA	1-naphthylacetic acid
NBT	nitroblue tetrazolium chloride
nptII	neomycin phosphotransferase II
ng	nanogram
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAHBAH	ρ-4-amino-2-hydroxybenzoicacid hydrazide
PCR	polymerase chain reaction
PEG	polyethylene glycol
PG	polygalacturonase
PGA	polygalacturonic acid
PGIP	polygalacturonase-inhibiting protein
pI	isoelectric point
PL	pectate lyase
PME	pectin methylesterase
PRI	porcine ribonuclease inhibitor
PS	panhandle structures
PVX	potato virus X
R-genes	resistance genes
RACE	rapid amplification of cDNA ends
Rf	rifampicin
RI	ribonuclease inhibitor
RNA	ribonucleic acid
ss	single stranded
SCN	soybean cyst nematode
SDS	sodium dodecyl sulphate

TAE	Tris-acetate ethylenediamine tetraacetic acid
T-DNA	transferred DNA
TE	Tris ethylenediamine tetraacetic acid
TEV	tobacco etch virus
Ti	tumour inducing
Tm	melting temperature
TNE	Tris-sodium chloride EDTA
UV	ultraviolet
vir	virulence
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-gluc	5-bromo-4-chloro-indolyl-β-D-glucuronide
YEP	yeast peptone

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PREFACE

Coniothyrium zuluense is the causal agent of a serious *Eucalyptus* stem canker disease in South Africa (Wingfield *et al.,* 1997). *Eucalypts* are the most important hardwood plantations in the world, and in South Africa these hardwoods occupy approximately 1.5 million hectares of plantation area, an area that is soon to be increased by an additional 600 000 hectares. As exotics, *Eucalyptus* plantations are constantly exposed to infection by fungal pathogens such as *C. zuluense,* which by secreting cell-wall degrading enzymes contribute to the degradation of plant cell walls and subsequent reduction and in the quality of timber produced. This ultimately affects the South African paper, pulp and timber industries.

Selection of resistant clones through traditional breeding methods is the most common method currently employed in overcoming the problem of fungal infection. The genetic manipulation of *Eucalyptus* trees for enhanced resistance to fungal diseases is an alternative to the time-consuming and tedious approach of conventional breeding. The identification of several antifungal proteins, particularly polygalacturonase-inhibiting proteins (PGIPs) from various plant species including *Eucalyptus,* lead to the hypothesis that over-expression of these proteins could potentially reduce pathogen attack. However, prior to the expression of PGIPs in plants, isolation and molecular characterization of these genes are required. The aims of this study were therefore (1) to clone and characterize the complete *Eucalyptus grandis pgip* gene, (2) to transform *Nicotiana tabacum* (tobacco) plants with the *E. grandis pgip* gene and (3) to test for inhibition of *C. zuluense* PGs by PGIPs extracted from transgenic tobacco plants. This forms the first step towards the generation of *E. grandis* clones that are more disease tolerant.

A review of the role of fungal endopolygalacturonases and polygalacturonase-inhibitors in plant-pathogen interactions are presented in chapter 1. Strategies employed to isolate and characterize *pgip* genes from a range of plant species are highlighted and the importance of PGIPs in disease resistance is discussed. In chapter 2, the molecular cloning and characterization of the *E. grandis pgip* gene is discussed. The work presented in this chapter is a follow up on work previously conducted by Chimwamurombe (2001). Previously, a partial *Eucalyptus pgip* gene sequence was

obtained with the use of degenerate oligonucleotide primers. In this study, the complete *Eucalyptus pgip* gene was obtained through the employment of genome walking strategies.

Transformation of *Nicotiana tabacum* cv LA Burley plants with the *Eucalyptus pgip* gene and the molecular characterization of transgenic tobacco plants is discussed in chapter 3. The transformation and expression of foreign genes in tobacco plants is a well-established protocol, making tobacco the most appropriate candidate plant for assessing the functionality of the plant transformation construct. The production of endopolygalacturonases from virulent *C. zuluense* isolates and the subsequent PGIP assays conducted to determine levels of PG inhibition are included in this chapter.

This thesis consists of three independent chapters representing studies on the molecular characterization of an *E. grandis pgip* gene and focusing on the potential for inhibition of PGs produced by *C. zuluense* by *Eucalyptus* PGIP extracted from transgenic tobacco plants. Repetition of certain aspects in the individual chapters has been unavoidable and the thesis is presented following a uniform style.

Chapter One

Literature Review

Fungal Endopolygalacturonases and Polygalacturonase-inhibitors in Disease Resistance

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Introduction

Elucidating the role of plant defence at the molecular level is one of the pressing issues facing modern plant biologists (Walton, 1997). Plants are constantly being exposed to variety of potential enemies, these include, bacteria, fungi, viruses and nematodes (Walton, 1994). Unlike vertebrates, however, plants lack an immune system and they therefore have to rely on alternative mechanisms to resist attack (De Lorenzo and Cervone, 1997). Oligomers released from the polysaccharide during pathogen penetration acts as recognition elicitors for the plant. Early detection of the pathogen is essential for plant survival and depends greatly on the speed at which pathogen signal molecules are perceived by the plant and how quickly appropriate biochemical reactions can begin (Agrios, 1988).

Following microbial attack, plants are able to induce a number of biochemical defences. These include the synthesis of antimicrobial phytoalexins, the reinforcement of plant cell walls by the deposition of lignins and the accumulation of pathogenesis-related proteins (Lamb *et al.,* 1989). In plant-pathogen interactions, two forms of reactions can occur between the host and a pathogen, which determine either resistance or susceptibility. In incompatible reactions, the pathogen fails to infect the host due to the presence of plant resistance (*R*) genes, which recognises the pathogen elicitors and rapidly induce biochemical responses (Agrios, 1988). In contrast, compatible reactions lead to the onset of disease symptoms (Collinge and Slusarenko, 1987). Disease resistance (*R*) genes control gene-for-gene resistance (Bent, 1996). The concept of gene-for-gene resistance implies that for every gene that confers virulence to a pathogen, there must be a corresponding gene in the host that confers resistance to the host (Agrios, 1988).

Prior to the development of genetic mapping and cloning technologies, classical breeding methods and recurrent selection of desirable traits were utilised for establishing disease resistant plants (Agrios, 1988). Although molecular cloning and the ability to construct transgenic organisms have only recently been introduced, it was previously hypothesised that some forms of resistance were due to single specific genes. With the advent of molecular biology, researchers have been able to identify

and clone these resistance genes in order to determine whether certain biochemical functions are indeed involved in physiological processes such as plant disease resistance (Keen, 1999).

Several plant resistance *(R)* genes have been cloned. These include the *Hm1* gene of maize, the *Pto*, *Prf*, *Cf-9 and Cf-2* genes of tomato, *RPM1* and *RPS2* genes of *Arabidopsis*, the *Xa21* gene of rice, the *N* gene of tobacco and the *L6* gene of flax (Jones *et al.*, 1994; Bent *et al.*, 1996; Dixon *et al.*, 1996). A number of these *R*-genes, particularly from dicotyledonous plants, encode proteins that are structurally similar to leucine-rich repeat (LRR) domains that are crucial for the recognition of non-self molecules (Warren *et al.*, 1998; Komjanc *et al.*, 1999).

The accumulation of pathogenesis-related proteins such as the leucine rich polygalacturonase-inhibiting proteins (PGIPs), which form part of the cell wall receptors that respond to fungal elicitors, is an important point in plant-pathogen interactions. In this chapter, the role of endopolygalacturonases in pathogenicity, biochemical and molecular characterisation of PGIPs and the role of PGIPs in disease resistance are discussed.

The Plant Cell Wall And Fungal Invasion Strategies

The external cuticle and the polysaccharide-rich cell wall are the major physical barriers to potential pathogens of plant tissues (Walton 1994; De Lorenzo *et al.*, 1997). The complexity of the polysaccharides provides cells with both mechanical support as well as protection against biotic stress factors that include infection by bacteria, fungi and viruses (Hahn *et al.*, 1989). The two types of polysaccharide matrices that make up the plant cell walls are the pectate network and the cellulose network (Herron *et al.*, 2000).

Fungal pathogens penetrate their hosts in order to establish infection. Like bacteria and viruses, fungal plant pathogens have evolved diverse strategies to ingress plant tissues. They can either enter through wounds, natural openings such as the stomata, or by penetrating the cell wall components directly (Hahn *et al.*, 1989; Schäfer, 1994). Direct penetration can be achieved either through mechanical forces, where the

deposition of melanins on the inner appressorial cell wall mediates the build-up of hydrostatic pressure that enables the fungus to puncture the plant epidermis, or by means of enzymatic degradation (Schäfer, 1994).

It is generally accepted that the enzymatic arsenal of fungal pathogens contributes, together with mechanical forces, to the degradation of both the cuticle as well as the polysaccharide-rich cell wall (De Lorenzo *et al.*, 1997). Pathogenic fungi need to degrade the plant cell wall not only for establishing infection, but also for releasing from the wall polysaccharides, nutrients necessary for their growth (De Lorenzo *et al.*, 1997). Studies on the enzymatic digestion of cell walls has led to the description of at least 20 different cell wall-degrading enzymes (CWDEs) (Walton, 1997). Most of these enzymes are extracellular, inducible glycoproteins that exist as multiple isoenzymes (Hahn *et al.*, 1989). Enzyme activities that have been identified include the pectic enzymes, cellulases, arabinase, xylanase, and galactanase (De Lorenzo *et al.*, 1997; Collmer and Keen, 1986; Hahn *et al.*, 1989; Walton, 1997; Ridley *et al.*, 2001; Fischer and Bennett, 1991).

Pectin Degrading Enzymes

Pectins belong to a group of complex polysaccharides that are often described in terms of 'smooth' and 'hairy' blocks (Fisher and Bennett, 1991). The 'smooth' blocks are defined by a linear copolymer of a- (1? 4)-linked galacturonic acid and its methyl ester, while the 'hairy' pectin blocks include rhamnogalacturonans I and II and are complex heteropolymers, comprising 12 different sugars (Fischer and Bennett 1991). These 'smooth' and 'hairy' pectin blocks cement the plant cells together and are thus responsible for maintaining plant tissue integrity (Varner and Lin, 1989; Ridley *et al.*, 2001).

The degradation of pectic polymers is one of the earliest functions performed by plant pathogenic fungi, making pectin-degrading enzymes considerably important for the plant infection process (Whitehead *et al.*, 1995; Caprari *et al.*, 1993a). Several types of pectinases are produced and these include the endo and exo-pectate lyases (PL), endo and exo-polygalacturonase (PG) and pectin methylesterase (PME) (Walton, 1994).

Pectin lyase, pectate lyase and exopolygalacturonate lyase cleave the glycosidic bonds of the pectin backbone by ß-elimination thus generating products with a 4,5-unsaturated galacturonosyl residue at the non-reducing end (Lima *et al.,* 2002; Collmer and Keen, 1986). The highly pectinolytic gram-negative bacteria, *Erwinia chrysanthemi* and *E. carotovora* are among the best known producers of pectate lyases (Davis *et al.,* 1984; Collmer and Keen, 1986). The role of pectate lyase in pathogenesis has been investigated by producing a mutant *E. chrysanthemi* strain in which four isozymes of pectate lyase was deleted. The mutant strain was not only able to grow on polygalacturonic acid as the sole carbon source, but it also caused significant plant tissue maceration, indicating that pectate lyase are not the only enzymes required for maceration (Hahn *et al.,* 1989; Collmer and Keen, 1986, Walton, 1994).

The cucumber pathogen *Cladosporium cucumerinum* produces pectate lyases that cause maceration in susceptible cucumber hypocotyls (Robertsen, 1989). The purified enzyme showed a pH optimum of about 9.7 as well as a dependance on Ca^{2+} ions for its activity (Robertsen, 1989). Similarly, the production of pectin lyase by *Colletotrichum lindemuthianum* in infected bean tissue, showed maximum activity at alkaline pH (Wijesundera *et al.,* 1984). However, unlike endopolygalacturonases, which are constitutively expressed, the lyases are largely regulated by induction and by cyclic AMP mediated catabolite repression (Keen *et al.,* 1984; Robertsen, 1989).

Polygalacturonases (PGs) are classified into endo-PGs and exo-PGs. They are more active in degrading demethylated than methylated pectin. Exo-polygalacturonases act on the non-reducing ends of the substrate chain, removing one or two sugars at a time (Walton 1994; Lima *et al.,* 2002). They do not cause plant tissue maceration, but have a role in degrading elicitor-active oligogalacturonides released by endoPGs and are generally not inhibited by polygalacturonase-inhibiting proteins (García-Maceira *et al.,* 2000). Endopolygalacturonases (Endo-PGs) are enzymes that catalyse the hydrolytic cleavage of a-(1? 4) galacturonan linkages of homogalacturonan and have been associated with tissue maceration and killing of plant cells (Fischer and Bennett, 1991; Hahn *et al.,* 1989).

Enzymatic secretion occurs in a time-specific manner depending on the accessibility of the cell wall polymers, nutrient suitability of the pathogen and the level of resistance to polymer degradation (Walton, 1994). The sequential occurrence of different classes of cell wall-degrading enzymes suggests that different signal molecules are responsible for inducing the specific enzymes (Dixon and Lamb, 1990).

Endopolygalacturonase activity appears to be crucial during the initial phase of fungal infection. At the onset of infection, the cell wall pH is low and the degradation is localised to the site of infection. As time progresses, the cell wall pH rises to above 6.5, at which time polygalacturonase activity is greatly reduced. Pectate lyase activity thus increases together with an increase in fungal growth (De Lorenzo *et al.*, 1997).

Role of Endopolygalacturonases in Pathogenicity

All plant pathogens secrete enzymes that are capable of degrading the polysaccharides of plant cell walls (Albersheim and Anderson, 1971). The fungal pathogen *C. lindemuthianum* has been shown to produce a wide range of cell wall-degrading enzymes that were secreted sequentially depending on the age of the culture (English *et al.,* 1972). A large variety of these polysaccharide-degrading enzymes have been isolated from plants, fungi and bacteria (Karr and Albersheim, 1970). Pectin degrading enzymes are invariably among the first of these enzymes to be secreted by pathogens grown in culture on host cell walls (Albersheim and Anderson, 1971; Scott-Craig *et al.,* 1990; Hahn *et al.,* 1989).

Endopolygalacturonases (Endo-PGs) are pectin-degrading enzymes. They are produced by numerous fungi and bacteria, higher plants and some plant-parasitic nematodes (Patino *et al.,* 1997). Endo-PGs, from a number of different fungi have been purified and characterised (Caprari *et al.,* 1993b). These chain-splitting glycoside hydrolases degrade the pectin polymers by hydrolysing the a-1,4 glycosidic linkages between galacturonic acid residues within the homolgalacturonan domain of pectin (Agrios, 1988; Herron *et al.,* 2000). Endopolygalacturonases are thus of particular importance due to the fact that they are produced in the largest amounts and are capable of macerating plant tissues and killing plant cells on their own (De Lorenzo and Cervone, 1997).

Polygalacturonases have several features, which indicate that these enzymes may have multiple roles during pathogenesis. Pre-treatment with endopolygalacturonases facilitates depolymerization of plant cell walls by other fungus-secreted cell wall-degrading enzymes (Karr and Albersheim, 1970; Cervone *et al.,* 1989). Products produced by enzymatic cleavage are utilized as nourishment by the fungus during the initial stages of colonization (Cervone *et al.,* 1989). In addition, several filamentous fungi produce multiple forms of secreted endopolygalacturonases and the redundancy of these enzymes may facilitate pathogenesis in various hosts and under a variety of conditions (Cooper, 1984; Caprari *et al.,* 1993a; De Lorenzo and Cervone, 1997).

According to Caprari *et al.* (1993b) analysis of chemically induced mutants provided the first genetic evidence for the involvement of endopolygalacturonases in the fungal diseases of plants. Scott-Craig *et al.* (1990) were the first to report the cloning and characterisation of an endopolygalacturonase gene designated as *PGN1* from the maize fungal pathogen, *Cochliobolus carbonum.* This enabled the construction of a precise gene disruption mutant in which only the PG gene is affected. The resulting mutant strain retained its ability to infect maize, indicating that endopolygalacturonases were not required in this disease interaction. In 1996, Caprari *et al.* provided the first molecular evidence for the role of endopolygalacturonases in cell wall degradation. A comparison between the endopolygalacturonase sequences from bacteria, fungi and plants showed the existence of highly conserved amino acid regions (Scott-Craig *et al.,* 1990). This conserved region contains a histidine residue that is essential for the activity of PGs (Caprari *et al.,* 1993b, 1996). Using site-directed mutagenesis on an endopolygalacturonase gene from *Fusarium moniliforme*, Caprari *et al.* (1996) were able to show that replacement of the His234 residue with a Lysine abolished enzymatic and macerating activity. Similarly, mutations of either the Ser234 or Ser240 residues to a Glycine, reduced enzymatic activity in *Fusarium moniliforme* PG.

Martel *et al.* (1998) reported the separation of 16 isoforms of polygalacturonases from the plant pathogen *Sclerotinia sclerotiorum.* They were able to identify seven genes constituting two subfamilies of endopolygalacturonases and hypothesised that the occurrence of multiple enzyme forms increases adaptive capacity and flexibility of

pathogens. Cook *et al.* (1999) went a step further and showed that different fungal PGs differ in their ability to degrade pectic substrates and that they exhibit different levels of susceptibility to inhibitory proteins. This implies that the underlying ability of a pathogen to degrade its host cell wall depends on whether the PGs produced are sufficiently active to initiate pathogenesis and also on the number of different inhibitory proteins present in the host.

One of the key features of fungal endoPGs is that they are elicitors of plant defence responses. This was initially observed in *Rhizopus stolonifer* endo-PGs, which were able to elicit phytoalexin accumulation in castor bean seedlings (Lee and West, 1981a). EndoPGs may, therefore, play two opposing roles during invasion, firstly as agents of fungal aggression and secondly as signal molecules (Cervone *et al.*, 1989). However, evidence suggests that endopolygalacturonases are not directly responsible for the induction of plant defence responses. Instead, the oligogalacturonide fragments released by the activity of endoPGs on plant cell walls are the true elicitors (De Lorenzo and Cervone, 1997).

Oligosaccharide Signals For Defence Response

Plants have evolved several mechanisms enabling ingress by a pathogen (Ridley *et al.*, 2001). These include the strengthening of the cell wall by deposition of lignin (Altamura *et al.*, 1998), the synthesis of phytoalexins and other antimicrobial compounds (Lee and West, 1981a; Walker-Simmons *et al.*, 1983; Jin and West, 1984; Davis *et al.*, 1986) and the production of several pathogenesis-related (PR) proteins (Cervone *et al.*, 1989). Fungal endopolygalacuronases have been implicated in the activation of a variety of these plant defence responses (Cervone *et al.*, 1989). Linear a-1,4-D-galactopyranosyluronic acid residues produced by the enzymatic cleavage of cell wall homogalacturonans by endopolygalacturonases, are biologically active carbohydrates. These carbohydrates are utilized by the pathogen as a carbon source and also function as signal molecules in the activation of defence responses (Ridley *et al.*, 2001; Cervone *et al.*, 1989).

The majority of elicitors of plant defence responses that have been characterised are derived from invading microorganisms (Nothnagel *et al.,* 1983). Lee and West (1981b) identified a glycoprotein polygalacturonase from *R. stolonifer* as an elicitor of casbene synthetase activity in castor beans. The elicitor activity was subsequently shown to be dependent on the catalytic activity of the polygalacturonase enzyme. Several lines of evidence support the idea that pectic fragments released from castor bean cell walls act as obligate intermediates in the elicitation process (Jin and West, 1984).

Hahn *et al.* (1981) described the release of endogenous elicitors from cell walls of soybeans by partial acid hydrolysis. The results of their study showed that the endogenous elicitor was a polysaccharide fragment as it contained the essential constituent, galacturonic acid. Studies to confirm and further characterize this endogenous elicitor, were later undertaken by Nothnagel *et al.* (1983). Consistent with results obtained by Hahn *et al.* (1981), treatment of purified pectin elicitors with highly purified endo-a-1,4-polygalacturonase, completely destroyed the elicitor activity.

Structural characterisation of elicitors has shown that the most active elicitor is a dodeca-a-1,4-D-galacturonide, which is a homo-oligomer of galacturonic acid (Jin and West, 1984). Linear a-(1? 4)-linked oligogalacturonides with a degree of polymerisation between 10 and 13, have thus been shown to be the most active elicitors of plant defence responses. Oligogalacturonides with shorter than 10 glycosyl residues have either little or no elicitor activity (Hahn *et al.,* 1989; De Lorenzo and Cervone, 1997).

Cervone *et al.* (1989) considered whether the degradation of elicitor-active oligogalacturonides into smaller inactive oligomers, could be manipulated such that the accumulation of active oligomers is prolonged. They investigated the effect of bean (*Phaseolus vulgaris*) PGIP on the digestion of polygalacturonic acid by either *Aspergillus niger* or *F. moniliforme* endopolygalacturonases. In the presence of PGIP, the half-life of elicitor-active oligogalacturonides is increased as opposed to when the inhibitory protein is absent (Cervone *et al.,* 1989). In the absence of the protein, endopolygalacturonases rapidly depolymerise the active oligogalacturonides

into shorter, inactive molecules. Bergmann *et al.* (1994) later showed that PGIPs are induced in suspension-cultured bean cells when elicitor-active oligogalacturonides or fungal glucan are added to the medium.

According to De Lorenzo and Cervone (1997), endopolygalacturonases and PGIPs form very specific interactions that lead to the accumulation of high concentrations of oligogalacturonides, ultimately activating plant defence responses. It has thus been hypothesised that PGIPs have a general role in reducing the activities of fungal PGs such that active oligogalacturonides are present for longer periods of time at the infection sites thus enhancing the signalling properties of plants (Ryan and Farmer, 1991).

Polygalacturonase-Inhibiting Proteins (PGIPs)

Polygalacturonase-Inhibiting Proteins (PGIPs), have been defined by their ability to inhibit fungal polygalacturonases (PGs) (De Lorenzo and Cervone, 1997). PGIP activity was found to occur in both infected (Abu-Goukh and Labavitch, 1983) and uninfected plant tissues (Albersheim and Anderson, 1971; Abu-Goukh *et al.*, 1983a) suggesting that they form part of the plant's pre-existing defence mechanism. They were initially described by Weurman (1953), but Albersheim and Anderson (1971) were the first to report the presence of a protein in *P. vulgaris* cell walls that could inhibit endoPGs of *C. lindemuthianum* (Lafitte *et al.*, 1984; Esquerré-Tugaýe *et al.*, 2000; Boudart and Dumas, 2000). PGIPs have since been shown to be associated with the cell walls of several dicotyledonous plants (De Lorenzo and Cervone, 1997). They have subsequently been purified from several plant species (Table 1).

Table 1.1 Summary of PGIPs purified from several plant species.

	Plant Species	Reference
Pear	*Pyrus communis* L. cv. Bartlett	Abu-Goukh *et al.* (1983a); Stotz *et al.* (1993)
Bean	*Phaseolus vulgaris* L. cv. Cannellino	Albersheim and Anderson (1971); Cervone *et al.* (1987); Salvi *et al.* (1990)
Soybean	*Glycine max* L. Merr.cv.Canton	Favaron *et al.* (1994)
Raspberry	*Rubus idaeus* L. cv. Autumn Bliss	Johnston *et al.* (1993)
Apple	*Malus domestica* cv. Golden Delicious	Yao *et al.* (1995)
Tomato	*Lycopersicon esculentum* cv. Sunny	Stotz *et al.* (1994)
Onion*	*Allium cepa* L.	Favaron *et al.* (1993)
Leek*	*Allium porrum* L.	Favaron *et al.* (1993) (1997)
Potato	*Solanum tuberosum* L. cv. Spunta	Machiandiarena *et al.* (2001)
Cotton	*Gossypium hirsutum* L.	James and Dubery (2001)
Guava	*Psidium guajava* Linn	Deo and Shastri (2003)

* monocotyledonous plant species

Tissue-specific Expression of PGIP genes

The accumulation of pgip mRNA is spatially and temporally regulated during development and in response to several stress stimuli (Devoto *et al.*, 1997; De Lorenzo *et al.*, 2001). Bioassays of PGIP activity from various *P. vulgaris* tissues indicated that the protein is ubiquitously expressed throughout the plant (Salvi *et al.*, 1990). The lowest level of PGIP expression was observed in the roots, while the vegetative apex displayed the highest activity. PGIP levels also increased in the stem tissue during growth but no appreciable difference could be observed at the different developmental stages of the leaves (Salvi *et al.*, 1990; Devoto *et al.*, 1997; Jones and Jones, 1997).

Both mature and immature flowers of *P. vulgaris* displayed high levels of PGIP activity (Salvi *et al.*, 1990). Northern blot analysis revealed that the stamens and the style displayed the highest *pgip* transcript levels (Salvi *et al.*, 1990; Devoto *et al.*,

1997). Biochemical analysis of the purified proteins from both flowers and the hypocotyls showed a molecular mass difference between the two proteins. This suggested that the different bean PGIPs are regulated in a tissue-specific manner (Salvi *et al.*, 1990).

Extraction and bioassay of PGIP activity from various soybean (*Glycine max*) tissues indicate that the protein is expressed at low levels in the roots and hypocotyls, with higher expression occurring in the leaves and the cotyledons (Favaron *et al.*, 1994; Jones and Jones 1997). In fruits such as pear, apple, raspberry and grape, *pgip* mRNA levels decrease as the fruits mature (Stotz *et al.*, 1993; Johnston *et al.*, 1993; Yao *et al.*, 1999; De Lorenzo *et al.*, 2001). Northern blot analysis of pear (*Pyrus communis*) *pgip* mRNA indicated that the transcript level in fruits was approximately 200-fold higher than in flowers and 1400-fold higher than in leaves (Stotz *et al.*, 1993). In contrast, no PGIP activity could be detected in raspberry (*Rubus idaeus*) flowers and in extracts from tomato (*Lycopersicon esculentum*) leaves (Johnston *et al.*, 1993; Stotz *et al.*, 1994).

Several stress stimuli have been reported to induce PGIP activity (De Lorenzo *et al.*, 2001). It has been observed that the accumulation of *pgip* transcripts could be induced in bean suspension-cultured cells by adding elicitor-active oligogalacturonides and fungal glucan to the medium (Bergmann *et al.*, 1994). In hypocotyls, the accumulation of *pgip* transcripts occurred in response to wounding or salicylic acid treatment (Bergmann *et al.*, 1994). Immunoblotting analysis of potato (*Solanum tuberosum*) PGIP revealed that the protein is induced by wounding, salicylic acid or by infecting the leaves with *Phytophtora infestans* (Machiandiarena *et al.*, 2001). The induction of PGIP by salicylic acid, which is a systemic stress signal, strengthens the hypothesis that PGIPs play a role in the resistance of plants to fungal attack (Machiandiarena *et al.*, 2001).

Northern blot analysis was used to study the temporal location of *pgip* mRNA in two near-isogenic lines of *P. vulgaris*. The two lines, one resistant and one susceptible to infection by *C. lindemuthianum,* was infected with race ? of *C. lindemuthianum*. Rapid accumulation of mRNA correlated with the appearance of the hypersensitive response in incompatible interactions, while a more delayed increase in *pgip* mRNA

in compatible interactions, was coincident with the formation of lesions (Nuss *et al.*, 1996; De Lorenzo *et al.*, 2001). By means of in-situ hybridization, Devoto *et al.* (1997) investigated the spatial distribution of *pgip* transcripts in infected hypocotyls and leaves. They found that the accumulation of *pgip* mRNA is mainly localised at the site of infection in the incompatible interactions.

Biochemical And Molecular Characterisation of PGIPs

Biochemical characterisation of several plant PGIPs have revealed the occurrence of two forms of protein namely, wall-bound PGIPs and soluble PGIPs (Lafitte *et al.*, 1984). Most PGIPs that have been characterised are relatively heat stable glycoproteins that are ionically bound to the plant cell wall (Salvi *et al.*, 1990; Johnston *et al.*, 1994; Jones and Jones, 1997). Several observations have suggested that almost all PGIP activity is released from cell debris following extraction with a high salt buffer (Machiandiarena *et al.*, 2001). However, soluble extracytoplasmic PGIP forms have also been described. In *P. vulgaris* both soluble and cell wall bound forms of the inhibitor are present, but they were indistinguishable from each other based on their elution profiles during ion-exchange chromatography or by gel filtration. They thus appeared to be the same protein (Lafitte *et al.*, 1984). This is in contrast to the results obtained for *Pisum sativum* (pea) inhibitors, which appeared to be different proteins (Hoffman and Turner; 1984 Lafitte *et al.*, 1984).

Preparative iso-electric focusing (IEF) showed that PGIPs from several plants have basic pI values, with the exception of apple (*Malus domestica*) PGIP, which has a pI value of 4.6, pear PGIP, which has isoforms with pIs of 4.5, 6.6 and 7.7, and guava (*Psidium guajava*) PGIP, with pI values ranging between 4.2 and 4.4 (Stotz *et al.*, 1993; Yao *et al.*, 1995; Glinka and Protsenko, 1998; Deo and Shastri, 2003). PGIPs characterised from bean and raspberry have very high pI values thus facilitating the binding of these positively charged proteins to the negatively charged pectin backbone of the plant cell walls (Toubart *et al.*, 1992; Johnston *et al.*, 1993).

Table 1.2 Summary of biochemical characteristics of some PGIP proteins as determined by SDS-PAGE and Isoelectric focusing analysis.

Plant Species	kDa	pI Value	Reference
Glycine max	37-40	9-10	Favaron *et al.* (1994)
Pyrus communis	34	4.6	Abu-Goukh *et al.* (1983); Stotz *et al.* (1993)
		6.6	
		7.7	
Malus domestica	34	4.6	Yao *et al.* (1995)
Lycopersicon esculentum	34	9	Stotz *et al.* (1994)
Actinidia deliciosa	34	8.2	Simpson *et al.* (1995)
Solanum tuberosum	41	ND	Machiandiarena *et al.* (2001)
Psidium guajava	29.5	4.2-4.4	Deo and Shastri, (2003)
	32.5		
	35		

Table 1.3 Summary of biochemical characteristics of some PGIP proteins as predicated from the gene sequences.

Plant Species	kDa	pI Value	Reference
Phaseolus vulgaris	34	8.81	Toubart *et al.* (1992); Leckie *et al.* (1999)
Pyrus communis	36.5	6.2	Stotz *et al.* (1993)
Glycine max	34	8.21	Favaron *et al.* (1994)
Rubus ideaus	38.5[a]	+10.0	Johnston *et al.* (1993); Ramanathan *et al.* (1997)

Molecular mass determination by either gel filtration or SDS-PAGE provided molecular mass estimations for PGIPs ranging from 37 to 54 kDa (Glinka and Protsenko, 1998). Potato PGIP, which exists as a monomer, has a molecular mass of 41 kDa. This value is consistent with molecular mass estimations of bean and raspberry, but shows little similarity to pear, which consists of two isoforms of 91 kDa each (Abu-Goukh *et al.*, 1983a). Leek (*Allium porrum*) contains three PGIP types, with molecular weights ranging from 39kDa to 42kDa (Favaron *et al.*, 1997). The three guava PGIPs recently reported seem to show comparatively lower molecular weights as compared to those previously characterised (Deo and Shastri,

2003). Molecular mass differences between PGIP proteins may be due to different levels of glycosylation (Glinka and Protsenko, 1998).

Isolation and characterisation of the *pgip* gene from bean

Trypsin digestion of purified bean PGIP enabled the identification of the N-terminal amino acid sequence and four internal peptide sequences of the protein. Based on the amino acid sequence of *P. vulgaris* cv. Pinto PGIP three degenerate oligonucleotides designated as N-A, N-B and Int were synthesised. Amplification with primers N-A and Int lead to the production of a 788bp fragment designated as PGIP-1, whereas primers N-B and Int gave a PGIP-2 fragment of 758bp. Southern analysis using labelled PGIP-2 as a probe showed that both PGIP-1 and PGIP-2 hybridised with the same intensitiy. The 758bp PGIP-2 fragment was thus cloned, sequenced and subsequently used as a hybridization probe in the isolation of the full-length PGIP gene from a commercial genomic library of *P. vulgaris* cv. Saxa. The isolated genomic clone consisted of a single, continuous open reading frame of 1026 nucleotides (342 amino acids) with four potential glycosylation sites and two in-frame ATGs (Toubart *et al.*, 1992).

A cDNA library of *P. vulgaris* cv. Pinto was screened using the genomic *pgip* clone isolated from *P. vulgaris* cv. Saxa (Leckie *et al.*, 1999). Seventeen clones were selected and subjected to restriction enzyme digestion and southern blot analysis. The 10 longest inserts were subcloned and sequenced. Sequence analysis revealed that two members (*pgip1* and *pgip2*) of the *pgip* gene family of *P. vulgaris* cv. Pinto was isolated. The *pgip1* gene was identical to the genomic clone of *P. vulgaris* cv. Saxa , which contained the two in-frame ATGs, while *pgip2* started with an ATG corresponding to the second ATG of *pgip1* (Leckie *et al.*, 1999). The mature PGIP had a molecular mass of 34kDa, and the presence of several lysines and arginines confer a high isoelectric point to the protein (Toubart *et al.*, 1992).

Isolation and characterisation of a *pgip* gene from pear

The gene encoding the pear PGIP was isolated using a combination of degenerate PCR and RACE-PCR (Stotz *et al.,* 1993). Degenerate oligonucleotide primers, designed based on partial amino acid sequence of the purified pear PGIP protein, were used to amplify a 569bp product from the pear cDNA. Sequence analysis of the degenerate-oligo primed cDNA, enabled the design of gene-specific primers that were subsequently used in RACE-PCR to amplify the 5' and 3' ends of the PGIP transcript (Stotz *et al.* 1993). A second set of gene-specific primers based on the sequence of the RACE-PCR products, were designed and used to amplify the complete PGIP coding sequence of 1059 bp. The isolated *pgip* gene encodes a protein of 330 amino acids, including a signal sequence of 24 amino acids and seven potential N-Glycosylation sites, of which only two are shared with bean. Amino acid sequence comparisons between the pear PGIP and that of bean, shows only moderate sequence identity of 50%. However, all eight cystein residues of the field bean PGIP are conserved in pear (Stotz *et al.,* 1993).

Isolation and characterisation of a *pgip* gene from soybean

Based on the homology between the field bean and soybean PGIPs, oligonucleotide primers corresponding to the N and C-terminal regions of the field bean PGIP gene were synthesised and used to amplify a fragment of approximately 950bp (Favaron *et al.,* 1994). The PCR product was cloned and subjected to nucleotide sequencing, which revealed that the gene contained a single open reading frame that encodes a polypeptide of 313 amino acids. The resulting amino acid sequence showed 66.8% and 47% homology to that of the previously isolated field bean and pear PGIPs, respectively. Nine cysteine residues are located in the N-terminal and C-terminal regions of the protein, eight of which are conserved in the field bean and pear PGIPs, suggesting that they may be involved in stabilizing the tertiary structure of the protein (Stotz *et al.,* 1993; Favaron *et al.,* 1994). Three potential N-glycosylation sites (Asn-X-Ser/Thr) located in the N-terminal region are conserved in the soybean, field bean and pear PGIPs (Favaron *et al.,* 1994).

Mahalingam *et al.* (1999) went a step further in the study and amplified an expected 950bp *pgip* fragment using oligonucleotide primers designed by Favaron *et al.* (1994). They used genomic DNA and root cDNAs of two soybean genotypes, PI437654 and a commercial cultivar Essex, in which parasitism of the host plant occurs by a soybean cyst nematode (SCN) as templates for amplification. Sequence analysis of the isolated *pgip* genes from both genotypes showed that they were 100% identical to each other at the nucleotide level. When compared to other published PGIP sequences, the PGIP from roots showed 88% amino acid identity to that of the field bean PGIP and only 72% amino acid identity to the soybean PGIP sequence published by Favaron *et al.* (1994). Previous data showed that the PGIP obtained by Favaron *et al.* (1994) was obtained from a single genomic clone that showed only 67% amino acid identity to the field bean PGIP. Based on the results presented by Mahalingam *et al.* (1999), they were able to verify that the PGIP isolated from the root cDNA represented a true copy of the soybean PGIP.

Isolation and characterisation of a *pgip* gene from tomato

Degenerate oligonucleotide primers have been synthesised from the amino acid sequence of purified tomato PGIP A (Stotz *et al.*, 1994). PCR reactions performed on genomic DNA as well as tomato fruit cDNA, yielded identical products of 417 bp, which were subsequently used as probes to screen a tomato genomic library. A full-length *PGIP* gene with an uninterrupted open reading frame of 984 nucleotides was identified. The gene encodes a protein of 328 amino acids and contains a putative signal sequence of 20 amino acids.

The predicted molecular mass (34.3 kDa) as well as the isoelectric point (8.4) of the mature polypeptide, corresponds to the values obtained for tomato PGIP A (Stotz *et al.*, 1994). Comparisons of the deduced tomato PGIP to that of pear and bean, showed that the mature polypeptide is more closely related to pear PGIP (68% identity) than to bean PGIP (50 % identity), and that eight cysteine residues are conserved between these species. Tomato PGIP contains seven potential N-glycosylation sites, two of which are conserved between the tomato, pear and bean

PGIPs, and three additional glycosylation sites that are shared with the pear PGIP (Stotz *et al.*, 1994).

Isolation and characterisation of a *pgip* gene from apple

The apple pgip gene has been isolated and characterised by two research groups. Arendse *et al.* (1999) employed a degenerate and inverse PCR technique while Yao *et al.* (1999) used degenerate PCR to isolate the full length apple *pgip* gene. The N-terminal amino acid sequence of the purified apple inhibitor was determined by protein sequencing (Yao *et al.*, 1995). Comparison of the apple PGIP proteins sequence to GenBank proteins sequences revealed that the apple PGIP shared 96% amino acid identity to the published pear PGIP protein sequence (Yao *et al.*, 1995). Three degenerate oligonucleotide primers based on the homology between pear and apple PGIP amino acid sequences could thus be designed. (Yao *et al.*, 1995; Yao *et al.*, 1999). One primer was designed from the N-terminal apple PGIP sequence (PGIP-N1), while the other two were designed from the C-terminus of pear PGIP (PGIP-C1 and PGIP-C2) (Yao *et al.*, 1999). Amplification of apple genomic DNA with primers PGIP-N1 and PGIP-C1 resulted in a fragment of 896bp, which was used as a probe to screen an apple cDNA library. A full-length *pgip* with a nucleotide sequence of 1162bp was identified. It contained a single open reading frame encoding a predicted protein of 330 amino acids, the first 24 amino acids being the signal peptide sequence.

Degenerate primers were designed to conserved internal regions between the bean (Toubart *et al.*, 1992) and pear (Stotz *et al.*, 1993) PGIP sequences (Arendse *et al.*, 1999). Amplification of apple genomic DNA gave an internal fragment that was 351bp in length. The internal apple PGIP fragment showed 94% identity on the DNA level and 97% similarity on the amino acid level to the corresponding pear *pgip* region (Arendse *et al.*, 1999). Gene-specific primers based on the internal fragment sequence were designed and used to amplify the flanking regions of the *pgip* by means of inverse PCR. Apple genomic DNA digested with *Bgl*II was used as a template in the inverse PCR reaction, and yielded a product of 820bp, which represented the 5' end of he gene. Based on the sequence similarity to the internal

PGIP fragment, the two sequences were joined to give a single open reading frame encoding 274 amino acids. A second set of inverse PCR primers were designed to amplify the 3' end of the gene. The complete sequence of the composite apple *pgip* gene was identical to the full-length *pgip* gene from Golden Delicious apples (Arendse *et al.*, 1999; Yao *et al.*, 1999).

The predicted molecular mass of the mature peptide is 34 kDa, which is in accordance with the estimated value for the native protein. However, the isoelectric point is 7, while that of the native protein was measured to be within the pH range of 3.0-5.9. This difference in pI values was attributed to the different levels of glycosylation of the seven potential N-glycosylation sites on the same protein (Yao *et al.*, 1999). When compared to several other PGIP sequences, apple PGIP showed 98, 83,82, 81, 67 and 63% identities at the amino acid sequence level with those from pear, orange, kiwifruit, tomato, bean and soybean, respectively (Yao *et al.*, 1999).

Isolation and characterisation of a *pgip* gene from raspberry

Primers designed to the conserved regions of bean (Toubart *et al.*, 1992) and pear (Stotz *et al.*, 1993), were used to amplify an expected 806bp *pgip* gene from raspberry genomic DNA. An unexpected PCR product of 1050bp was obtained instead. Sequence analysis of the PCR product showed a high degree of sequence identity to pear PGIP at both ends of the insert. However, the 990bp insert also contained an internal sequence region that showed no similarity to previously isolated *pgip* genes. Raspberry-specific primers were used to amplify a 260bp probe that was subsequently labelled and used to screen a cDNA library prepared from immature raspberry fruit RNA (Ramanathan *et al.*, 1997). Two of nine positive clones were sequenced, and a full-length *pgip* cDNA of 1325bp was identified (Ramanathan *et al.*, 1997). The cDNA clone was identical to the genomic clone, with the exception of the 293bp internal intron present in the genomic clone.

The raspberry *pgip* cDNA contains an open reading frame that is predicted to encode a 331 amino acid protein. The polypeptide contains a 22 amino acid N-terminal signal sequence, and eight conserved cysteine residues (Ramanathan *et al.*, 1997).

The raspberry PGIP contains four potential N-glycosylation sites and the deduced amino acid sequence shows a high degree of similarity to the predicted amino acid sequences from pear, tomato and kiwifruit (Ramanathan *et al.,* 1997).

Isolation and characterisation of a partial *pgip* gene from *Eucalyptus spp.*

Degenerate oligonucleotide primers, one designed from the pear PGIP sequence (Stotz *et al.,* 1993) and the other designed by inspection of the C-termini of aligned PGIP polypeptides (Chimwamurombe *et al.*,2001), were used to amplify a 909bp fragment from genomic DNA of *E. grandis*. The partial *Eucalyptus pgip* gene isolated encodes a protein of 298 amino acid residues that is uninterrupted by intron sequences. The partial *pgip* gene shows relatively high sequence similarity to apple (95%), pear (94%), kiwifruit (69%) and tomato (65%) *pgip* genes (Chimwamurombe *et al.,* 2001). The *E. grandis* PGIP polypeptide contains seven potential N-glycosylation sites that are comparable to those found in pear PGIP (Stotz *et al.,* 1993). In contrast to other reported PGIPs, however, the partial mature peptide of *E. grandis* contains only seven cysteine residues located in the N- and C-terminal regions of the polypeptide (Chimwamurombe *et al.,* 2001). Analysis of the amino acid composition of several cloned PGIPs, indicates the presence of a high content of leucine residues (De Lorenzo and Cervone, 1997; Glinka and Protsenko, 1998). *Eucalyptus* PGIPs also fall into the category of the leucine-rich repeat class of proteins (Chimwamurome *et al.,* 2001). A modified 24 amino acid leucine-rich repeat motif is conserved in all *pgip* genes sequenced thus far (De Lorenzo *et al.,* 1994). In plants, LRR proteins are responsible for many protein-protein interactions that are relevant in both plant development and defence (De Lorenzo and Cervone, 1997; Leckie *et al.,* 1999).

Leucine-Rich Repeat Proteins (LRRs)

Leucine-Rich Repeats (LRRs) were initially discovered in the leucine-rich human serum protein, a2-glycoprotein (Kobe and Deisenhofer, 1993). More than a hundred different LRR proteins with differing functions and cellular locations have now been

described (Kobe and Deisenhofer, 1994; Leckie *et al.,* 1999). LRRs are usually present in tandem, and are distinguished by a consensus sequence consisting predominantly of leucine residues. They are commonly 24 residues in length, but LRRs between 20 and 30 residues have been identified (Kobe and Deisenhofer, 1994). LRRs belong to a superfamily of proteins that appear to be involved in protein-protein interactions, and to some extent, some may even be involved in signal transduction pathways (Kobe and Deisenhofer, 1994; Jones and Jones, 1997).

The existence of LRRs in animals and fungi has been known for some time, but LRR proteins have only recently been described in plants (Jones and Jones, 1997). The first plant LRR proteins identified were the Polygalacturonase-Inhibiting Proteins (PGIPs) and receptor kinases (Jones and Jones, 1997). Many plant *R*-genes have LRR domains (Dixon *et al.,* 1998; Marino *et al.,* 2000). Proteins containing LRRs differ greatly in structure and function and many are part of the cell wall receptors that respond to elicitors (Ramanathan *et al.,* 1997; Marino *et al.,* 2000). Plant *R*-genes confer gene-for-gene resistance to a wide array of natural enemies (Stahl and Bishop, 2000). The *R*-gene products thus serve as receptors for pathogen-encoded avirulence (avr) proteins and they lead to the activation of downstream defence response pathways (Leckie *et al.,* 1999). The first disease resistance (*R*) gene cloned was a *Pto* gene, which confers resistance in tomato to a bacterial spot disease caused by *Pseudomonas syringae* (Dixon *et al.,* 1998). Several other genes, including the tomato *Cf9* gene, which confers resistance to races of *Cladosporium fulvum;* and the rice gene *Xa21*, which confers resistance to the bacterium *Xanthomonas oryzae* pv. *oryzae* have since been cloned (Jones and Jones, 1997; Hammond-Kosack *et al.,* 1998).

Kobe and Deisonhofer (1993) determined the three-dimensional structure of porcine ribonuclease-inhibitor protein (PRI) as a model for other LRR proteins. They were able to show that the LRR of the RI molecule consists of a-helixes that are approximately parallel to short ß-sheets. This gives rise to a non-globular, horseshoe shaped molecule. The binding of PRI to the ribonuclease involved multiple amino acids, which were probably located on the exposed surface of the parallel ß-sheet. It was thus concluded that like the PRIs, leucine-rich repeats of PGIPs could also form three-dimensional structures capable of inhibiting fungal polygalacturonases by

extensive binding to exposed amino acids (Kobe and Deisonhofer, 1993; Yao *et al.*, 1999).

Each of the PGIPs that have been isolated consist primarily of 10 imperfect LRR, with an average of 24 amino acids. The consensus sequence, LxxLxxLxxLxLxxNxLxGxIPxx, remains conserved in all PGIPs currently sequenced and it also shows significant homology to several disease resistance (*R*) genes (Leckie *et al.*, 1999; Jones and Jones, 1997).

PGIPs are the only LRR's for which a ligand has been demonstrated. Models for determining the specificity with which they are able to differentially inhibit PGs from different fungal sources have thus been proposed (Stotz *et al.*, 2000; Leckie *et al.*, 1999). Leckie *et al.* (1999) hypothesised that sequence variations with the LRR domains could influence recognition specificity. The interaction of plant PGIPs and fungal PGs at the molecular level was thus investigated.

Like most *R*-genes, PGIPs consist of multigene families with different inhibitory specificities. In *P. vulgaris* cv. Pinto, two members of the *pgip* gene family have been isolated and shown to have only eight amino acid differences in their mature peptides (Leckie *et al.*, 1999). Using site-directed mutagenesis and surface plasmon resonance (SPR), Leckie *et al.* (1999), showed that single amino acid mutations of residue Q253 within the LRR domain of the *pgip-2* gene, into corresponding residues of the *pgip-1* gene, resulted in the loss of affinity for *F. moniliforme* PG. Q253 therefore, plays an essential role in the binding capacity of the complex. To test this hypothesis, residue K253 of the *pgip-1* gene was mutated to a glutamine, resulting in the protein acquiring new recognition specificities (Leckie *et al.*, 1999). Their results clearly proved that PGIP specificity and affinity for fungal PGs are determined by amino acids residing within the solvent-exposed ß-sheet/ß-turn structure. Non-synonymous variations within the LRR domains explained the ability of PGIPs from different plant sources to recognize specific ligands. This, therefore, gives a plausable explanation for the observed differential inhibition of PGIP to fungal PGs.

The Role of PGIPs in Disease Resistance

PGIP proteins purified from a vast array of plant species show differential inhibition towards pathogenic fungi (Leckie *et al.,* 1999). Several plant PGIPs are encoded by a family of genes and it is possible that each member of the PGIP family encodes a protein that differs from other members in terms of regulation and specificity for fungal PGs (Desiderio *et al.,* 1997; De Lorenzo *et al.,* 2001). The inhibition patterns from various plant sources were thus established when tested against several fungal PGs.

Abu-Goukh and Labavitch (1983) demonstrated that purified pear PG inhibitors are effective against PGs from *Apergillus niger, Botrytis cinerea* and *Dothiorella gregaria*, slightly inhibitory to PGs from *Penicillim expansum,* but that it does not affect PG activity from *Fusarium oxysporum.* Tomato PGIPs were reported to completely inhibit PG activity from *Glomerella cingulata*, but they were incapable of inhibiting *Botrytis cinerea* PGs (Brown and Adikaram, 1983). Contradictory to these results, Stotz *et al.* (1994) could identify two isoforms of the tomato PGIPs that were able to inhibit PGs from *B. cinerea,* even though their inhibition was less effective than that of the pear PGIP genes.

Purified bean PGIP was shown to have inhibitory activity against PGs produced by *C. lindemuthianum* (Lafitte *et al.,* 1983). Cervone *et al.* (1989) later showed that purified bean PGIP slows down the degradation of sodium polypectate by purified *A. niger* or *F. moniliforme* PGs, leading to the production of longer oligogalacturonides that were able to induce plant defence responses. They thus hypothesised that in addition to preventing degradation of plant cell walls by fungal PGs, PGIPs also have a role in cell signalling.

Experiments were conducted by Cook *et al.* (1999) to determine whether PGs from various fungal sources, could be inhibited by PGIPs isolated from bean, tomato and pear, which were previously reported to differ in their ability to inhibit PGs. Their results indicated that PGIPs from bean have a broad inhibition range, whereas those isolated from tomato and pear, show differential inhibition to fungal PGs. These results were confirmed by experiments conducted by Stotz *et al.* (2000), who directly

compared the effectiveness of PGIPs among these different plant species. *B. cinerea* PGs were inhibited with similar efficiency by bean, tomato and pear PGIPs, but only tomato and bean PGIPs could inhibit *A. niger* PGs. *F. moniliforme* PG in contrast was inhibited only by bean PGIPs. Similarly, the recently isolated potato PGIP also has a broad spectrum of interactions and has been shown to inhibit PGs from *A. niger, F. moniliforme* and *F. solani.* (Machinandiarena *et al.,* 2001).

PGIPs from a single plant source have also been reported to show differential inhibition towards PGs from different fungi (De Lorenzo and Cervone, 1997). At least five PGIP genes are present in *P. vulgaris*, and the two that have been characterized, differ in their abilities to inhibit PGs from *F. moniliforme* and from *Aspergillus niger* (Stotz *et al.,* 2000). Contrary to the results obtained by Cook *et al.* (1999), Di Pietro and Roncero (1996) were able to show that the tomato PGIP effectively inhibited PGs from *F. moniliforme.* Therefore, in addition to plants having multiple PGIP genes, most plant pathogens also produce multiple forms of PGs (Sharrock and Labavitch, 1994).

As single plant species may contain multiple forms of PGIP, each with nearly identical biochemical properties. It is therefore, necessary to express cloned genes in heterologous systems in order to overcome the problem of purifying single PGIPs from the bulk PGIP (De Lorenzo *et al.,* 2001). Although several expression systems such as yeast and other fungi have been used, they proved to be inadequate. Plants, however, have been shown to successfully express functional PGIPs via plant transformation or transient expression using a PVX vector (De Lorenzo *et al.,* 2001).

In an attempt to demonstrate the influence of inhibitor activity in disease development, Desiderio *et al.* (1997), expressed high levels of bean PGIP-1 in transgenic tomato plants. PGIP-1 from *P. vulgaris* showed limited ability to inhibit PGs from *F. oxysporum* f.sp *lycopersici, B. cinerea* and *Alternaria solani.* In comparison to untransformed plants, transgenic tomato plants over expressing the PGIP-1 gene did not exhibit enhanced resistance to these pathogenic fungi. This further strengthened the idea that multiple PGIP genes exist in *P. vulgaris,* each with its own PG specificity.

Powell *et al.* (2000) hypothesised that any alteration of internal plant PGIPs should reduce plant tissue maceration and necrosis by fungal endopolygalacturonases. A heterologous pear PGIP was thus expressed in tomato plants in order to evaluate whether resistance to *B. cinerea* could be increased. Their results showed that transgenic plants expressing the pear PGIP had attenuated disease symptoms in comparison to the control plants (Powell *et al.*, 2000). They further showed that over expression of PGIPs does not necessarily prevent the initial plant-pathogen interaction, but it controls subsequent development of the fungus in the plant tissues. It was thus concluded that the use of heterologous defence factors in certain host plants might effectively reduce its susceptibility to a pathogen.

The fungal pathogen of maize, *Stenocarpella maydis* causes epidemics of both ear and stalk rot in certain regions of South Africa. It has previously been reported that *F. moniliforme,* another pathogen of maize, could be inhibited by bean PGIP-2 (Leckie *et al.*, 1999). Berger *et al.* (2000) thus investigated whether the PGs produced by *S. maydis* could also be inhibited by bean PGIP. Recombinant DNA technology was used to clone the PGIP-1 gene into an *Agrobacterium*-based binary vector. Tomato cotyledons were transformed by means of *Agrobacterium*-mediated transformation and transgenic plants selected on kanamycin, were subsequently hardened off in the greenhouse. Reducing sugar assays verified that the transgenic tomato tissue extracts that do not exhibit high levels of endogenous PGIPs, expressed the bean PGIP-1 gene and biochemical assays showed that PGs from both *A. niger* as well as *S. maydis* were inhibited. However, pathogenicity tests were not conducted, as tomato is not a host for the maize pathogen *S. maydis* (Berger *et al.*, 2000).

Conclusion

This review attempted to highlight the importance of polygalacturonase-inhibiting proteins in plant defence responses. Several lines of evidence show that PGIPs are cell wall associated proteins that function to limit fungal invasion. They prevent the degradation of the pectin-rich plant cell walls by fungal polygalacturonases, by forming a stable complex with the PG thus blocking its enzymatic activity and allowing the release of oligogalacturonides that act as elicitors of defence responses.

PGIPs from various plant sources have been purified to homogeneity and several genes have also been cloned, sequenced and biochemically characterized. They exhibit characteristics similar to several reported pathogenesis related proteins and have LRR motifs that are typical of several disease resistance (*R*) genes. Multiple PGIP genes have been reported in some plant species, each having different inhibition specificities to various fungal polygalacturonases.

Several plant PGIPs have thus been cloned and expressed in heterologous plant species in order to establish the role of these proteins in resistance. The aims of the studies presented in this thesis were, therefore, to clone and characterise the full-length *pgip* gene from *E. grandis* (Chapter 2) and subsequently to transform *Nicotiana tabacum* with the *Eucalyptus pgip* gene (Chapter 3). Transgenic tobacco plants expressing the *Eucalyptus grandis pgip* gene were assessed to determine whether they could inhibit PGs produced by the *Eucalyptus* pathogen *Coniothyrium zuluense* (Chapter 3).

Chapter Two

Cloning and Characterisation of the *Eucalyptus grandis pgip* gene

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Abstract

Polygalacturonase-inhibiting Proteins (PGIPs), associated with the cell wall of many plant species form part of the plant's active defence against phytopathogenic fungi. PGIPs are leucine-rich repeat proteins that are evolutionarily related to several plant resistance (*R*) genes, involved in gene-for-gene reactions. The objective of this study was to clone and characterize the complete *Eucalyptus pgip* gene. Based on the alignments between the pear *pgip* and partial *Eucalyptus pgip* isolated in a previous study, gene-specific primers were designed and used to amplify a *pgip* fragment of 1020bp from *Eucalyptus grandis* genomic DNA. Cloning and sequence analysis of the fragment revealed that it did not contain sequences upstream of the start codon or downstream of the 3' termination codon. Genome-walking PCR was, therefore, applied to amplify the unknown regions of the *pgip* gene. Two positive clones designated as pCR-*Sca*IGW and pCR-*Dra*IGW were sequenced and the resulting sequences represented the unknown 5' and 3' regions respectively. The complete *Eucalyptus pgip* gene contains a single open reading frame, which encodes a protein of 331 amino acids and contains domains that are typical of PGIPs.

Introduction

Polygalacturonase-Inhibiting proteins (PGIPs) are extracellular glycoproteins that are ionically bound to plant cell walls and are capable of inhibiting fungal, but not bacterial, endopolygalacturonases (PGs) (De Lorenzo and Ferrari, 2002). PGIPs form specific, reversible, saturable, high-affinity complexes with fungal endopolygalacturonases, thereby activating plant defense responses (Cervone *et al.*, 1989). They have been shown to be structurally related to several plant disease resistance genes, and they belong to a super-family of leucine-rich repeat proteins that are specialized for the recognition of non-self molecules and the rejection of pathogens (Jones and Jones, 1997).

PGIPs have been purified to homogeneity from several dicotyledonous plants, including bean, soybean, tomato, pear, apple, raspberry, potato, and very recently, cotton and guava (Salvi *et al.*, 1990; Abu-Goukh *et al.*, 1993; Johnston *et al.*, 1993; Stotz *et al.*, 1993, 1994; Favaron *et al.*, 1994; Yao *et al.*, 1995; Machiandiarena *et al.*, 2001; James and Dubery, 2001; Deo and Shastri, 2003). They have also been identified in the pectin-rich monocotyledonous plants such as, onion and leek (Favaron *et al.*, 1993, 1997).

The first gene encoding a PGIP was cloned from *Phaseolus vulgaris* (Toubart *et al.*, 1992). *Pgip* genes have since been cloned from many other plant species, with the implementation of several PCR techniques. Most *pgip* genes that have been characterized contain a single open reading frame, approximately 1kb in length that is not interrupted by introns (Toubart *et al.*, 1992; Stotz *et al.*, 1993; Chimwamurombe *et al.*, 2001). However, the raspberry (*Rubus idaeus*) PGIP, contains an internal sequence region that showed no similarity to previously isolated PGIP clones (Ramanathan *et al.*, 1997). A highly hydrophobic region corresponds to the N-terminal signal peptide, which targets PGIPs to the endomembrane system for export to the extracellular space (Toubart *et al.*, 1992; Stotz *et al.*, 1993; Powell *et al.*, 1994; Ramanathan *et al.*, 1997). The positions of eight cysteine residues are conserved in all *pgip* genes (Toubart *et al.*, 1992; Stotz *et al.*, 1993, 1994; Yao *et al.*, 1995).

Several PCR techniques have been employed to isolate *pgip* genes from plants. PCR is a powerful tool developed and used to amplify microgram amounts of a specific, targeted gene (Parker *et al.,* 1991). Typical PCR reactions utilize oligonucleotide primers that hybridize to opposite strands, so that extension proceeds inwards between the two primers (Ochman *et al.,* 1988). A limitation to the technology is the inability to amplify regions that lie outside the boundaries of known sequences. In this case, several major molecular modifications are required, which include cloning and ligation of adaptors to the ends of amplified sequences (Parker *et al.,* 1991). Several methods have been developed for 'walking' from a known DNA region to an unknown region in both cloned and uncloned DNA. These include inverse PCR, randomly primed PCR, adaptor-ligation PCR and genome walking PCR (Siebert *et al.,* 1995).

Siebert *et al.* (1995) have developed an improved gene walking technique for walking in uncloned genomic DNA regions (Figure 2.1). The method is based on the ligation of a special adaptor to genomic DNA, which has been digested with restriction enzymes that generate blunt-ends. The sequences of the genome-walking adaptor and adaptor primers are shown in Table 1. The blunt-end of the adaptor ligates to any blunt-end of the genomic DNA. The desired genomic region is amplified with an adaptor primer, which is specific to the outer part of the adaptor and a second gene-specific primer. The adaptor primers are much shorter than the adaptors, thus allowing the amplification of only the desired fragments. PCR products that contain double-stranded adaptor sequences at both ends, form panhandle structures (PS-effect) that prevent the exponential amplification of the desired product.

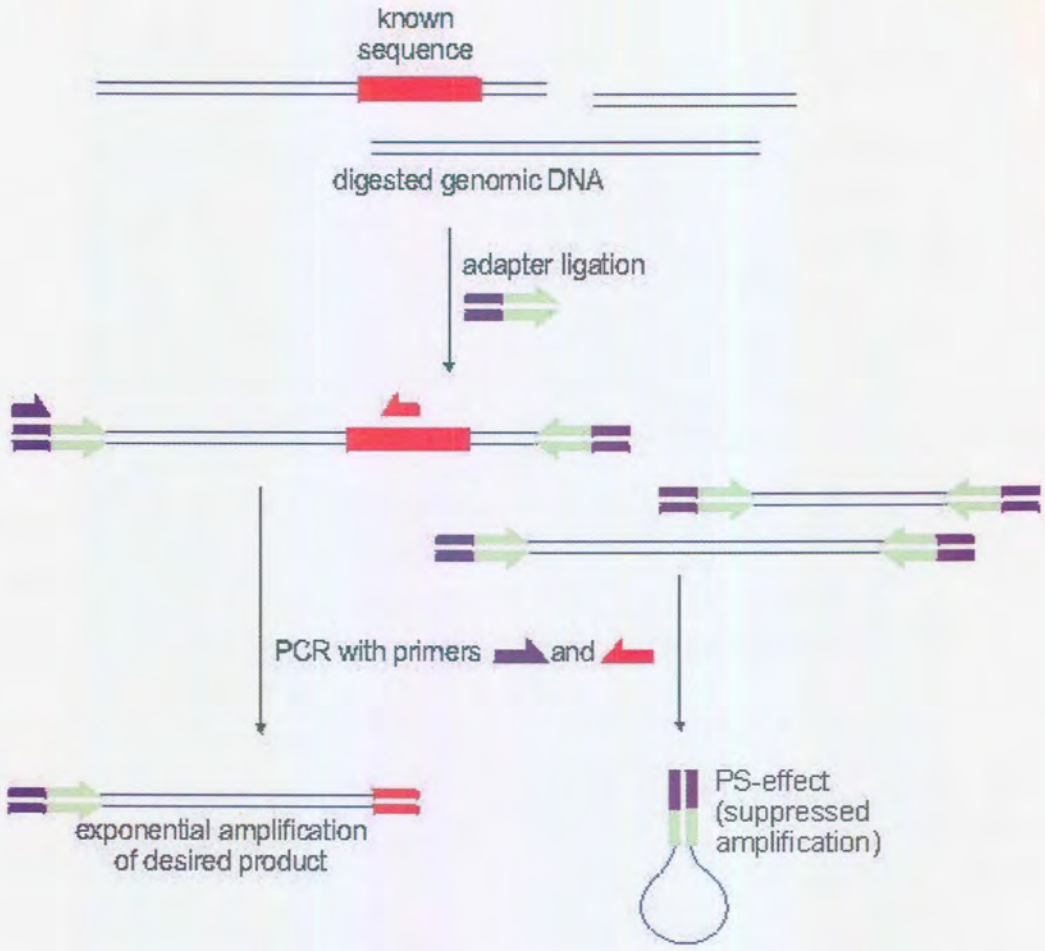

Figure 2.1 Schematic representation of the amplification of unknown genomic DNA segments by means of Genome Walking adapted from www.euregene.com.

In this chapter, a study focused on cloning and characterisation of a *Eucalyptus pgip* gene is presented. In a previous study by Chimwamurombe *et al.* (2001) a partial *Eucalyptus pgip* gene was isolated. Sequence analysis revealed the absence of the putative signal peptide and the downstream termination codon. The isolated PGIP also showed a high degree of amino acid identity to the pear *pgip* gene. Using the genome-walking technology, I chose to 'walk' upstream as well as downstream of the known PGIP sequence, in order to determine the signal peptide sequence and thus the complete *Eucalyptus* PGIP sequence. In addition, I was interested to know whether the same degree of amino acid identity is maintained between the complete *Eucalyptus* and the pear *pgip* gene sequences.

Materials and Methods

Genomic DNA Extraction

One hundred milligrams of fresh, young leaves were collected from a *Eucalyptus grandis* TAG5 clone. Genomic DNA was extracted from the leaves using the DNeasy Plant Mini Kit (QIAGEN) as recommended by the manufacturer's protocols. The plant material was ground to a fine powder in liquid nitrogen followed by the addition of a lysis buffer containing RNaseA. The mixture was incubated at 65°C to allow the reaction to proceed. Proteins, polysaccharides and detergents were subsequently salt precipitated. The cell debris were removed by a brief spin through QIAshredder ™ filtration unit. The cleared lysate was transferred to a new tube to which a binding buffer and ethanol were added. The mixture was applied to a DNeasy mini spin column placed in a 2ml collection tube, which was then centrifuged enabling the binding of the DNA to the DNeasy membrane. The DNeasy spin column was placed into a new 2ml collection tube and the membrane was washed twice allowing contaminants such as proteins and polysaccharides to be efficiently removed. Following the wash steps, the DNeasy spin column was placed into a sterile 1,5ml tube and purified DNA was eluted in 100µl of TE buffer.

DNA Amplification

Gene specific primers, PC6 (5'-ACATCTCTCAGGCTCTCAACC-3', Stotz *et al.,* 1993) and SPPGIP2 (5'-GCAGTGTGGAGGGGTGCACCACACAGGCA-3', Chimwamurombe *et al.,* 2001) were designed from the conserved regions of pear and *Eucalyptus* PGIP sequences respectively. Polymerase chain reactions were performed in 0,2ml thin walled tubes in the GeneAmp PCR system 9700 (Applied Biosystems). The following reaction mixture was used: oligonucleotide primer SPPGIP2 (0.2µM), oligonucleotide primer PC6 (0.2µM), 40ng *Eucalyptus* genomic DNA, 10 X PCR buffer, 0.25mM dNTPs, 1.5mM $MgCl_2$, 5 units *Taq* polymerase (Roche Diagnostics, Germany) in 50µl. PCR was performed for 30 cycles (one cycle = 1 min at 94ºC, 2min at 55ºC, 2min at 72ºC). The

reaction had an initial denaturation step of 2min at 94°C and a final elongation step of 7min at 72°C. Control PCR amplification was performed using 1ng of clone P1 plasmid DNA containing a partial *Eucalyptus* PGIP fragment (obtained from P. Chimwamurombe). The PCR products were separated along with a λIII marker and a 100bp DNA ladder (Promega), on a 1% (w/v) agarose/TAE (pH 8.0) gel stained with ethidium bromide (Sambrook *et al.,* 1989) and visualised under an ultraviolet light.

Cloning of the *Eucalyptus* PGIP fragment

Ligation into pGEM-T-Easy

An approximately 1.2kb *Eucalyptus* PGIP PCR fragment was purified from the gel, using the QIAquick Gel Extraction Kit Protocol (Qiagen, Germany). Ligation was performed with a 3:1 insert-to-vector molar ratio. A 10μl ligation reaction was prepared containing 50ng pGEM-T-Easy vector (Promega), 60ng PCR product, 2X Rapid Ligation Buffer and 3U T4 DNA ligase. A positive control using the control insert DNA (Promega) provided was included to assess transformation efficiency. A ligation reaction in which the insert DNA was omitted was included as a background control. The reactions were incubated for 16 hours at 4°C.

Transformation and Plasmid DNA isolation

The ligated plasmids were transformed into competent *E.coli* (JM109) cells (Promega). 25μl of competent cells were added to 2μl of each ligation reaction and the mixture was incubated on ice for 20 min. This was followed by a heat-shock treatment for 45 sec at 42°C. A vol. of 975μl SOC medium containing 50mM glucose and 1M Mg^{2+} was added to each reaction and incubated at 37°C for one hour. Transformants were screened on LB (Luria Bertani) plates containing 100μg/ml ampicillin, 0,5mM isopropyl-β-D-thiogalactopyranoside (IPTG)(Boehringer Mannheim) and 80μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The plates were incubated at 37°C overnight.

Isolation of plasmid DNA was achieved using a modification of the alkaline lysis method of Sambrook *et al.* (1989). Recombinant white colonies were selected and individually inoculated into 5ml LB medium supplemented with 50μg/ml ampicillin. Following an overnight incubation at 37°C, the bacterial cells were collected by centrifugation and re-suspended in 100μl of Solution 1 (Appendix B). Cells were lysed by the addition of 200μl of Solution 2 (Appendix B) and incubation on ice for a period of 5min. The sample was then neutralized by adding 150μl of Solution 3 (Appendix B) and the resulting cell debris were removed by centrifuging the sample for 15 min at 4°C. The clear supernatant solution was precipitated with 2.5 vol.s of cold, absolute ethanol and the DNA was then pelleted at 4°C. Excess salts were washed from the sample with 70% ethanol and the pellet was dried and the plasmid DNA was re-suspended in TE buffer (pH 8). An RNaseA (10mg/ml) digestion was performed for each sample at 37°C for 30 min. The purified plasmid DNA products were separated on a 1% (w/v) agarose gel.

Screening of Positive Transformants

The putative recombinant clones were screened by restriction enzyme digestion. A 20μl reaction was set up containing 200ng (10μl) plasmid DNA, 2μl restriction enzyme Buffer H and 10 U *Eco*RI (Roche). Digestion was allowed to proceed for 3 hours at 37°C followed by separation on a 1% (w/v) agarose gel. The isolated plasmid DNA was used as a template in PCR reactions and a 25μl PCR reaction was set up as described in Chapter 3. Gene specific primes EN-PGIP-FWD and REV-PB-PGIP were designed in this study and used to amplify the pgip fragment from the putative recombinant clones. Thirty PCR cycles were used with one cycle of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min, an initial denaturation step of 94°C for 2 min and a final elongation step of 72°C for 7 min. PCR products were analysed by electrophoresis through a 1% (w/v) agarose gel in 1 X TAE buffer (pH 8.0) (Appendix B).

Nucleotide sequencing of the *pgip* fragment

A positive recombinant clone designated as pGEM-*Eucpgip*110B was selected and sequenced using the BIG Dye terminator cycle sequencing kit with an ABI Prism 3100 sequencer (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA). T7 and SP6 promoter primers were used to sequence the double-stranded DNA insert. The sequences were analysed using the ABI Prism® SeqScape Software Version 1.0 (Applied Biosystems) program and a BLASTX search was done to determine regions of homology to other published PGIP polypeptide sequences (http://www.ncbi.nlm.nih.gov). The partial *Eucalyptus* peptide sequence was aligned to the pear PGIP peptide using the CLUSTAL (EBI) software (http://www2.ebi.uk).

Genome Walking

Restriction Enzyme Digestion

Eighty nanograms of *Eucalyptus grandis* TAG5 genomic DNA was digested at 37°C with 10U/µl blunt end restriction enzymes that included *Hae*III, *Dra*I, *Sca*I and *Sma*I, in a total vol. of 50µl. An aliquot was analysed on a 1% (w/v) agarose gel in 1 x TAE buffer (pH 8.0) to determine whether complete digestion had been achieved. The total digested DNA was precipitated by adding 1/10 th vol. 3M NaOAc (pH 5.3) and 2.5 vol. of 96% EtOH. The samples were incubated on ice for 30 min followed by centrifugation at 15000 rpm in a microcentrifuge for 15 min. The pellets were washed with 70% EtOH and immediately centrifuged for 5 min at 15000 rpm. After air-drying, the pellets were re-suspended in 50µl of 1 x TE buffer.

Adaptor Ligation

The adaptors and adaptor primers used for genome walking in this study were designed as described by Siebert *et al.* (1995). A 5µM genome walking adaptor (Siebert *et al.*, 1995; Table 1) was ligated to 8µl of digested DNA overnight at 16°C with 5 U T4 DNA ligase in

a total vol. of 30µl. The ligation reaction was terminated by incubation of the samples at 70°C for 5 min. The samples were then diluted to a final vol. of 100µl with sterile distilled water.

Table 2.1 Nucleotide sequence of the genome walking adaptor and adaptor primers used in this study.

Primer	Nucleotide Sequence
Adaptor	5'CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT 3'
	3' H_2NCCCGTCCA 5'
ADP1	5'GGATCCTAATACGACTCACTATAGGGC 3'
ADP2	5'AATAGGGCTCGAGCGGC 3'

Genome Walking PCR

Genome walking primer pairs, N-PGIP-L1 and PGIP-Inv-L2 and N-PGIP-R1-1 and PGIP-Inv-R3 were designed in opposite orientation to normal PCR primers using the pGEM-*Eucpgip*110B sequencing data. Characteristics of the adaptor primers used for genome walking are shown in Table 2.1. All PCR reactions were performed in 0.2ml thin walled tubes in the GeneAmp PCR system 9700 (Applied Biosystems). Primary PCR reactions were performed using ADP1 and N-PGIP-L1 for amplifying the upstream 5' region of the gene and primers ADP1 and N-PGIP-R1-1 were used for amplifying the downstream 3' region. PCR conditions were as follows: 0.5µl of ligated and diluted DNA, 0.45µM adaptor primer ADP1; 0.45µM N-PGIP-L2 or N-PGIP-R1-1; 0.2µM dNTPs; 1U of Expand *Taq* polymerase (Roche). The cycle parameters included an initial denaturation at 96°C for 1 min followed by 35 cycles of denaturation at 94°C for 30 sec and annealing/extension at 68°C for 6 min, and a final elongation at 68°C for 15 min. Secondary PCR reactions were performed using ADP2 and PGIP-Inv-L2 for the upstream region and primers ADP2 and

PGIP-Inv-R3 for amplifying the downstream 3' region. PCR conditions were the same as for the primary reactions, but the cycle parameters differed slightly. Twenty cycles of 94°C for 20sec, 62°C for 30 sec and 68°C for 1 min with an initial denaturation step at 96°C for 2 min and a final elongation at 68°C for 1 min, was performed. A negative control containing all PCR reagents, except template DNA was also included in both primary and secondary PCR reactions. PCR products were analysed on a 1% (w/v) agarose gel in 1 x TAE buffer (pH 8.0).

Cloning of the genome walking PCR products

Ligation into pCR®2.1

The QIAquick gel extraction kit protocol (Qiagen, Germany) was used to purify the PCR products from a 1% (w/v) agarose gel. The PCR fragments were ligated into the multiple cloning site of the pCR®2.1 vector provided in the TA® Cloning Kit (Invitrogen). A 10µl ligation reaction was prepared containing 50 ng PCR product, 25ng pCR®2.1 and 4 Weiss units T4 DNA Ligase. Ligation reactions were incubated overnight at 14°C.

Transformation

The ligated samples were transformed into One Shot®cells (TOP10F'). Two microlitres of each ligation reaction were added to 50 µl of One Shot®cells and gently mixed with the pipette tip. The vials were incubated for 30 min on ice, followed heat shocking the samples for exactly 30 sec in a 42°C water bath. SOC medium was added to each sample, which was then incubated at 37°C for 1h on a shaking incubator at 225rpm. A hundred microlitres of each transformation reaction was then spread on LB agar plates supplemented with 100µg/ml ampicillin, 0.5µM IPTG (Boehringer Mannheim) and 80µg/ml X-gal. The plates were incubated overnight at 37°C.

Plasmid DNA isolation and screening of putative transformants

Thirty, white recombinant colonies were selected and subjected to plasmid DNA isolation. The colonies were inoculated individually in 5ml LB medium supplemented with 50μg/ml ampicillin and grown overnight at 37°C. Plasmid DNA was isolated from the bacterial cultures using the Wizard®Plus SV Miniprep DNA Purification System (Promega). The putative recombinants were analysed by restriction enzyme digestion as described by Sambrook *et al.* (1989) as well as PCR analysis using M13 forward and M13 reverse primers. Two positive clones designated as pCR-*ScaI*GW-4 (Upstream fragment) and pCR-*DraI*-GW2 (Downstream fragment) were sequenced as described in Chapter 3, using the M13 forward and M13 reverse primers. Sequences were analysed using the ABI Prism ® SeqScape Software Version 1.0 (Applied Biosystems) program.

Table 2.2 Characteristics of all oligonucleotide primers used in this study

Primer	Size (bp)	Sequence 5'-3'	Tm	%GC
PC6	21	ACATCTCTCAGGCTCTCAACC	59	52
SPPGIP2	29	GCAGTGTGGAGGGGTGCACCACACAGGCA	74[a]	65
N-PGIP-L1	20	CCATGAGGCCAAGACATAGG	61	55
N-PGIP-R1-1	20	CTGAACGTGAGCTACAACAG	60	50
PGIP-Inv-L2	20	GGACGGAGGAGAAGAGTAGG	62	60
PGIP-Inv-R3	20	CCATAACCGATGCCTGTGTG	62	55
ADP1	27	GGATCCTAATACGACTCACTATAGGGC	66[a]	48
ADP2	17	AATAGGGCTCGAGCGGC	56	65
T7	20	ATTATGCTGAGTGATATCCC	56	40
SP6	21	ATTCTATAGTGTCACCTAAAT	54	30
M13 F	18	GGTTTTCCCAGTCACGAC	56	55
M13 R	20	AGCGGATAACAATTTCACAC	56	40

[a] Tm calculated using the website : http://www.basic.nwv.edu/biotools/oligocalc.html.

The melting temperature (Tm) was calculated using the Wallace formula :

Tm = 4 (G + C) + 2 (A + T)

G, C, A, T – number of respective nucleotides in the primer.

Results

Isolation of the partial *Eucalyptus grandis pgip* gene

In a previous study, Chimwamurombe *et al.* (2001) used degenerate oligonucleotide primers PC1 (5' GGAATTCAAYCCNGAYGAYAARGT 3') (Stotz *et al.*, 1993) and Per 3 (5'RCANWSNGGNARNGGNGCNCCRCANARRCA 3') (Chimwamurombe *et al.*, 2001) to amplify a partial *Eucalyptus pgip* gene of 909bp (Figure 2.2). The predicted 298 amino acid polypeptide lacked both the putative signal sequence as well as the downstream translation termination signal. It showed 95% amino acid sequence identity to the published pear PGIP, thus allowing the design of gene-specific primers corresponding to the N-terminal region of pear pgip (PC6) and the C-terminal region of the partial *Eucalyptus* pgip (SPPGIP2). The SPPGIP2 primer was designed from the sequence of the degenerate primer, Per3 of pear PGIP (Stotz *et al.*, 1993) and the partial *Eucalyptus pgip* sequence obtained by Chimamurombe *et al.* (2001). The gene-specific primer PC6 was designed from a region upstream of the first ATG and it thus lead to the amplification of the signal peptide sequence.

```
                                                                                PC6 ⇒
Pear DNA                                                                ACA TCT CTC AGG CTC TCA ACC AAA ACC AAA ACA    [33]

Pear AA        M   E   L   K   F   S   T   F   L   S   L   T   L   L   F   S   S   V   L   N   P   A   L   S   D    [25]
Pear DNA      ATG GAA CTC AAG TTC TCC ACC TTC CTC TCC CTA ACC CTA CTC TTC TCC TCC GTC CTA AAC CCC GCT CTC TCC GAT   [108]

             G GAA TTC AAY CCN GAY GAY AAR AAR GT  PC1 ⇒
Euc DNA       GAA TTC AAT CCG GAC GAC AAG AAG GTC CTC CTA CAA ATC AAG AAA GCC TTC GGC GAC CCC TAT GTC TTG GCC TCA
Euc AA                                         V   L   L   Q   I   K   K   A   F   G   D   P   Y   V   L   A   S

Pear AA        L   C   N   P   D   D   K   K   V   L   L   Q   I   K   K   A   F   G   D   P   Y   V   L   A   S    [50]
Pear DNA      CTC TGC AAC CCC GAC GAC AAA AAA GTC CTC CTA CAA ATC AAG AAA GCC TTC GGC GAC CCC TAC GTC TTG GCC TCA   [183]

Euc DNA       TGG AAA TCA GAC ACC GAC TGT TGT GAT TGG TAC TGC GTC ACC TGT GAC TCA ACC ACA AAC CGC ATC AAC TCC CTC
Euc AA         W   K   S   D   T   D   C   C   D   W   Y   C   V   T   C   D   S   T   T   N   R   I   N   S   L

Pear AA        W   K   S   D   T   D   C   C   D   W   Y   C   V   T   C   D   S   T   T   N   R   I   N   S   L    [75]
Pear DNA      TGG AAA TCA GAC ACT GAC TGC TGC GAT TGG TAC TGC GTC ACC TGT GAC TCC ACC ACA AAC CGC ATT AAC TCC CTC   [258]

Euc DNA       ACC ATC TTT GCC GGC CAG GTA TCC GGC CAA ATC CCC GCC CTA GTT GGA GAC TTG CCG TAC CTT GAA ACC CTT GAA
Euc AA         T   I   F   A   G   Q   V   S   G   Q   I   P   A   L   V   G   D   L   P   Y   L   E   T   L   E

Pear AA        T   I   F   A   G   Q   V   S   G   Q   I   P   A   L   V   G   D   L   P   Y   L   E   T   L   E   [100]
Pear DNA      ACC ATC TTT GCC GGC CAG GTG TCA GGC CAA ATC CCC GCC CTA GTA GGA GAC TTG CCA TAC CTT GAA ACC CTT GAA   [333]

Euc DNA       TTC CAC AAG CAA CCC AAT CTC ACT GGC CCA ATC CAA CCC GCC ATT GCC AAG CTC AAA GGA CTC AAG TTT CTC AGG
Euc AA         F   H   K   Q   P   N   L   T   G   P   I   Q   P   A   I   A   K   L   K   G   L   K   F   L   R

Pear AA        F   H   K   Q   P   N   L   T   G   P   I   Q   P   A   I   A   K   L   K   G   L   K   S   L   R   [125]
Pear DNA      TTC CAT AAG CAA CCC AAT CTC ACT GGC CCA ATC CAA CCC GCC ATT GCC AAG CTC AAA GGA CTC AAG TCT CTC AGG   [408]
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Euc DNA   CTC AGC TGG ACC AAC CTC TCA GGC TCT GTC CCT GAC TTC CTC AGC CAA CTC AAG AAC CTC ACA TTC CTC GAC CTC
Euc AA     L   S   W   T   N   L   S   G   S   V   P   D   F   L   S   Q   L   K   N   L   T   F   L   D   L

Pear AA    L   S   W   T   N   L   S   G   S   V   P   D   F   L   S   Q   L   K   N   L   T   F   L   D   L   [150]
Pear DNA  CTC AGC TGG ACC AAC CTC TCA GGC TCT GTC CCT GAC TTC CTC AGC CAA CTC AAG AAC CTC ACA TTC CTC GAC CTC   [483]


Euc DNA   TCC TTC AAC AAC CTC ACC GGC GCC ATC CCC AGC TCG CTT TCT CAG CTC CCA AAC CTC AAC GCT CTT CAT CTA GAC
Euc AA     S   F   N   N   L   T   G   A   I   P   S   S   L   S   Q   L   P   N   L   N   A   L   H   L   D

Pear AA    S   F   N   N   L   T   G   A   I   P   S   S   L   S   E   L   P   N   L   G   A   L   R   L   D   [175]
Pear DNA  TCC TTC AAC AAC CTC ACC GGT GCC ATC CCC AGC TCG CTT TCT GAG CTC CCA AAC CTC GGC GCT CTT CGT CTA GAC   [558]


Euc DNA   CGC AAT AAG CTC ACA GGT CAT ATT CCG AAA TCG TTT GGG CAG TTC ATT GGC AAC GTT CCA GAC CTG TAT CTC TCC
Euc AA     R   N   K   L   T   G   H   I   P   K   S   F   G   Q   F   I   G   N   V   P   D   L   Y   L   S

Pear AA    R   N   K   L   T   G   H   I   P   I   S   F   G   Q   F   I   G   N   V   P   D   L   Y   L   S   [200]
Pear DNA  CGC AAT AAG CTC ACA GGT CAT ATT CCG ATA TCG TTT GGG CAG TTC ATT GGC AAC GTT CCA GAC CTG TAT CTC TCC   [633]


Euc DNA   CAC AAC CAG CTC TCG GGC AAC ATT CCA ACC TCA TTT GCC CAG ATG GAC TTC GGC AAG CAT AGA CTA TCA CGG AAC
Euc AA     H   N   Q   L   S   G   N   I   P   T   S   F   A   Q   M   D   F   G   K   H   R   L   S   R   N

Pear AA    H   N   Q   L   S   G   N   I   P   T   S   F   A   Q   M   D   F   T   S   I   D   L   S   R   N   [225]
Pear DNA  CAC AAC CAG CTT TCT GGT AAC ATT CCA ACC TCA TTC GCT CAG ATG GAC TTC ACC AGC ATA GAC TTA TCA CGG AAC   [708]


Euc DNA   AAG CTC GAG  -  GAC GCA TCA GTG ATA TTT GGG CTG AAC AAG ACA ACC CAG ATT GTG GAC CTA TCC AGG AAC TTG
Euc AA     K   L   E   -   D   A   S   V   I   F   G   L   N   K   T   T   Q   I   V   D   L   S   R   N   L

Pear AA    K   L   E   G   D   A   S   V   I   F   G   L   N   K   T   T   Q   I   V   D   L   S   R   N   L   [250]
Pear DNA  AAG CTC GAA GGT GAC GCA TCC GTG ATA TTT GGG CTG AAC AAG ACA ACC CAG ATT GTG GAC CTG TCC AGG AAC TTG   [783]


Euc DNA   CTG GAA TTT AAT CTG TCA AAG GTG GAG TTT CCG ACA AGC TTG ACC TCA CTG GAT GTA AAC CAC AAT AAG ATC TAC
Euc AA     L   E   F   N   L   S   K   V   E   F   P   T   S   L   T   S   L   D   V   N   H   N   K   I   Y

Pear AA    L   E   F   N   L   S   K   V   E   F   P   T   S   L   T   S   L   D   I   N   H   N   K   I   Y   [275]
Pear DNA  CTG GAA TTT AAT CTG TCA AAG GTG GAG TTT CCG ACA AGC TTG ACC TCG CTG GAT ATC AAC CAC AAT AAG ATC TAC   [858]
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Euc DNA   GGG AGT ATC CCA GTG GAG TTT ACC CAA CTG AAT TTC CAG TTC CTG AAC GTG AGC TAC AAC AGG CTG TGT GGT CAG
Euc AA     G   S   I   P   V   E   F   T   Q   L   N   F   Q   F   L   N   V   S   Y   N   R   L   C   G   Q

Pear AA    G   S   I   P   V   E   F   T   Q   L   N   F   Q   F   L   N   V   S   Y   N   R   L   C   G   Q   [300]
Pear DNA  GGG AGT ATC CCA GTG GAG TTT ACG CAA CTG AAT TTC CAG TTC CTG AAC GTG AGC TAC AAC AGG CTG TGT GGT CAG   [933]


Euc DNA   ATT CCA GTG GGC GGA AAG TTG CAA AGC TTC AAC GAG TAT TCT TAT TTC CAT AAC CGA
Euc AA     I   P   V   G   G   K   L   Q   S   F   N   E   Y   S   Y   F   H   N   R

Pear AA    I   P   V   G   G   K   L   Q   S   F   D   E   Y   S   Y   F   H   N   R                       [319]
Pear DNA  ATT CCT GTG GGT GGA AAG TTG CAG AGC TTC GAC GAG TAT TCT   TAT TTC CAT AAC CGA                       [990]

Euc DNA   TGC CTG TGT GGT GCA CCC CTC CAC ACT GC
       3'ACR RAN ACR CNC GNG GNR ANG GNS WNA CR-5' ⇐ Per3

Pear AA    C   L   C   G   A   P   L   P   S   C   K   *                                                   [330]
Pear DNA  TGC TTG TGC GGT GCT CCA CTC CCA AGC TGC AAG TAA                                                   [1026]
       3' ACG GAC ACA CCA CGT GG- GGA GGT GTG ACG -5' ⇐ SPPGIP2
```

Figure 2.2 Sequence information used for PCR primer design to amplify the complete *Eucalyptus grandis pgip* gene. The nucleotide and amino acid sequences of the partial *Eucalypus pgip* gene (Chimwamurombe *et al.*,2001) and the published pear *pgip* gene (Stotz *et al.*, 1993) that were available at the start of the study are shown.

Eucalyptus grandis (TAG5) genomic DNA was used in a PCR reaction with gene-specific primers PC6 and SPPGIP2 to amplify a 1020bp fragment. An annealing temperature of 55°C enabled the exponential amplification of an expected fragment of approximately 1000bp. Amplification products are illustrated in figure 2.3.

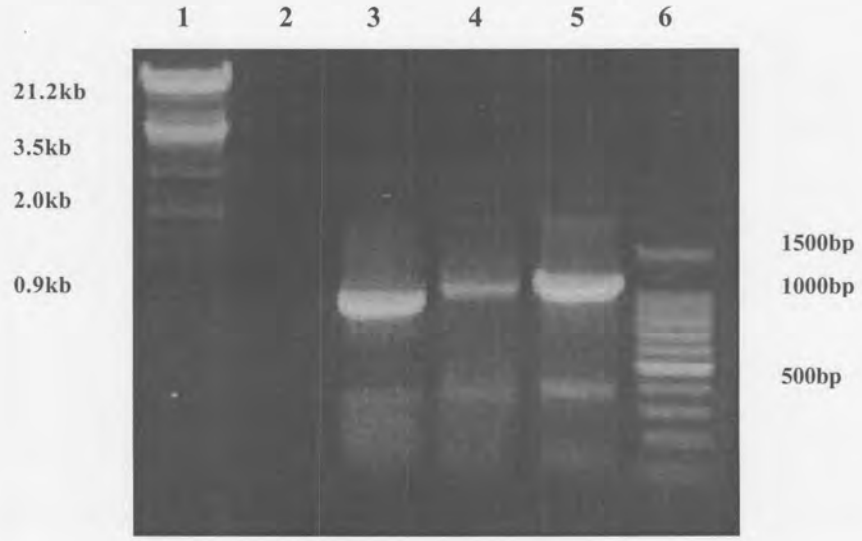

Figure 2.3 PCR amplification of the *pgip* gene from *Eucalyptus grandis* genomic DNA. Lane 3 contains the product of amplification with primers PC1 and Per3 using the plasmid P1 containing the partial *Eucalyptus* pgip (Chimwamurombe *et al.*, 2001). Lanes 4 and 5 contain PCR fragments amplified from genomic DNA using gene-specific primers PC6 and SPPGIP2. The fragments obtained are approximately 100bp larger than those obtained for the positive control. Lane 2 represents the negative control in which no template DNA was included. Lanes 1 and 6 are the molecular markers. Lane 1 contains λ DNA digested with *Hind*III and *Eco*RI, while lane 6 contains the commercial 100bp ladder (Promega).

The 1020bp pgip fragment was cloned into the commercial pGEM-T-Easy cloning vector (Promega) and twelve putative recombinant clones designated as pGEM-*Eucpgip*110A to 110L were subjected to restriction analysis using *Eco*RI. The restriction profile is

illustrated in figure 2.4. Five of the 12 clones contained the *Eucalyptus* pgip insert, which was cut into two fragments indicating the occurrence of an *Eco*RI restriction site within the *pgip* gene.

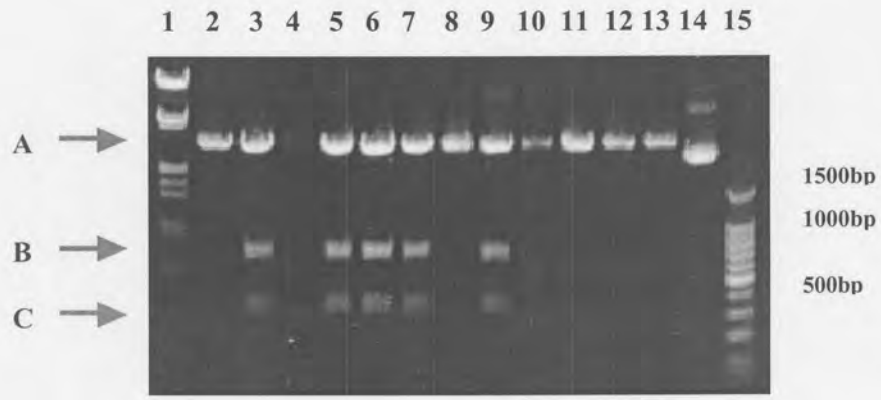

Figure 2.4 Restriction enzyme digest of putative recombinant clones with *Eco*RI. Lanes 2-13 represents clones pGEM-*Eucpgip*110A to 110L digested with *Eco*RI. Lane 14 represents an undigested clone and Lanes 1 and 15 contain the λ *Hind*III/*Eco*R1 marker and 100bp DNA ladder, respectively. Digestion with *Eco*RI gave three fragments in the recombinant clones. These are represented as, **(A)** 3015bp linearized pGEM-T-Easy vector, **(B)** 695bp fragment and **(C)** 337bp fragment.

The recombinant clones were screened by PCR analysis using a second set of gene-specific primers designed from the nucleotide sequences of the pGEM-*Eucpgip* clone and the published pear *pgip* gene sequence. The EN-FWD-PGIP primer was designed from the 5' region of the partial eucalyptus *pgip* gene and contains an *Nco*I restriction site. The reverse primer, REV-PB-PGIP was designed from the 3' region of the pear *pgip* gene and contains a *Bam*HI site. The design and use of these primers are discussed in detail in chapter 3. Clones containing the inserts, as established from restriction analysis, were selected and used as templates in PCR reactions with the EN-FWD-PGIP and REV-PB-PGIP primers. A fragment of approximately 1kb was amplified from each of the clones screened (Figure

2.5). The plasmid map of the constructed recombinant pGEM-*Eucpgip*110B clone is indicated in figure 2.6.

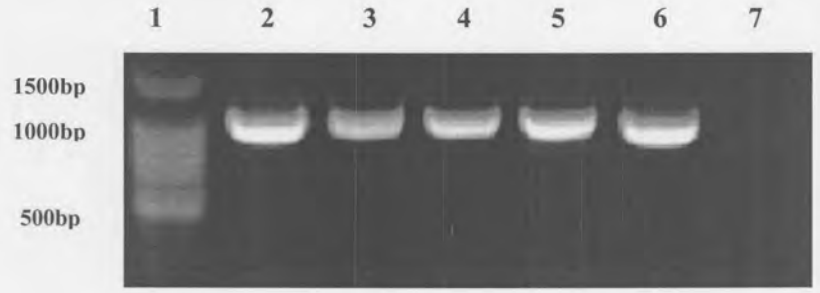

Figure 2.5 PCR amplification of recombinant pGEM-*Eucpgip* clones. Lane 1 contains the 100bp DNA ladder (Promega). Lanes 2-6 contains amplification products of the recombinant pGEM-*Eucpgip* clones 110B, 110D, 110E, 110F and 110H, respectively, using gene-specific primes EN-PGIP-FWD and REV-PB-REV. Lane 7 contains the negative ddH_2O control.

Sequence Analysis of the partial *Eucalyptus* PGIP

A recombinant PCR clone pGEM-*Eucpgip*110B was selected and sequenced with T7 and SP6 primers. Direct sequencing using gene-specific primers EN-FWD-PGIP and REV-PB-PGIP was also performed on the PCR product amplified from genomic DNA (TAG5), yielding the same internal nucleotide sequence as that of the pGEM-*Eucpgip*110B clone (Figure 2.7). Amplification with the gene-specific PC6 primer, designed from the N-terminal region of the pear PGIP, enabled determination of the start codon (ATG) and signal sequence of PGIP, which was not included in the partial *Eucalyptus* PGIP fragment sequenced by Chimwamurombe *et al.* (2001).

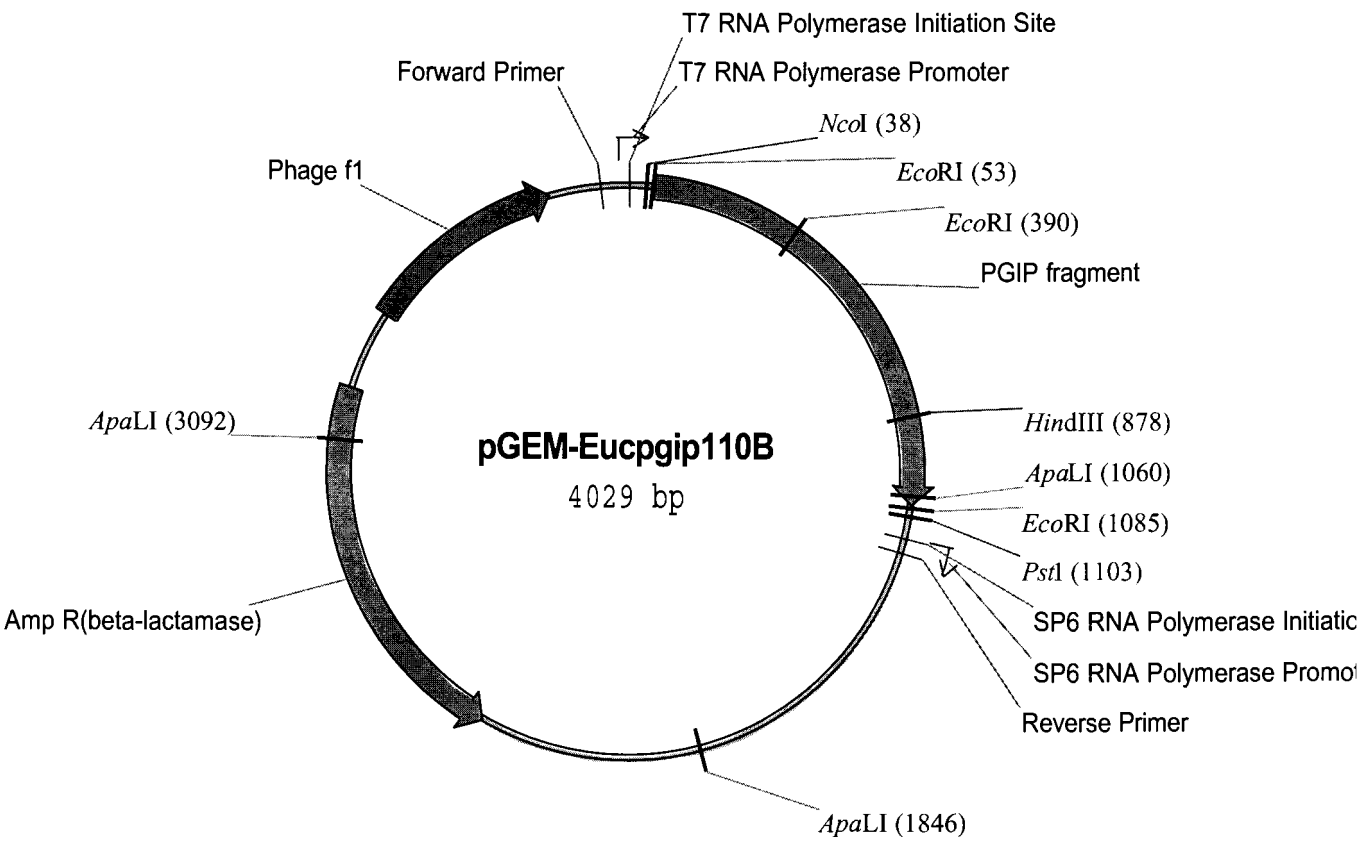

Figure 2.6 Plasmid map of the constructed pGEM-*Eucpgip*110B clone containing the *Eucalyptus pgip* fragment.

PC6 ⇒

pGEM-*Eucpgip*110B ACATCTCTCA GGCTCTCAAC CAAAACCCAA AACAATGGAG CTCAAGTTCT TCACCTTCCT CTCCCTAACC CTACTCTTCT CCTCCGTCCT AAACCCCGCT
Eucalyptus ....CCCCGCT
Pear ACATCTCTCA GGCTCTCAAC CAAAACC.AA AACAATGGAA CTCAAGTTCT CCACCTTCCT CTCCCTAACC CTACTCTTCT CCTCCGTCCT AAACCCCGCT
Pear Amino Acid M E L K F S T F L S L T L L F S S V L N P A

pGEM-*Eucpgip*110B CTCTCCGATC TCTGCAACCC CGACGACAAA AAAGTCCTCC TACAAATCAA GAAAGCCTTC GGCGACCCCT ATGTCTTGGC CTCATGGAAA TCAGACACCG
Eucalyptus CTCTCCGATC TCCGCAACCC CGACGACAAA AAAGTCCTCC TACAAATCAA GAAAGCCTTC GGCGACCCCT ATGTCTTGGC CTCATGGAAA TCAGACACCG
Pear CTCTCCGATC TCTGCAACCC CGACGACAAA AAAGTCCTCC TACAAATCAA GAAAGCCTTC GGCGACCCCT ACGTCTTGGC CTCATGGAAA TCAGACACTG
Pear Amino Acid L S D L C N P D D K K V L L Q I K K A F G D P Y V L A S W K S D T

pGEM-*Eucpgip*110B ACTGTTGTGA TTGGTACTGC GTCACCTGTG ACTCAACCAC AAACCGCATC AACTCCCTCA CCATCTTTGC CGGCCAGGTA TCCGGCCAAA TCCCCGCCCT
Eucalyptus ACTGTTGTGA TTGGTACTGC GTCACCTGTG ACTCAACCAC AAACCGCATC AACTCCCTCA CCATCTTTGC CGGCCAGGTA TCCGGCCAAA TCCCCGCCCT
Pear ACTGCTGCGA TTGGTACTGC GTCACCTGTG ACTCCACCAC AAACCGCATT AACTCCCTCA CCATCTTTGC CGGCCAGGTG TCAGGCCAAA TCCCCGCCCT
Pear Amino Acid D C C D W Y C V T C D S T T N R I N S L T I F A G Q V S G Q I P A L

pGEM-*Eucpgip*110B AGTTGGAGAC TTGCCGTACC TTGAAACCCT TGAATTCCAT AAGCAACCCA ATCTCACTGG CCCAATCCAA CCCGCCATTG CCAAGCTCAA AGGACTCAAG
Eucalyptus AGTTGGAGAC TTGCCGTACC TTGAAACCCT TGAATTCCAT AAGCAACCCA ATCTCACTGG CCCAATCCAA CCCGCCATTG CCAAGCTCAA AGGACTCAAG
Pear AGTAGGAGAC TTGCCATACC TTGAAACCCT TGAATTCCAT AAGCAACCCA ATCTCACTGG CCCAATCCAA CCCGCCATTG CCAAGCTCAA AGGACTCAAG
Pear Amino Acid V G D L P Y L E T L E F H K Q P N L T G P I Q P A I A K L K G L K

pGEM-*Eucpgip*110B TCTCTCAGGC TCAGCTGGAC CAACCTCTCA GGCTCTGTCC CTGACTTCCT CAGCCAACTC AAGAACCTCA CATTCCTCGA CCTCTCCTTC AACAACCTCA
Eucalyptus TCTCTCAGGC TCAGCTGGAC CAACCTCTCA GGCTCTGTCC CTGACTTCCT CAGCCAACTC AAGAACCTCA CATTCCTCGA CCTCTCCTTC AACAACCTCA
Pear TCTCTCAGGC TCAGCTGGAC CAACCTCTCA GGCTCTGTCC CTGACTTCCT CAGCCAACTC AAGAACCTCA CATTCCTCGA CCTCTCCTTC AACAACCTCA
Pear Amino Acid S L R L S W T N L S G S V P D F L S Q L K N L T F L D L S F N N L

pGEM-*Eucpgip*110B CCGGTGCCAT CCCCAGCTCG CTTTCTGAGC TCCCAAACCT CGGCGCTCTT CGTCTAGACC GCAATAAGCT CACAGGTCAT ATTCCGATAT CGTTTGGGCA
Eucalyptus CCGGTGCCAT CCCCAGCTCG CTTTCTGAGC TCCCAAACCT CGGCGCTCTT CGTCTAGACC GCAATAAGCT CACAGGTCAT ATTCCGATAT CGTTTGGGCA
Pear CCGGTGCCAT CCCCAGCTCG CTTTCTGAGC TCCCAAACCT CGGCGCTCTT CGTCTAGACC GCAATAAGCT CACAGGTCAT ATTCCGATAT CGTTTGGGCA
Pear Amino Acid T G A I P S S L S E L P N L G A L R L D R N K L T G H I P I S F G Q

pGEM-*Eucpgip*110B GTTCATTGGC AACGTTCCAG ACCTGTATCT CTCCCACAAC CAGCTTTCTG GTAACATTCC AACCTCATTC GCTCAGATGG ACTTCACTAG CATAGACTTA
Eucalyptus GTTCATTGGC AACGTTCCAG ACCTGTATCT CTCCCACAAC CAGCTTTCTG GTAACATTCC AACCTCATTC GCTCAGATGG ACTTCACTAG CATAGACTTA
Pear GTTCATTGGC AACGTTCCAG ACCTGTATCT CTCCCACAAC CAGCTTTCTG GTAACATTCC AACCTCATTC GCTCAGATGG ACTTCACCAG CATAGACTTA
Pear Amino Acid F I G T V P D L Y L S H N Q L S G N I P T S F A Q M D F T S I D L

pGEM-*Eucpgip*110B TCACGGAACA AGCTCGAAGG TGACGCATCC GTGATATTTG GGCTGAACAA GACAACCCAG ATTGTGGACC TGTCCAGGAA CTTGCTGGAA TTTAATCTGT
Eucalyptus TCACGGAACA AGCTCGAAGG TGACGCATCC GTGATATTTG GGCTGAACAA GACAACCCAG ATTGTGGACC TGTCCAGGAA CTTGCTGGAA TTTAATCTGT
Pear TCACGGAACA AGCTCGAAGG TGACGCATCC GTGATATTTG GGCTGAACAA GACAACCCAG ATTGTGGACC TGTCCAGGAA CTTGCTGGAA TTTAATCTGT
Pear Amino Acid S R N K L E G D A S V I F G L N K T T Q I V D L S R N L L E F N L

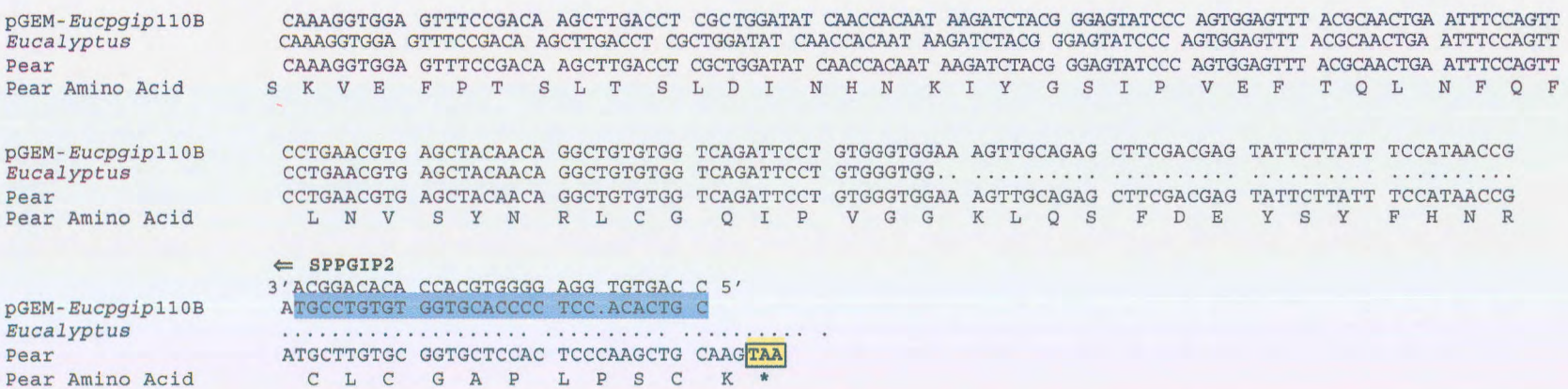

Figure 2.7 Nucleotide sequence alignments between amplified genomic DNA (*Eucalyptus*), the pGEM-*Eucpgip*110B clone and the published pear *pgip* sequence (Stotz *et al.*, 1993). The genomic amplified *pgip* gene (*Eucalyptus)* was subjected to direct sequencing using the gene-specific primers EN-FWD-PGIP and REV-PB-PGIP. T7 and SP6 primers enabled the sequencing of the entire cloned pgip fragment from the pGEM-*Eucpgip*110B clone. The regions used for synthesizing the PC6 and SPPGIP2 primers are indicated in blue. The pear PGIP stop codon (TAA) is indicated in yellow and is absent from the nucleotide sequence of the *Eucalyptus pgip* gene. Nucleotide differences between the amplified genomic DNA (*Eucalyptus*), the pGEM-*Eucpgip*110B clone and the published pear *pgip* sequence are highlighted in yellow.

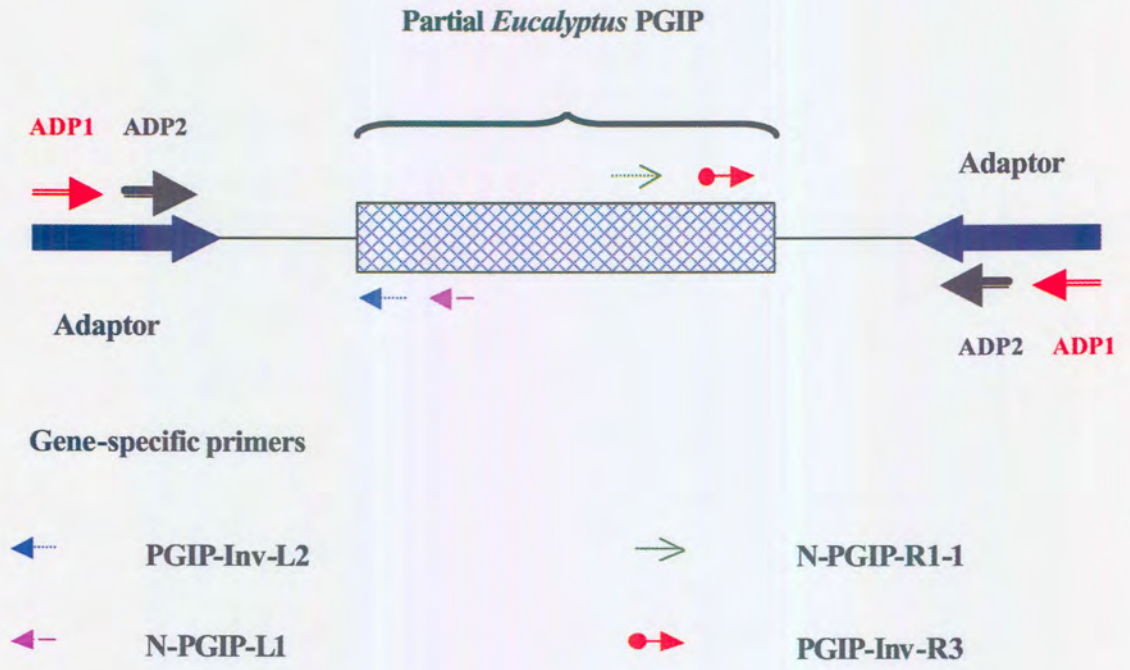

Figure 2.8 Schematic representation of the Genome Walking strategy used to clone the unknown 5' and 3' ends of the *Eucalyptus pgip* gene. Two sets of gene-specific primers were designed and used in conjunction with adaptor primers (ADP1 and ADP2) in the primary and secondary PCR walk reactions. In the primary PCR reactions, ADP1 and N-PGIP-L1 were used for walking upstream, while ADP1 and N-PGIP-R1-1 were used for walking towards the 3' region. The PCR products from the primary walk reactions were used in a secondary PCR, where ADP2 and PGIP-Inv-L2 were used for walking towards the 5' region and ADP2 and PGIP-Inv-R3, were used for the 3' region.

Isolation of the 5' and 3' ends of the *Eucalyptus pgip* gene by Genome Walking

The general procedure for genome walking has been illustrated in figure 1 and the genome walking cloning strategy for the *Eucalyptus pgip* gene is outlined in figure 8. *E. grandis* (EUCALYPTUS) genomic DNA was digested with a selection of blunt-end restriction enzymes. The restriction profile is represented in figure 2.9.

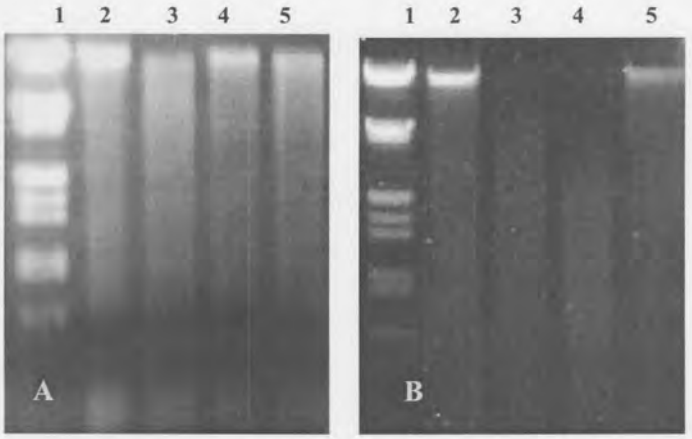

Figure 2.9 Restriction enzyme digest of TAG5 genomic DNA. **(A)** Lane 1 contains the λIII marker. Lane 2 represents undigested TAG5 genomic DNA and lanes 3, 4, and 5 contain genomic DNA digested with *Nco*I, *Pst*I and *Sca*I respectively. **(B)** Lane 1 contains the λIII marker and lane 2 contains the undigested genomic DNA. Lanes 3, 4 and 5 contain *Dra*I, *Hae*III and *Sma*I digested genomic DNA, respectively.

Restriction enzyme digestion with *Sca*I enabled the amplification of the 5' upsteam region, using adaptor primer (ADP2) and PGIP-Inv-L2, while digestion with *Dra*I lead to the amplification of the downstream 3' region when ADP2 and PGIP-Inv-R3 were used. Secondary PCR reactions were carried out at 62∘C, allowing the amplification of two distinct PCR fragments for the upstream region and a fragment of approximately 600bp, which represented the downstream region of the gene (Figure 2.10).

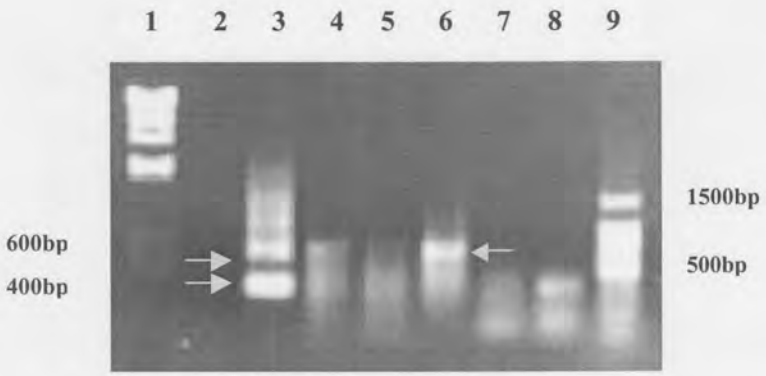

Figure 2.10 Genome Walking PCR analysis of genomic DNA using ADP2 and PGIP-Inv-L2 to walk upstream (Lanes 3, 7 and 8) and ADP2 and PGIP-Inv-R3 to walk downstream (Lanes 4,5 and 6). Lanes 3 and 4 represent amplification using genomic DNA digested with *Sca*I. In lanes 5 and 7 the template DNA was digested with *Hae*III and in lanes 6 and 7, *Dra*I digested DNA was used. Molecular weight marker λIII is shown in lane 1 and lane 9 contains a 100bp ladder (Promega). The arrows indicate PCR products that were cloned and sequenced.

Each fragment was individually cloned into the pCR®2.1 vector (TA®Cloning Kit, Invitrogen) and subjected to PCR screening and restriction enzyme analysis. Recombinant clones were digested with *Eco*RI to release the inserts, which are represented in figure 2.11. Recombinant plasmids containing the 700bp *Sca*I fragment were designated as pCR-*Sca*IGW-1 and pCR-*Sca*IGW-2, while plasmids containing the 400bp *Sca*I fragment were designated as pCR-*Sca*IGW-3 and pCR-*Sca*IGW-4. Plasmids pCR-*Dra*I-GW1 and pCR-*Dra*I-GW2 contain the 700bp downstream PCR fragment. The recombinant plasmids pCR-*Sca*IGW-3 and pCR-*Sca*IGW-4 show inserts that are approximately 280bp in length, indicating that the cloned PCR product possibly contains internal *Eco*RI sites. Restriction digestion of the pCR-*Sca*IGW-1 plasmid did not result in the release of the insert, while digestion of the pCR-*Sca*IGW-2 plasmid lead to the release of a 700bp insert.

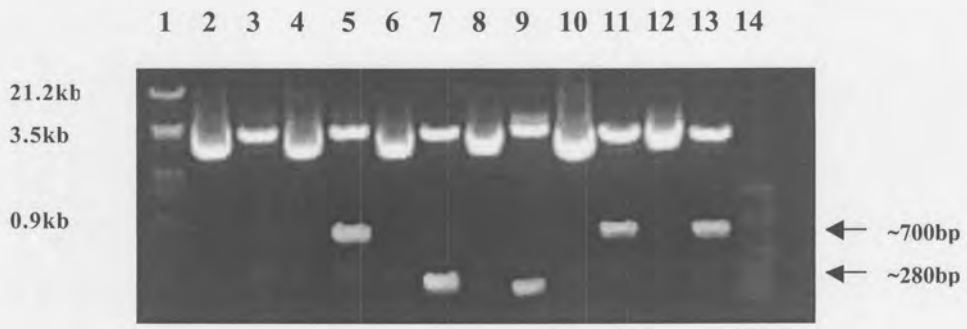

Figure 2.11 Restriction analysis of recombinant plasmids with *Eco*RI. Lanes 1 and 14 contain the molecular marker λIII and 100bp ladder (Promega) respectively. Lanes 2, 4, 6 and 8 contain the undigested plasmids pCR-*Sca*IGW-1 to pCR-*Sca*IGW-4 respectively, while lanes 3, 5, 7 and 9 contain plasmids pCR-*Sca*IGW-1 to pCR-*Sca*IGW-4 digested with *Eco*RI. Similarly, lanes 10 and 12 contain the undigested plasmids pCR-*Dra*I-GW1 and pCR-*Dra*I-GW2 respectively and lanes 11 and 13 represent *Eco*RI digested pCR-*Dra*I-GW1 and pCR-*Dra*I-GW2 respectively.

Each plasmid was used as a template in a PCR reaction with M13 forward and M13 reverse oligonucleotide primers. The PCR reaction was carried out at an annealing temperature of 64°C and the resulting PCR products are represented in figure 2.14. Inserts could not amplified from plasmids pCR-*Sca*IGW-1 and pCR-*Sca*IGW-2, however, a product of approximately 600bp could be amplified from plasmids pCR-*Sca*IGW-3 and pCR-*Sca*IGW-4. Recombinant plasmids pCR-*Dra*I-GW1 and pCR-*Dra*I-GW2 lead to the amplification of a fragment of approximately 900bp.

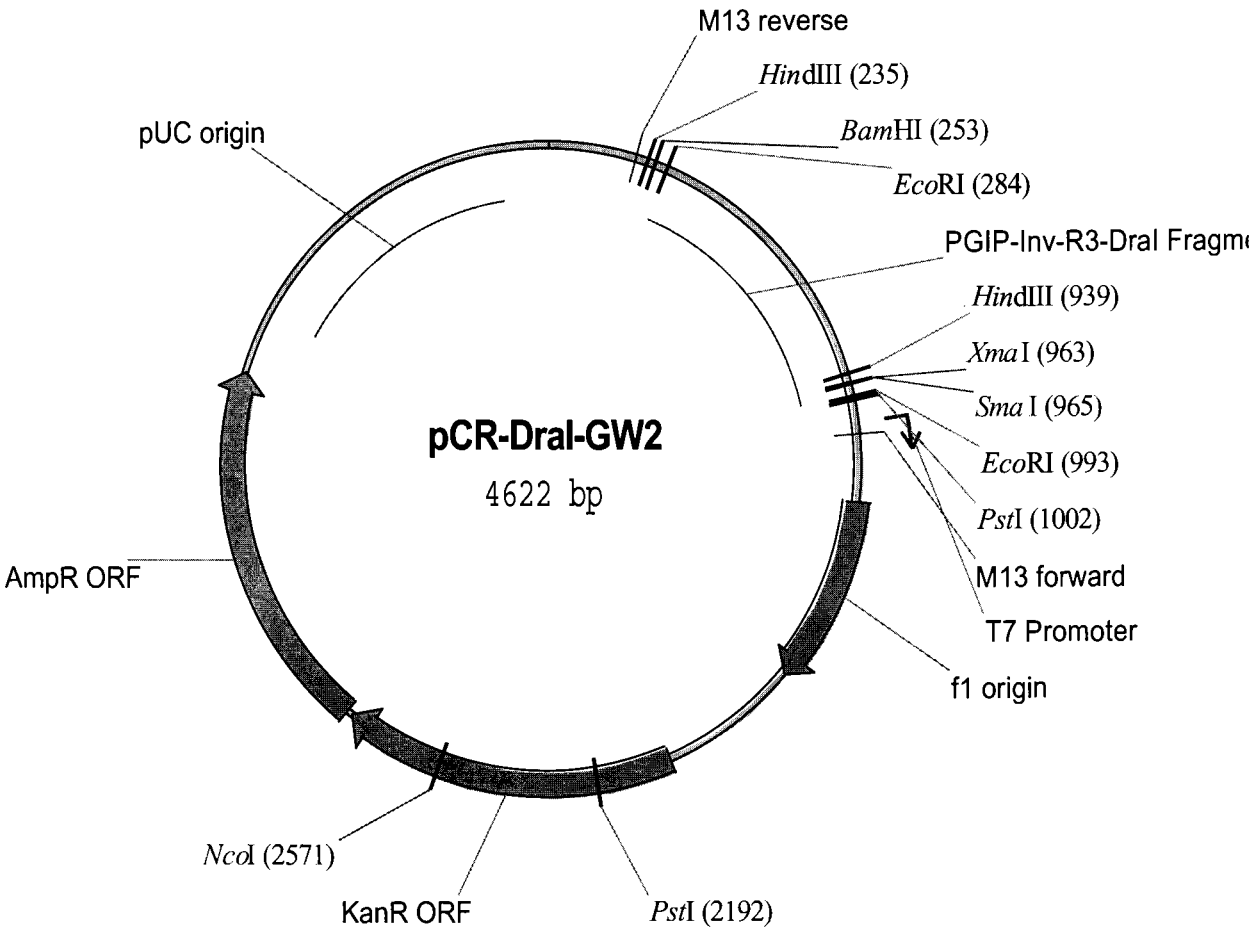

Figure 2.12 Plasmid map of the genome walking pCR-*Dra*I-GW2 construct containing the 3' downstream *Eucalyptus pgip* fragment.

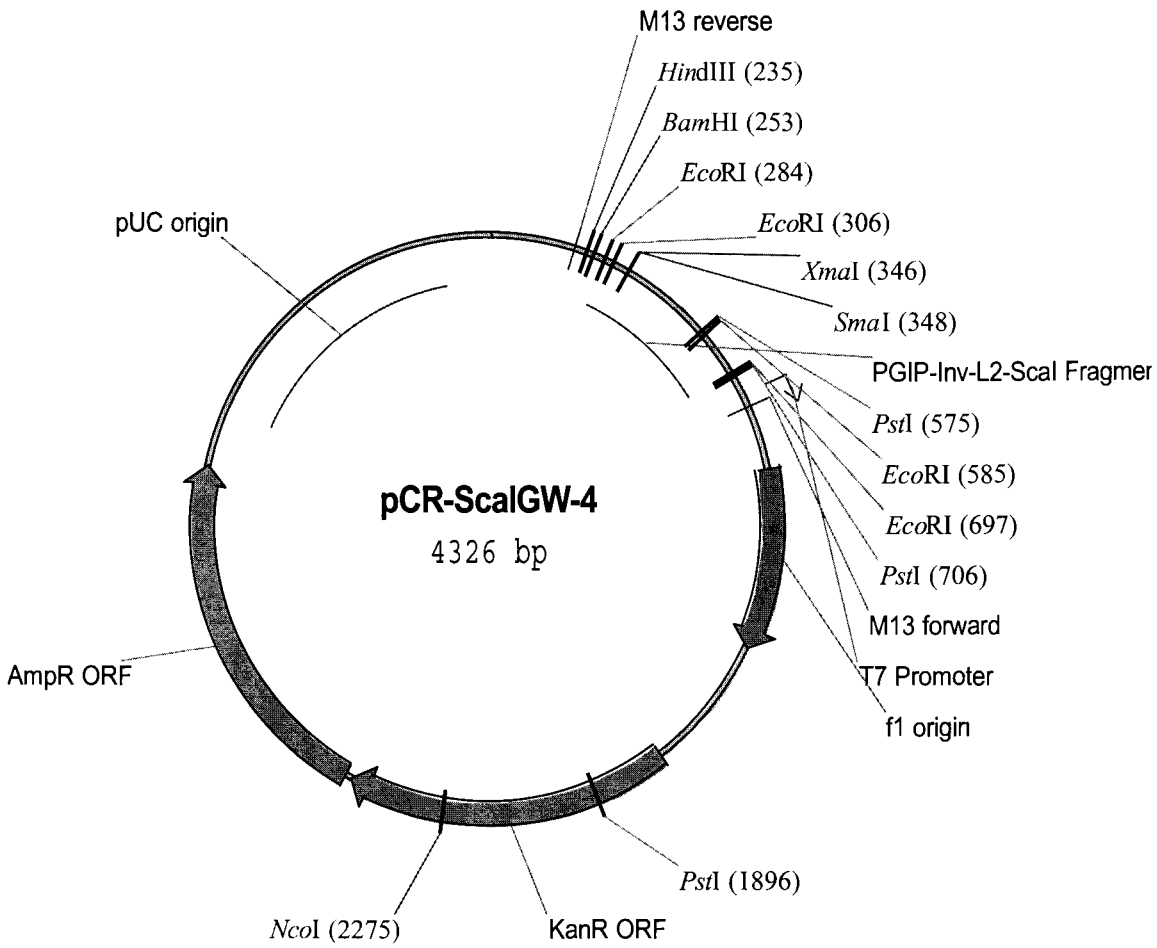

Figure 2.13 Plasmid map of the genome walking pCR-*ScaI*GW-4 construct containing the 5' upstream *Eucalyptus pgip* fragment.

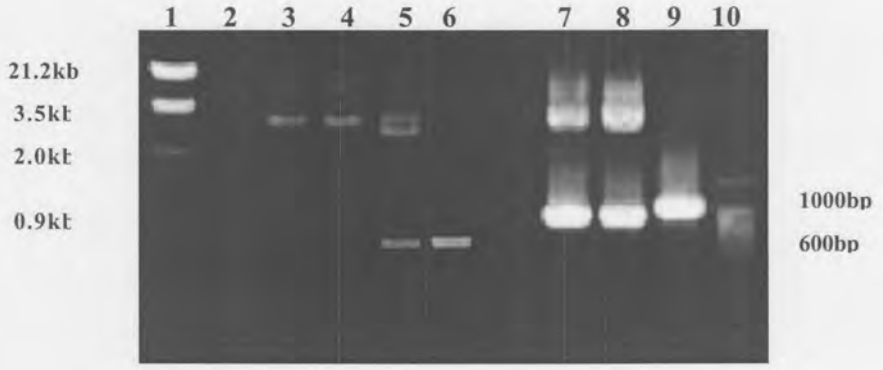

Figure 2.14 PCR analysis of genome walking plasmids using M13 forward and M13 reverse primers. Lanes 1 and 11 contain the molecular markers λIII and a 100bp DNA ladder (Promega) respectively. Lane 2 contains the negative water control and lane 9 contains a positive control that represents the amplification of the pgip fragment from the pGEM-*Eucpgip*110B clone with EN-FWD-PGIP and REV-PB-PGIP primers. Lanes 3 and 4 contain reactions in which pCR-*Sca*IGW-1 and pCR-*Sca*IGW-2 clones were used as templates, respectively. Lanes 5 represents amplification using pCR-*Sca*IGW-3 and lane 6 contains PCR products amplified from pCR-*Sca*IGW-4. Lanes 7 and 8 contain PCR products amplified from pCR-*Dra*I-GW1 and pCR-*Dra*I-GW2, respectively.

Clones pCR-*Sca*IGW-4, which represented the 5' upstream sequence and pCR-*Dra*I-GW2, which represents the 3' downstream sequence, were selected and subjected to nucleotide sequencing. The clones were sequenced in both directions using the M13 forward and M13 reverse primers. Plasmid maps of the pCR-*Dra*I-GW2 and pCR-*Sca*IGW-4 constructs are represented in figures 2.12 and 2.13, respectively.

Sequence Analysis of the complete *Eucalyptus pgip* gene

Based on inspection of the sequences to the upstream and downstream regions of the pGEM-*Eucpgip*110B clone it was concluded that the inserts from pCR-*Sca*IGW-4 and pCR-*Dra*I-GW2 represented the 5' and 3' regions of the *Eucalyptus pgip* gene respectively.

A 397bp fragment could be sequenced from the pCR-*Sca*IGW-4 clone using the M13 forward and M13 reverse primers (Figure 2.15). Alignment with the partial *Eucalyptus* sequence showed that the genome-walking clone shared an overlap of 91bp with the *Eucalyptus* sequence at the 5' region of the gene. Only one nucleotide difference, which results in a silent mutation could be observed within the signal sequence region. Further analysis of the additional 360bp of the pCR-*Sca*IGW-4 clone revealed that the upstream, untranslated region did not contain any transcription signals or promoter sequences.

The pCR-*Dra*I-GW2 clone contained a 693bp sequence of which only 39bp shared an overlap with the pGEM-*Eucpgip*110B clone (Figure 2.16). The overlap occurred in the downstream region of the partial eucalyptus *pgip* gene. The translation termination codon (TAG) could be observed in the nucleotide sequence of the genome-walking pCR-*Dra*I-GW2 clone. The 5' and 3' DNA sequences obtained from genome-walking PCR were linked to the partial *Eucalyptus pgip* sequence to give the composite *Eucalyptus* PGIP sequence. The complete nucleotide and deduced amino acid sequences of the composite *Eucalyptus pgip* gene are illustrated in figure 2.17.

```
                                                                ADP2 ⇒                ?
pCR-ScaI-GW4                                                    AATAGGGC TCGAGCGGCC GCCCGGGCAC TGAGCGCAAC GCAATTAATG  [80]
pGEM-Eucpgip110B      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

pCR-ScaI-GW4          TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT TGTGTGGAAT TGTGAGCGGA [160]
pGEM-Eucpgip110B      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

pCR-ScaI-GW4          TAACAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG CCAAGCTATT TAGGTGACAC TATAGAATAC TCAAGCTATG [240]
pGEM-Eucpgip110B      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

pCR-ScaI-GW4          CATCCAACGC GTTGGGAGCT CTCCCATATG GTCGACCTGC AGGCGGCCGC GAATTCACTA GTGATTACTT CTCTCAGGCT [320]
pGEM-Eucpgip110B      ---------- ---------- ---------- ---------- ---------- ---------- ------ACAT CTCTCAGGCT [14]
                                                                                              ↑

pCR-ScaI-GW4          CTCAACCAAA ACCCAAAACA ATGGAGCTCA AGTTCTCCAC CTTCCTCTCC CTAACCCTAC TCTTCTCCTC CGTCCAA--- [397]
pGEM-Eucpgip110B      CTCAACCAAA ACCCAAAACA ATGGAGCTCA AGTTCTTCAC CTTCCTCTCC CTAACCCTAC TCTTCTCCTC CGTCCTAAAC [94]
                                                                                GGATG AGAAGAGGAG GCAGG
                                                                              ⇐ PGIP-Inv-L2

pCR-ScaI-GW4          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pGEM-Eucpgip110B      CCCGCTCTCT CCGATCTCTG CAACCCCGAC GACAAAAAAG TCCTCCTACA AATCAAGAAA GCCTTCGGCG ACCCCTATGT [174]

pCR-ScaI-GW4           ---------- ----------
pGEM-Eucpgip110B      CTTGGCCTCA TGGAAATGCA
```

Figure 2.15 Alignment of the nucleotide sequence of pCR-ScaI-GW4 with the sequence of the pGEM-*Eucpgip*110B clone. The genome-walking oligonucleotide primers used to generate the upstream sequence are highlighted in yellow. The blue arrow indicates the beginning of the *Eucalyptus pgip* insert in the pGEM-*Eucpgip*110B clone and the start (ATG) codon is indicated in blue. The sequences overlap by 91bp in the region surrounding the ATG codon. The partial *Sca*I site is indicated by the ? symbol. The sequence indicated in pink is derived from the adaptor.

Figure 2.16 Alignment of the nucleotide sequence of pCR-*Dra*I-GW2 with the downstream sequence of the pGEM-*Eucpgip*110B clone. Oligonucleotide primers PGIP-Inv-R3 and ADP2 are highlighted in yellow. The stop (TAG) codon is indicated in blue and by the asterisk (*). The pCR-*Dra*I-GW2 clone shows only 39bp overlap with the sequence of the pGEM-*Eucpgip*110B clone. The partial DraI site is indicated by the ? symbol. SPPGIP2 primer, which shares an overlap of 10bp with the PGIP-Inv-R3 primer, is indicated in green. The sequence indicated in pink is derived from the adaptor.

⇐ PGIP-Inv-L2

```
1    Atg GAG CTC AAG TtC TCC ACC TTC CTC TCC CTA ACC CTA CTC TTC  45
1     M   E   L   K   F   S   T   F   L   S   L   T   L   L   F  15

46   TCC TCC GTC CTA AAC CCC GCT CTC TCC GAT CTC TGC AAC CCC GAC  90
16    S   S   V   L   N   P   A   L   S   D   L   C   N   P   D  30

91   GAC AAA AAA GTC CTC CTA CAA ATC AAG AAA GCC TTC GGC GAC CCC  135
31    D   K   K   V   L   L   Q   I   K   K   A   F   G   D   P  45

136  TAT GTC TTG GCC TCA TGG AAA TCA GAC ACC GAC TGT TGT GAT TGG  180
46    Y   V   L   A   S   W   K   S   D   T   D   C   C   D   W  60

181  TAC TGC GTC ACC TGT GAC TCA ACC ACA AAC CGC ATC AAC TCC CTC  225
61    Y   C   V   T   C   D   S   T   T   N   R   I   N   S   L  75

226  ACC ATC TTT GCC GGC CAG GTA TCC GGC CAA ATC CCC GCC CTA GTT  270
76    T   I   F   A   G   Q   V   S   G   Q   I   P   A   L   V  90

271  GGA GAC TTG CCG TAC CTT GAA ACC CTT GAA TTC CAT AAG CAA CCC  315
91    G   D   L   P   Y   L   E   T   L   E   F   H   K   Q   P  105

316  AAT CTC ACT GGC CCA ATC CAA CCC GCC ATT GCC AAG CTC AAA GGA  360
106   N   L   T   G   P   I   Q   P   A   I   A   K   L   K   G  120

361  CTC AAG TCT CTC AGG CTC AGC TGG ACC AAC CTC TCA GGC TCT GTC  405
121   L   K   S   L   R   L   S   W   T   N   L   S   G   S   V  135

406  CCT GAC TTC CTC AGC CAA CTC AAG AAC CTC ACA TTC CTC GAC CTC  450
136   P   D   F   L   S   Q   L   K   N   L   T   F   L   D   L  150

451  TCC TTC AAC AAC CTC ACC GGT GCC ATC CCC AGC TCG CTT TCT GAG  495
151   S   F   N   N   L   T   G   A   I   P   S   S   L   S   E  165

496  CTC CCA AAC CTC GGC GCT CTT CGT CTA GAC CGC AAT AAG CTC ACA  540
166   L   P   N   L   G   A   L   R   L   D   R   N   K   L   T  180
```

⇐ N-PGIP-L1

```
541   GGT CAT ATT CCG ATA TCG TTT GGG CAG TTC ATT GGC AAC GTT CCA   585
181    G   H   I   P   I   S   F   G   Q   F   I   G   N   V   P   195

586   GAC CTG TAT CTC TCC CAC AAC CAG CTT TCT GGT AAC ATT CCA ACC   630
196    D   L   Y   L   S   H   N   Q   L   S   G   N   I   P   T   210

631   TCA TTC GCT CAG ATG GAC TTC ACT AGC ATA GAC TTA TCA CGG AAC   675
211    S   F   A   Q   M   D   F   T   S   I   D   L   S   R   N   225

676   AAG CTC GAA GGT GAC GCA TCC GTG ATA TTT GGG CTG AAC AAG ACA   720
226    K   L   E   G   D   A   S   V   I   F   G   L   N   K   T   240

721   ACC CAG ATT GTG GAC CTG TCC AGG AAC TTG CTG GAA TTT AAT CTG   765
241    T   Q   I   V   D   L   S   R   N   L   L   E   F   N   L   255

766   TCA AAG GTG GAG TTT CCG ACA AGC TTG ACC TCG CTG GAT ATC AaC   810
256    S   K   V   E   F   P   T   S   L   T   S   L   D   I   N   270

811   CAC AAT AAG ATC TAC GGG AGT ATC CCA GTG GAG TTT ACG CAA CTG   855
271    H   N   K   I   Y   G   S   I   P   V   E   F   T   Q   L   285

                                N-PGIP-R1-1 ⇒
856   AAT TTC CAG TTC CTG AAC GTG AGC TAC AAC AGG CTG TGT GGT CAG   900
286    N   F   Q   F   L   N   V   S   Y   N   R   L   C   G   Q   300

901   ATT CCT GTG GGT GGA AAG TTG CAG AGC TTC GAC GAG TAT TCT TAT   945
301    I   P   V   G   G   K   L   Q   S   F   D   E   Y   S   Y   315

                      PGIP-Inv-R3 ⇒
946   TTC CAT AAC CGA TGC CTG TGT GGT CCT CCC CTC GGT CCC TGC AAG   990
316    F   H   N   R   C   L   C   G   A   P   L   G   P   C   K   330

991   AGT TAG   996
331    S   *
```

Figure 2.17 Nucleotide and predicted amino acid sequence of the complete composite *Eucalyptus pgip* gene. The red arrow indicates the initiation codon and the asterisk indicates the termination codon (TAG). The open arrow indicates the end of the putative signal peptide and the predicted start of the mature PGIP protein. The cysteine residues are

indicated in blue and the poteintial N-glycosylation sites are highlighted in green. The regions used for synthesizing genome walking PCR primers are presented in blue coloured blocks.

Discussion

Polygalacturonase-inhibiting Proteins (PGIPs), associated with the cell wall of many plant species form part of the plants active defense against phytopathogenic fungi. Genes encoding PGIPs have been isolated from various plant species that include, bean, soybean, tomato, pear, apple, raspberry and recently, guava (Toubart *et al.,* 1992; Stotz *et al.,* 1993, 1994; Favaron *et al.,* 1994; Yao *et al.,* 1999; Ramanathan *et al.,* 1997; Deo and Shastri, 2003). In this chapter, the isolation and molecular characterisation of a full-length *pgip* gene from *Eucalyptus grandis* using conventional PCR and genome-walking PCR techniques is reported.

The reported composite *Eucalyptus pgip* gene is comprised of a single open reading frame of 996bp that is uninterrupted by introns. It encodes a protein of 331 amino acids with a predicted molecular mass of 36.5 kDa. A theoretical isoelectric point of 6.2 was calculated (Table 2.3). A comparison with published sequences of pear, apple, bean and soybean PGIPs, revealed the conservation of the potential processing site for signal peptidase (Toubart *et al.,* 1992) (Figure 2.18). The N-terminal highly hydrophobic region of the composite *Eucalyptus pgip* gene corresponds to the putative signal peptide, which is identical to that of the pear PGIP signal peptide sequence. The signal peptide has been proposed to have a role in targeting the PGIP through the endomembrane system (Stotz *et al.,* 1993).

The complete polypeptide sequence differs from the published pear polypeptide sequence by only three amino acids at the C-terminal region. When compared to the partial *Eucalyptus* polypeptide sequence, however, there is an observed difference of four amino acids (GKHR) within the peptide sequence (Figure 2.18). The *Eucalyptus* sequence obtained in this study was TSID, which is identical to that found in both pear and apple

PGIP1 (Figure 2.18). Several reasons can be attributed to the observed difference in amino acid sequence between the partial *Eucalyptus* PGIP and the complete *Eucalyptus* PGIP obtained in this study. The most obvious reason could be the use of different *E. grandis* clones in each study, which could possibly result in a difference in the polypeptide sequence. Alternatively, the differences observed could be attributed to the sequencing of a different PGIP allele or PGIP gene family member as compared to the one obtained in this study. The type of polymerase used to amplify the *pgip* gene also plays a significant role as to whether any mutations are incorporated in the gene sequence. In the current study, the DNA sequence printouts were analysed carefully in this region. Furthermore, the sequence of this region was confirmed in this study by direct sequencing from genomic *E. grandis* (TAG5) DNA (Figure 2.7). A possibility is that in the previous study by Chimwamurombe *et al.* (2001), this region may have been difficult to read by the automated sequence prediction software. This would have resulted in a frameshift over this region by the insertion or deletion of bases before and after this region. However, Chimwamurombe *et al.* (2001) obtained the same (GKHR) polypeptide region for each of the five *Eucalyptus* PGIP clones sequenced, thus strengthening the hypothesis that compared to this study, an alternative PGIP allele or PGIP gene family member was previously obtained.

The complete *Eucalyptus* PGIP polypeptide sequence shows relatively high sequence similarity to pear, apple, and tomato PGIPs, and in comparison to bean and soybean PGIPs (Table 2.4). It is interesting to note that the PGIP from *Eucalyptus*, which belongs to the family Myrtaceae, shows a high degree of sequence similarity to pear and apple PGIPs, which both reside in the Rosaceae. These families are phylogenetically rather distant from each other.

According to Chimwamurombe *et al.* (2001), the five *Eucalyptus* species studied each have more than one *pgip* gene. For each species only one of these *pgip* genes were partially characterized. Phylogenetic analysis of PGIPs shows that those from the Myrtaceae group together with those from the Rosaceae (Chimwamurombe *et al.*, 2001). A possible explanation for such a grouping could be that the partially characterized *Eucalyptus pgip* gene included in the phylogenetic analysis was non-homologous to the *pgip* genes from the

other plant species. The high similarity of PGIPs between the distantly related Myrtaceae and Rosaceae could also imply a possible divergence of these PGIPs from a common ancestral PGIP. However, it is tenuous to infer phylogenies based on single gene sequences since multigene analyses that include chloroplast and nuclear genes for example, is usually required before true relatedness of plants can be inferred.

The *Eucalyptus* PGIP contains nine cysteine residues, eight of which are conserved between the apple, pear, tomato, bean and soybean PGIPs. The cysteine residues are clustered at the N- and C-terminal regions of the mature protein and their location, therefore, indicates a potential role in stabilising the secondary structure of the protein (Stotz *et al.*, 1993, Favaron *et al.*, 1994). As in all other characterised PGIPs, the proposed *Eucalyptus* PGIP structure contains seven potential N-glycosylation sites (Asn-X-Ser/Thr) that are shared with the apple and pear PGIPs. Five of the sites are shared with the tomato PGIP, two with bean PGIP and only one site is shared with soybean PGIP.

Table 2.3 Predicted molecular mass and isoelectric point values of some cloned *pgip* genes.

Species	kDa	pI Value	Reference
Phaseolus vulgaris	34	8.81	Toubart *et al.* (1992); Leckie *et al.* (1999)
Pyrus communis	36.5	6.2	Stotz *et al.* (1993)
Glycine max	34	8.21	Favaron *et al.* (1994)
Lycopersicon esculentum	34.3	8.4	Stotz *et al.* (1994)
Eucalyptus grandis	36.5*	6.2*	This study

* http://www.expasy.ch

Table 2.4 Nucleotide and amino acid sequence comparison of the complete *Eucalyptus* PGIP with other published PGIP polypeptide sequences.

Plant Species	Accession No.	%DNA identity	%AA identity	%AA similarity
Soybean	X78274	59	46	60
Bean	X64769	59	49	66
Tomato	L26529	77	66	78
Partial Eucalyptus	AF159167	95	95	96
Apple PGIP1	U77041	97	93	66
Pear	L09264	98	99	99

```
                          10         20         30         40         50         60         70         80         90        100
Pear                .....MELKF STFLSLTLLF SSVLNPALSD LCNPDDKKVL LQIKKAFGDP YVLASWKSDT DCCD..WYCV TCDSTTN..R INSLTIFAGQ VSG..QIPAL [89]
Composite_Eucalyptus.....MELKF STFLSLTLLF SSVLNPALSD LCNPDDKKVL LQIKKAFGDP YVLASWKSDT DCCD..WYCV TCDSTTN..R INSLTIFAGQ VSG..QIPAL [89]
Partial_Eucalyptus                                    VL LQIKKAFGDP YVLASWKSDT DCCD..WYCV TCDSTTN..R INSLTIFAGQ VSG..QIPAL [64]
Apple_PGIP1         .....MELKF SIFLSLTLLF SSVLKPALSD LCNPDDKKVL LQIKKAFGDP YVLTSWKSDT DCCD..WYCV TCDSTTN..R INSLTIFAGQ VSG..QIPAL [89]
Tomato              .........M NLSLLLVVIF LCFASPSLSV RCNPKDKKVL LQIKKDLGNP YHLASWDPNT DCCY..WYVI KCDRKTN..R INALTVFQAN ISG..QIPAA [85]
Bean               MTQFNIPVTM SSSLSIILVI LVSLRTALSE LCNPQDKQAL LQIKKDLGNP TTLSSWLPTT DCCNRTWLGV LCDTDTQTYR VNNLDLSGHN LPKPYPIPSS 100]
Soybean             .......... .......... .........E LCNPQDKQTL LQIKKELGNP TTLSSWHPKT DCCNNSWVGV SCDTVTPTYR VDNLDLSELN LRKPYPIPPS [71]
                                                    ↑

                         110        120        130        140        150        160        170        180        190        200
Pear               VGDLPYLETL EFHKQPNLTG PIQPAIAKLK GLKSLRLSWT NLSGSVPDFL SQLKNLTFLD LSFNNLTGAI PSSLSELPNL GALRLDRNKL TGHIPISFGQ [189]
Composite_Eucalyptus VGDLPYLETL EFHKQPNLTG PIQPAIAKLK GLKSLRLSWT NLSGSVPDFL SQLKNLTFLD LSFNNLTGAI PSSLSELPNL GALRLDRNKL TGHIPISFGQ [189]
Partial_Eucalyptus VGDLPYLETL EFHKQPNLTG PIQPAIAKLK GLKFLRLSWT NLSGSVPDFL SQLKNLTFLD LSFNNLTGAI PSSLSQLPNL NALHLDRNKL TGHIPKSFGQ [164]
Apple_PGIP1        VGDLPYLETL EFHKQPNLTG PIQPAIAKLK GLKFLRLSWT NLSGSVPDFL SQLKNLTFLD LSFNNLTGAI PSSLSQLPNL NALHLDRNKL TGHIPKSLGQ [189]
Tomato             VGDLPYLETL EFHHVTNLTG TIPPAIAKLT NLKMLRLSFT NLTGPIPEFL SQLKNLTLLE LNYNQFTGTI PSSLSQLPNL LAMYLDRNKL TGTIPESFGR [185]
Bean               LANLPYLNFL YIGGINNLVG PIPPAIAKLT QLHYLYITHT NVSGAIPDFL SQIKTLVTLD FSYNALSGTL PPSISSLPNL GGITFDGNRI SGAIPDSYGS [200]
Soybean            VGSLPCLKFL YITNNPNIVG TIPTTITKLT KLRELNIRYT NISGQIPHFL SQIKALGFLD LSNNKLSGNL PSWLPSLPDL YGISFDNNYI SGPIPDLFAS [171]

                         210        220        230        240        250        260        270        280        290        300
Pear               FIG.NVPDLY LSHNQLSGNI PTSFAQMDFT SIDLSRNKLE GDASVIFGLN KTTQIVDLSR NLLEFNLSKV EFPTSLTSLD INHNKIYGSI PVEFTQLN.F [287]
Composite_Eucalyptus FIG.NVPDLY LSHNQLSGNI PTSFAQMDFT SIDLSRNKLE GDASVIFGLN KTTQIVDLSR NLLEFNLSKV EFPTSLTSLD INHNKIYGSI PVEFTQLN.F [287]
Partial_Eucalyptus FIG.NVPDLY LSHNQLSGNI PTSFAQMDFG KHRLSRNKLE .DASVIFGLN KTTQIVDLSR NLLEFNLSKV EFPTSLTSLD VNHNKIYGSI PVEFTQLN.F [260]
Apple_PGIP1        FIG.NVPDLY LSHNQLSGNI PTSFAQMDFT SIDLSRNKLE GDASVIFGLN KTTQIVDLSR NLLEFNLSKV EFPTSLTSLD INHNKIYGSI PVEFTQLN.F [287]
Tomato             FKGPNIPDLY LSHNSLTGHV PASLGDLNFS TLDFSRNKLE GDVSFLFGKN KTSQVIDLSR NLLEFDISKS EFAESLISLD LNHNRIFGSL PPGLKDVP.L [284]
Bean               FSK.LFTAMT ISRNRLTGKI PPTFANLNLA FVDLSRNMLE GDASVLFGSD KNTKKIHLAK NSLAFDLGKV GLSKNLNGLD LRNNRIYGTL PQGLTQLKFL [299]
Soybean          VSE.RFGFIS LSGNRLIGKI PASLGKPDMK IVDLSRNMLE GDASVLFGSE KHTERIYLAN NLFAFDLGKV RLSKTLGLLD VGHNLIYGTL PKGLTSLKDL [270]

                         310        320        330        340
Pear               QFLNVSYNRL CGQIPVGGKL QSFDEYSYFH NRCLCGAPLP SCK. [330]
Composite_Eucalyptus QFLNVSYNRL CGQIPVGGKL QSFDEYSYFH NRCLCGAPLG PCKS [331]
Partial_Eucalyptus QFLNVSYNRL CGQIPVGGKL QSFNEYSYFH NR.......... [301]
Apple_PGIP1        QFLNVSYNRL CGQIPVGGKL QSFDEYSYFH NRCLCGAPLP SCK. [330]
Tomato             QFFNVSYNRL CGQIPQGGTL QSFDIYSYLH NKCLCGSPLP KCK. [327]
Bean               QSLNVSFNNL CGEIPQGGNL KRFDVSSYAN NKCLCGSPLP SCT. [342]
Soybean            YYLDVSYNNL CGEIPRGGKL QEFDASLYAN NKCLCGSPLP SCT. [313]
```

Figure 2.18 Comparison of the deduced amino acid sequences of PGIPs from apple, pear, eucalyptus, tomato, bean and soybean (Yao *et al.*, 1999; Stotz *et al.*, 1993; Chimwamurombe *et al.*, 2001; Stotz *et al.*, 1994; Toubart *et al.*, 1992; Favaron *et al.*, 1994). The arrow indicates the N-terminal position for each of the mature peptides. The cysteine residues are highlighted in blue and the potential N-glycosylation sites are indicated in green. Amino acids that differ between the pear PGIP and the partial *Eucalyptus* PGIP are highlighted in green. Amino acids that differ between the composite *Eucalyptus* PGIP and the pear PGIP are highlighted in yellow.

Like all other PGIPs isolated to date, *Eucalyptus* PGIP is almost entirely composed of 10 imperfect Leucine-rich repeats (LRRs) characterised by the consensus sequence LxxLxxLxxLxLxxNxLxGxIPxx (Jones and Jones, 1997) (Figure 2.19). LRR motifs that are similar to those found in PGIPs, occur in both bacterial and eukaryotic proteins (De Lorenzo *et al.,* 1997; Marino *et al.,* 2000). In eukaryotes, LRR motifs are found in proteins encoding the disease resistance (*R*) genes of the plant immune system and by the *toll* and *toll*-like genes of *Drosophila* and mammals (Marino *et al.,* 2000). The consensus sequence of the repeating units is strikingly similar in all described LRR proteins, indicating a selection pressure for the conservation of this structure (De Lorenzo and Cervone, 1997). Many of these proteins are part of the cell wall receptors that respond to elicitors and are thus involved in protein-protein interactions and signal transduction pathways (Kobe and Deisenhofer, 1994; Ramanathan *et al.,* 1997).

Most plant resistance (*R*) genes encode leucine-rich repeat (LRR) proteins, which function as putative receptors that are able to trigger defence responses upon recognition of a pathogen (Jones and Jones, 1997). Similarly, PGIPs, which recognise extracellular polygalacturonases secreted by pathogenic fungi, contain LRR domains that are homologous to proteins encoded by plant disease (*R*) resistance genes that include tomato Cf-9, Cf-4 and Cf-4, and the rice Xa21 gene (Jones and Jones, 1997; Komjanc *et al.,* 1999). Within each LRR domain is a specific sequence xxLxLxx occurs, which is predicted to form a solvent exposed β-sheet/β-turn structure and is directly involved in ligand interaction (Leckie *et al.,* 1999; De Lorenzo and Ferrari, 2002). The amino acids of PGIP that determine specificity and affinity for fungal PGs reside within the β-sheet/β-turn structure. Thus, information on the structural and functional basis of the interactions between PGIPs and PGs can be obtained from manipulations of LRR receptor proteins. This information can ultimately lead to a better understanding of how plants recognise non-self molecules from pathogenic microorganisms.

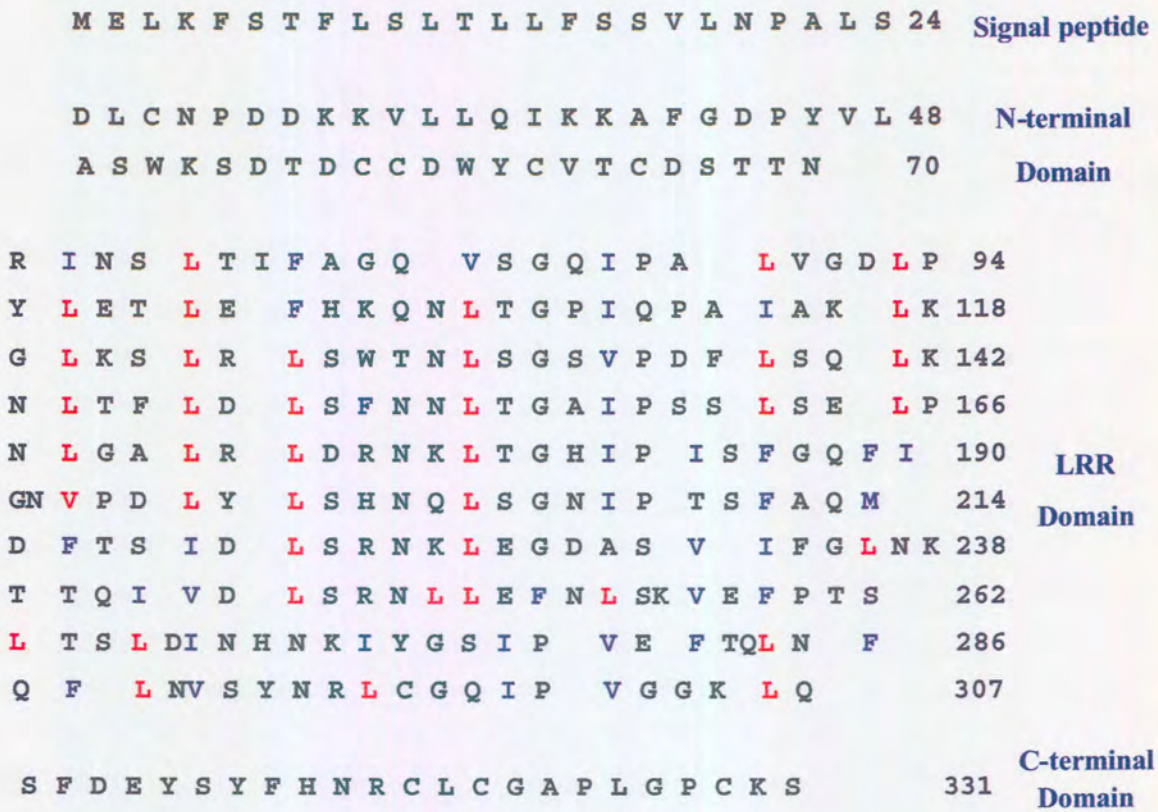

Figure 2.19 Predicted domain structure of the complete *Euclayptus* PGIP. The amino acid sequence is shown in single-letter code. Leucine residues are indicated in red. Changes in the consensus sequence, where L and I tend to be substituted by each other and by M, V and F are indicated in blue.

This chapter reports on the cloning and characterisation of a *Eucalyptus pgip* gene. The full-length *Eucalyptus pgip* gene is uninterrupted by intron sequences and shows 99% amino acid identity to pear PGIP (Stotz *et al.*, 1993). The high sequence homology to pear PGIP could imply that they have evolved from the same ancestral PGIP, however such conclusions can only be derived from comparative analysis of all PGIP copies from both plant species.

Chapter Three

Transformation of *Nicotiana tabacum* cv. La Burley with a *Eucalyptus pgip* gene and inhibition of *Coniothyrium zuluense* polygalacturonases

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Abstract

Transgenic tobacco plants expressing the *Eucalyptus* PGIP were produced via *Agrobacterium*-mediated plant transformation. The intermediate pRTL2 vector provided an enhanced CaMV dual 35S promoter, for transcription of the *Eucalyptus pgip* gene and a TEV leader sequence for enhanced protein translation. The expression cassette was cloned into the multiple cloning site of the pCAMBIA 2301 *Agrobacterium*-based plant transformation system. The resulting construct was used to transform *Nicotiana tabacum* cv LA Burley plants. Transgenic plants were regenerated on medium containing kanamycin and displayed phenotypic characteristics identical to non-transformed plants. PCR analysis of eight transgenic lines revealed the presence of the *Eucalyptus pgip* gene, the *nptII* gene as well as the GUS-intron gene, however no hybridisation signals could be observed by Southern analysis. GUS analysis of the eight transgenic lines revealed the expression of the GUS-intron gene by the blue staining of the plant tissue. PGIP expression studies of the eight transgenic events showed no inhibition from *Eucalyptus* PGIP extracts towards PGs of the fungal pathogen, *Coniothyrium zuluense.*

Introduction

The incorporation of disease resistance genes for the development of improved crop cultivars is one of the major challenges facing plant breeders. Conventional breeding methods involving crosses made between resistant and susceptible parents has been successfully used for the introduction of disease resistance genes into plants (Punja, 2001). The advent of recombinant DNA technology in the 1970's allowed for the powerful combination of genetic engineering and conventional breeding programs making possible the introduction of useful genetic traits into commercial crops within economically viable time frames (Hansen and Wright, 1999).

Applications of plant genetic engineering lie beyond crop improvement. Among the first transgenic plants produced were those that are resistant to viruses and insects, tolerant to herbicides and plants that are being used as 'bioreactors' to produce pharmaceutical products and food additives (Gelvin, 1998). Transgenic plants also provide an informative tool for study of gene function within a plant system and to monitor physiological and developmental changes (Hansen and Wright, 1999).

Modern plant genetic engineering involves the transfer of the desired genes into the plant genome, and then the regeneration of the whole plant from the transformed tissue. The successful transformation of plants depends on the availability of certain essential requirements that include (a) target tissues competent for propagation and regeneration, (b) a method to deliver the DNA into the plant cells and (c) suitable agents to select and regenerate transgenic tissues (Birch, 1997; Hansen and Wright, 1999). Several approaches for the genetic modification of plant species have been developed. These range from the exploitation of the natural gene transfer system of *Agrobacterium* (Hooykaas and Schilperoort, 1992; Zambryski, 1992; Hooykaas and Mozo, 1994) to direct gene transfer techniques that include the chemical treatment of isolated protoplasts by polyethylene glycol (PEG) and the physical introduction of DNA by electroporation (Bilang *et al.,* 1994) and microprojectile bombardment (Christou, 1994; Southgate *et al.,* 1995).

Transformation Systems

Plant protoplasts are commonly transformed via treatment with polyethylene glycol (PEG). Treatment with PEG alters the properties of the plasma membrane by causing reversible permeabilization thereby enabling external macromolecules to enter the plant cytoplasm (Songstad *et al.,* 1995). PEG-mediated direct transformation can be affected by several parameters that include the concentration of magnesium and calcium ions in the incubation mixture and molecular weight and concentration of the PEG (Bilang *et al.,* 1994). PEG has been successfully used as a DNA delivery vehicle for a range of dicots as well as a number of monocot plants, where multiple copy integration and DNA sequence rearrangements have been observed (Bilang *et al.,* 1994; Songstad *et al.,* 1995). Efficient transformation with PEG requires a protoplast system, giving this technique a less broader application range than that of direct DNA transfer via electroporation (De Block, 1993).

Electroporation involves subjecting the plant cells or tissues to electrical pulses that cause reversible permeabilization of the plasma membrane allowing efficient transport of the DNA into the plant cell (Songstad *et al.,* 1995). The primary advantage of electroporation is the reproducibility of DNA delivery and the simplicity of the technique (De Block, 1993). However, multiple gene integrations are common and most inserted copies are tightly linked and may thus lead to gene silencing (De Block, 1993).

Microprojectile bombardment or biolistics has been used to transfer DNA into cells of plants, animals, yeast, fungi and bacteria (Kikkert *et al.,* 1999). The procedure involves the delivery of microprojectiles, usually of tungsten or gold to which purified DNA has been precipitated and propelled at a high velocity into target cells (Hansen and Wright, 1999). Advantages of particle bombardment are that DNA can be delivered into virtually any tissue and transformation is genotype-independent (Walden and Wingender, 1995). Successful transformation depends on the ability of the target tissue to proliferate and produce fertile plants. As with other transformation systems, biolostics is not without limitations. These being the need for expensive

equipment, the production of chimeric plants, and multiple insertion events that leads to gene silencing by co-suppression (Southgate *et al.*, 1995).

The natural ability of the gram-negative soil-borne pathogenic bacteria, *Agrobacterium-tumefaciens* to transfer its DNA into a plant's genome has been exploited and is now the most widely used method for transferring genes into plants (Zupan and Zambryski, 1995). The general strategy for *Agrobacterium*-mediated transformation involves the incubation of plant cells or tissues with the bacterial culture, followed by regeneration of plants from transformed cells (Walden and Wingender, 1995). The *A. tumefaciens* system is simple, efficient and inexpensive. The DNA transferred to the plant genome is defined and therefore rearrangements that rarely occur can be readily detected. Integration occurs as a single copy in many cases (De Block, 1993; Walden and Wingender, 1995). A limitation is that several plant species are recalcitrant to transformation with *Agrobacterium*, but recent modifications to the transformation system has enabled the transformation of some monocots even though they are not generally their natural hosts (Hansen and Wright, 1995; Gelvin, 2003).

The *Agrobacterium*-mediated plant transformation strategy

Wild type *A. tumefaciens* results in the formation of crown gall tumors at the wound sites of infected dicotyledonous plants (Hooykaas and Schilperrort, 1992). During infection, a piece of DNA called the T-DNA (transfer DNA) is transferred from the bacterium to the plant cell. The T-DNA is carried on a large (~200kb) plasmid called the Ti (tumor inducing) plasmid and is delimited by 25bp direct repeats that flank the T-DNA. Any DNA between these borders will be transferred to the plant cell (Zupan and Zambryski, 1995).

The Ti plasmid contains a *vir* (virulence) region that encodes products necessary to mediate the transfer of the T-DNA (Zambryski, 1992). The *vir* region is 40kb in size and is organised into eight complementary groups, *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*F, *vir*G and *vir*H (Hooykaas and Beijersbergen, 1994). Bacterial elements required for the attachment of the *A. tumefaciens* to the plant cell and subsequent bacterial

colonization are located in the *Agrobacterium* chromosome. These chromosomal-determined elements include the *chv*A, *chv*B, *psc*A and the *att* genes (Hooykaas and Beijersbergen, 1994; de la Riva *et al.,* 1998).

The transfer of genes from *Agrobacterium* to the plant cell requires several important steps. These include (1) bacterial colonization, (2) induction of the virulence system, (3) generation of the T-DNA transfer complex, (4) T-DNA transfer and (5) integration of the T-DNA into the plant genome (de la Riva *et al.,* 1998). A schematic representation of the process of *Agrobacterium*-mediated transformation is depicted in figure 3.1.

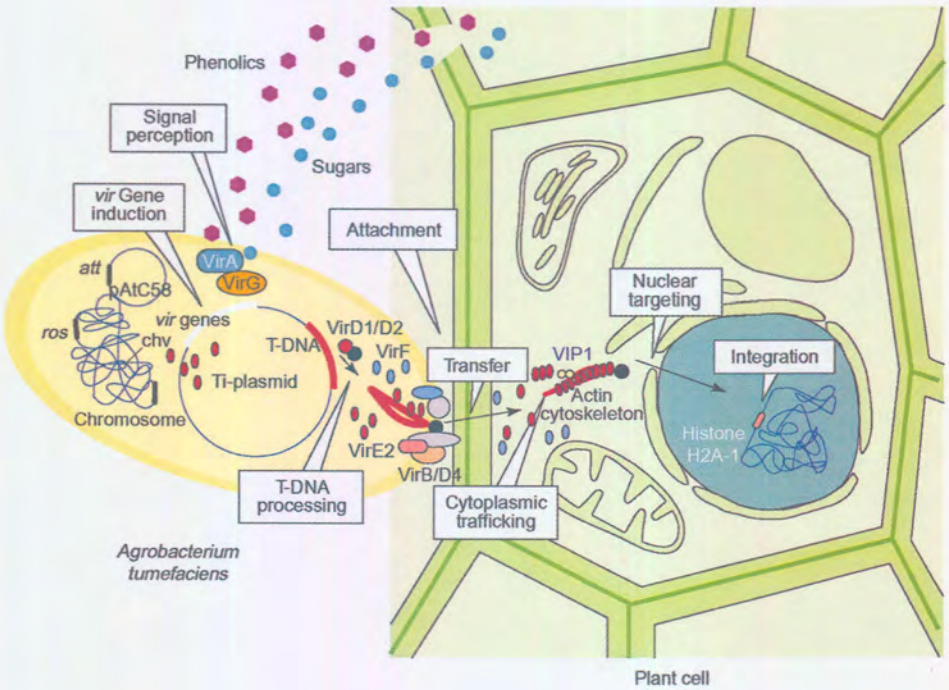

Figure 3.1 Schematic representation of the *Agrobacterium*-mediated transformation process taken from Gelvin (2003).

The first step in tumor induction is the attachment of *Agrobacteria* to the plant cells at the wound site. Wounded cells release low-molecular-weight phenolic compounds

such as acetosyringone that induces the *Agrobacterium vir* genes (Zambryski, 1992; Hooykaas and Beijersbergen, 1994). The VirA and VirG proteins are a two-component regulatory system that controls gene expression. VirA is a transmembrane sensor protein that detects the phenolic compounds released by wounded cells and in so doing becomes autophosphorylated. The activated VirA then phosphorylates VirG, which then functions as a transcription factor regulating the expression of the *vir* genes (Zupan and Zambryski, 1995; de la Riva *et al.,* 1998). Upon activation of the *vir* genes, the VirD1 and VirD2 proteins recognise the T-DNA border sequences and via their endonuclease activity, start to nick the bottom T-DNA strand. Following endonuclease activity, the VirD2 protein remains attached to the 5'-end of the single stranded (ss) T-strand thus distinguishing the 5'-end as the leading end of the T-DNA transfer complex. The ss-T-DNA-VirD2 complex is coated with the 69kDa VirE2 protein, which protects the complex from nuclease attack. The VirB proteins start forming a channel involving both the *Agrobacterium* as well as the plant cell membranes thereby allowing the ss-T-DNA complex to enter the plant cell where it is targeted to the nucleus. The final step of T-DNA transfer is its integration into the plant genome (Zambryski, 1992; Zupan and Zambryski, 1995; de la Riva *et al.,* 1998).

The natural ability of *A. tumefaciens* to genetically transform plant cells during infection identified the Ti (tumour-inducing) plasmid of the plant pathogen as a potential gene vector for the transformation of higher plants (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995). Infection by the bacterium causes crown gall disease that is characterised by the formation of tumours (Hooykaas and Schiloperoort, 1992). The ability to produce tumours on plants was found to be associated with the possession by the bacterium of a large Ti plasmid (Zambryski, 1992). Genetic experiments conducted to establish whether the entire Ti plasmid or just the T-DNA is introduced into plant cells via *Agrobacterium* showed that no physical linkage between the Ti plasmid and the T-DNA region is required for efficient transfer to occur (Hooykaas and Schilperoort, 1992). These results lead to the development of two vector systems for the transformation of plants.

The co-integrate system allows the incorporation of a gene of interest into an artificial T-DNA already present on the Ti plasmid, via homologous recombination (Hooykaas

and Schilperoort, 1992; Walkerpeach and Velten, 1994). The binary vector system makes use of a shuttle vector containing the gene of interest between T-DNA borders and a 'helper' Ti plasmid containing the *vir* gene products necessary for gene transfer (Walkerpeach and Velten, 1994). The Ti plasmids are disarmed by removing the oncogenic genes that cause tumour formation, but are still capable of providing the necessary gene products required for transferring the T-DNA to the host plant cell. The binary vector system is a more commonly used system that does not require *in vivo* recombinational events to be stably maintained within the *A. tumefaciens* strain. Additionally, the *in vivo* and *in vitro* DNA manipulations of small binary vectors are much easier than DNA manipulations of large co-integrate systems and higher transformation efficiencies are obtained with the use of binary vector systems (Walkerpeach and Velten, 1994).

Transgene selectable and scorable markers

Selection forms an important process in plant transformation. Generally, the gene of interest is co-transformed with a selectable marker that enables the identification of transgenic events. Selectable markers are generally based on the sensitivity of plants to antibiotics and herbicides thus allowing the direct selection of transgenic cells by their ability to proliferate under selective conditions (Hooykaas and Schilperoort, 1992; Birch, 1997). A wide variety of selectable markers are available, but the most commonly used markers include aminoglycoside 3'-phosphotransferase II (NPTII), which confers resistance to the antibiotic kanamycin. The hygromycin phosphotransferase (*hpt*) gene originally derived from *Escherichia coli* was modified for expression in plant cells and has subsequently been widely used as a resistance gene (Herrera-Estrella *et al.,* 1988; Angenon *et al.,* 1994).

Transient gene expression can be studied with the use of several reporter genes. These include β-glucuronidase (GUS), chloroamphenicol acetyltransferase (CAT), luciferase and genes involved in anthocyanin biosynthesis (Chee *et al.,* 1991; Hansen and Wright, 1999). The histochemical assay for β-glucuronidase (GUS) activity has been the method of choice as many higher plants lack endogenous β-glucuronidase

(Jefferson *et al.*, 1987). There are various β-glucuronic acid substrates available for GUS detection. All of these substrates contain a D-glucopyranosiduronic acid sugar that is linked to a hydroxyl group of a chromogenic, fluorogenic or other molecule by means of a glycosidic linkage. The β-glucuronidase enzyme cleaves the glycosidic bond and releases the detectable molecules. X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) is the substrate of choice for the histochemical localisation of GUS activity. The colourless substrate produces a blue precipitate at the site of enzyme activity (Jefferson *et al.*, 1987; Hooykaas and Schilperoort, 1992).

Molecular identification of transgenic plants

Selectable markers and reporter genes has been successfully used in the identification of transgenic plants. However, additional screening methods based on molecular analysis of transgenic plants provides further verification of transgene insertion (Chee *et al.*, 1991). Determination of the structural integrity of transferred target genes can be conducted by PCR analysis with gene-specific primers and DNA Southern blot analysis (Southern, 1975). Southern analysis provides information on the number of transgene insertion events but does not necessarily provide information on whether the gene is being expressed. Gene expression can be studied using Northern blot analysis and Western blots are generally used to determine the presence of the protein product of the gene.

Transformation of plants for improved resistance to fungal diseases

Since the beginning of the plant molecular biology era in the late 1980's, a major research focus has been on the ability to identify, clone and characterise various disease resistance genes (Punja, 2001). Several approaches have been utilised by researchers to incorporate resistance to diseases. Of particular interest is the expression of gene products that can destroy or neutralise pathogen virulence products such as polygalacturonases (PGs) (Punja, 2001; Ferrari *et al.*, 2003).

Plants have been engineered to express glycoproteins called polygalacturonase-inhibiting proteins (PGIPs) that can inhibit the activity of fungal endopolygalacturonases (Desiderio *et al.,* 1997; Powell *et al.,* 2000). *Agrobacterium*-mediated transformation of tomato plants expressing a bean PGIP gene did not enhance resistance to the fungal pathogens *Fusarium, Botrytis* or *Alternaria* but tomato plants transformed with a pear PGIP gene showed reduced colonization of leaves and fruits by *Botrytis* (Desiderio *et al.,* 1997; Powell *et al.,* 2000). Similarly, tomato cotyledons transformed with a bean PGIP-1 gene showed inhibition of PGs from both *Aspergillus niger* as well as *Stenocarpella maydis* (Berger *et al.,* 2000). Resistance to these fungi was however, not reported. Experiments describing the production of transgenic plants for enhanced fungal resistance are discussed in further detail in Chapter 1.

The *Eucalyptus* canker pathogen *Coniothyrium zuluense*

Coniothyrium canker caused by *Coniothyrium zuluense* is an important *Eucalyptus* stem canker pathogen (Wingfield *et al.,* 1997; Van Zyl, 1999). The disease was first noted in South Africa in 1988 on a single *Eucalyptus grandis* clone. Subsequent to that, *C. zuluense* has been reported to infect a large number of *Eucalyptus* species, clones and hybrids (Wingfield *et al.,* 1997). Early pathogen infection can be observed by the occurrence of small necrotic lesions on young branches and stems. These eventually develop into large girdling cankers that reduce the wood quality and may eventually lead to the death of the tree (Wingfield *et al.,* 1997; Van Zyl, 1999).

Cell-wall degrading enzymes are essential factors for the virulence of plant pathogens to their hosts (Collmer and Keen, 1988; Schafer, 1994; Alghisi and Favaron, 1995). Previous studies on the pathogenicity of *C. zuluense* to susceptible *Eucalyptus* clones showed the positive involvement of polygalacturonases (PGs) to the virulence of the fungus (Van Zyl, 1999). Chimwamurombe (2001) conducted experiments to test the inhibition of polygalacturonases from four tree pathogens by PGIP extracts from two *E. grandis* clones. Their results showed that the fungal pathogens *C. zuluense* and *Botryosphaeria dothidea* produced polygalacturonases with a higher activity than those from *Cryphonectria cubensis* and *Phytophthora cinnamomi.* PGIP extracts

from TAG5, a moderately tolerant *E. grandis* clone and ZG14, a susceptible clone, were tested for their ability to inhibit PGs produced by the fungal pathogens. Inhibition studies revealed that PGs from *C. zuluense* were inhibited to a greater capacity by PGIP extracts from the moderately tolerant TAG5 clone than the susceptible ZG14 clone. This suggested that PGIPs are involved in the general ability of TAG5 to protect itself from fungal invasion by *C. zuluense.*

Assays for Polygalacturonase activity and PGIP inhibition

Several methods have been developed to determine the activity of polygalacturonases in culture filtrates. These include a simple, rapid qualitative agarose diffusion assay based on the degradation of the polygalacturonic acid (PGA) substrate by fungal endopolygalacturonases present in the culture filtrate (Taylor and Secor, 1988). Culture filtrates are applied to wells in an agarose assay medium containing the PGA substrate. The enzymatic activity of the endopolygalacturonase is detected by staining the plates with a ruthenium red dye that interacts with unhydrolysed PGA. Areas of enzymatic activity and thus areas where the PGA hydrolysis has occurred appear as clear zones. In the inhibition assay itself, equal amounts of the culture filtrate and PGIP extract are mixed and the mixture is added to the wells. A reduction in the zone size is an indication of PG inhibition by the PGIP extract.

A second method that measures the amount of reducing sugars present, is a quantitative assay called the reducing sugar assay (York *et al.,* 1985). In this assay the polygalacturonic acid substrate is degraded by the fungal endopolygalcturonases to produce reducing sugars. Addition of a colour reagent such as ρ-4-amino-2-hydroxybenzoic acid hydrazide (PAHBAH) reacts with the reducing sugars and under alkaline conditions, produces yellow anions. The anions produced by this reaction can then be measured spectrophotometrically at a wavelength of 410nm. The product of this reaction is proportional to the amount of reducing sugars present (York *et al.,* 1985).

This chapter deals with the transformation of *Nicotiana tabacum* cv. LA Burley plants with a *Eucalyptus pgip* gene and the subsequent analysis of transgenic plants for their

ability to inhibit polygalcturonases produced by the fungal pathogen *C. zuluense*. An expression cassette containing the *pgip* gene amplified from *E. grandis* genomic DNA and placed under control of plant expression signals was cloned into a pCAMBIA plant transformation vector. Transgenic tobacco plants carrying the *Eucalyptus pgip* gene were produced via *Agrobacterium*-mediated plant transformation. Construction of the PGIP expression cassette and molecular characterisation of the transgenic plants are discussed. This chapter further describes the preparation of polygalacturonases from culture filtrates of *C. zuluense* as well as the extraction of PGIP proteins from the transgenic tobacco plants. Several assays to test the expression of PGIP activity and its ability to inhibit the fungal polygalacturonases are presented.

Materials and Methods

Construction of a recombinant plant transformation vector

The construction of a recombinant plant transformation vector required several intermediate steps (Figure 3.2). A *Eucalyptus pgip* gene amplified from genomic DNA was initially cloned into the commercial cloning vector, pGEM-T-Easy (Appendix C). Sequence analysis of the fragment enabled the design of gene-specific primers containing suitable restriction ends for cloning into the intermediate pRTL2 vector (Appendix C). The recombinant cassette contained the *Eucalyptus pgip* gene under the control of a dual Cauliflower Mosaic Virus (CaMV) 35S promoter. The expression cassette was subsequently coned into the *Agrobacerium*-based plant transformation vector, pCAMBIA 2301 (Appendix C).

Construction of a *Eucalyptus pgip* gene cassette

Primer design and PCR amplification of the *Eucalyptus pgip* gene

Sequence analysis of the partial *Eucalyptus pgip* gene discussed in Chapter 2, enabled the design of two gene-specific primers, EN-PGIP-FWD and REV-PB-PGIP. PCR was performed in 0.2ml thin-walled tubes in the GeneAmp PCR system 9700

(Applied Biosystems). The PCR reaction mixture included 2.5 units of Expand *Taq* polymerase (Roche Diagnostics, Germany), 10 X reaction buffer, 40ng of pGEM-*Eucpgip*110B plasmid DNA as template, 0.25mM dNTPs and 0.2µM of each primer in a 50 µl reaction volume. A negative water control was included to test for the presence of any contaminants.

The thermal cycler was programmed to include one denaturing cycle at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at 65°C and then primer extension at 72°C for 30 sec. A final step at 72°C for seven min was included to ensure complete elongation of the fragment. The PCR products were visualised on a 1 % (w/v) agarose gel, containing ethidium bromide, under an ultraviolet light. The PCR products were purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Germany).

Restriction Enzyme digestion of the PCR product

PCR amplification resulted in the production of a 1kb fragment flanked by *Nco*I and *Bam*HI sites at the 5' and 3' ends respectively. A ten microliter volume of purified PCR product was digested at 37 °C for 3 h with 2U *Nco*I and 5U *Bam*HI in a total reaction volume of 20µl. The digested products were separated on a 1% (w/v) agarose gel and visualised under UV light.

Preparation of the pRTL2 vector

The pUC18-derived plasmid vector, pRTL2 is an intermediate vector used in the construction of the plant transformation vector. It contains a dual CaMV35S promoter as well as the tobacco etch potyvirus (TEV) leader sequence for enhanced translation (Restrepo *et al.*, 1990).

Restriction enzyme digestion

Four hundred nanograms of the pRTL2 plasmid DNA was subjected to double digestion with the enzymes *Nco*I (Roche) and *Bam*HI (Roche). The reaction mixture was made up to a final volume of 20 µl with an appropriate amount of restriction enzyme Buffer B and water. The reaction was allowed to proceed for 3 h at 37°C before being subjected to gel electrophoresis in order to determine whether complete digestion had occurred.

Cloning of the *Eucalyptus pgip* gene into the pRTL2 vector

Ligation of the *Eucalyptus pgip* PCR fragment into the digested pRTL2 vector was performed with a 3:1 insert-to-vector molar ratio. A 10µl ligation reaction was set up containing 22ng of the digested pRTL2 vector, 54ng of the *Nco*I/*Bam*HI digested *Eucalyptus pgip* PCR product, 2X Rapid ligation buffer and 3 Weiss units/µl T4 DNA ligase. The reactions were incubated for 16 h at 4°C.

The ligated plasmids were transformed into competent *E. coli* (JM109) cells (Promega) as described in Chapter 2. Ten colonies were selected randomly and screened for the presence of the insert by isolating the plasmid using the Wizard®Plus SV Miniprep DNA Purification System (Promega) according to the manufacturer's specifications and then subjecting the plasmid DNA to PCR screening.

Screening of positive transformants by PCR and restriction digests

Putative recombinants were tested for the presence of the *Eucalyptus pgip* insert by means of PCR and restriction enzyme digestion. Standard PCR was performed using gene-specific EN-PGIP-FWD and REV-PB-PGIP oligonucleotide primers. Thirty PCR cycles were carried out with denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The reaction had an initial denaturation step of 94°C for 3 min and a final elongation step of 72°C for 7 min. PCR products

were analysed by gel electrophoresis using a 1% (w/v) agarose gel stained with ethidium bromide.

Restriction enzyme digestion was also carried out to further verify the insertion of the *Eucalyptus pgip* gene into the pRTL2 vector. The isolated plasmid DNA was digested with *Nco*I (Roche) and *Bam*HI (Roche) in a final reaction volume of 20µl. The digested products were separated together with a λIII (*Hin*dIII/*Eco*RI) marker and a 100bp DNA ladder on a 1% (w/v) agarose gel stained with ethidium bromide.

Nucleotide screening of p*Euc*RTL2

The recombinant plasmid designated as p*Euc*RTL2-2 was selected and sequenced using a pBI121 sequencing primer #2 (5'-GACGCACAATCCCACTATCC-3') designed to sequences within the CaMV 35S promoter. This primer enabled the sequencing of the 5'-junction region between the pRTL2 vector and the *Eucalyptus pgip* gene in order to establish whether the sequences were in frame. Sequencing reactions were set up using the BIG Dye terminator cycle sequencing kit and the samples were subjected to automated sequencing with an ABI Prism model 3100 sequencer (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA). Sequence analysis and alignments were done using the ABI Prism® SeqScape Software Version 1.0 (Applied Biosystems) computer software program.

Preparation of the plant transformation vector pCAMBIA 2301

The pCAMBIA 2301 plasmid is a pUC18-derived, *Agrobacterium*-based plant transformation vector (http://www.cambia.org). It contains unique restriction sites in the multiple cloning site (MCS) that is located within a β-galactosidase α-segment thus allowing blue/white screening for fragment insertion. The vector contains a kanamycin resistance gene for bacterial selection and it has a neomycin phosphotransferase II (*nptII*) gene that encodes kanamycin resistance for selection in

plants, within the T-DNA border region. It also contains a GUS-intron gene as a marker for selection of transformants.

The pCAMBIA 2301 plant transformation vector was digested with 5U of *Pst*I (Roche) made up to a final reaction volume of 20µl with Restriction buffer H and water. Digestion of the plasmid was allowed to proceed for 3 h at 37°C. An aliquot of the digested products were analysed on a 1% (w/v) agarose gel stained with ethidium bromide and visualised under a UV light.

Construction of the pECambia4 plant transformation vector

Excision of the *Eucalyptus pgip* cassette from p*Euc*RTL2

The expression cassette contained within the recombinant p*Euc*RTL2-2 vector had to be spliced out with appropriate restriction enzymes for successful ligation into the *Pst*I site of the linearized pCAMBIA 2301 vector. Attempts to release the expression cassette by digestion with *Pst*I were unsuccessful. A double digestion with *Sca*I and *Pst*I was thus attempted. Two hundred nanograms of p*Euc*RTL2-2 was digested with 5U of each restriction enzyme in a total volume of 20µl and this resulted in the removal of the expression cassette from the recombinant vector.

The samples were separated on a 1% (w/v) agarose gel stained with ethidium bromide and visualised under UV light. A 2132bp expression cassette was purified from the 1% (w/v) agarose gel using the QIAquick Gel Extraction Kit Protocol (Qiagen, Germany) according to the manufacturer's protocols.

Cloning of the expression cassette into pCAMBIA 2301

The gel purified expression cassette was ligated into the *Pst*I site of the linearised plant transformation vector pCAMBIA 2301. Ligation was performed with a 3:1 insert-to-vector molar ratio and a 10µl ligation reaction was set up in which approximately 76ng of insert and 25ng of pCAMBIA 2301vector DNA was used.

Three units of T4 DNA ligase and 5µl of a 2X Rapid ligation buffer was included in the reaction mixture that was incubated at 4°C for 16 h.

The ligation reactions were transformed into competent JM109 cells as described in Chapter 2. The cells were plated onto LB Agar plates supplemented with 10mg/ml Kanamycin, 50mg/ml X-gal and 0.1M IPTG. Following an overnight incubation at 37°C, single colonies were selected and inoculated into LB medium supplemented with 10mg/ml kanamycin. Plasmid DNA was isolated using the Wizard®Plus SV Miniprep DNA Purification System (Promega) according to the manufacturer's protocol.

PCR screening and restriction enzyme digestion of the pECambia recombinants

The putative pECambia recombinants were subjected to PCR screening using the EN-FWD-PGIP and REV-PB-PGIP primers. A 20µl PCR reaction was set up containing 50ng plasmid DNA as template, 0.25mM dNTPs, 10 X reaction buffer and 0,2µM of each primer. PCR was performed for 30 cycles (one cycle = 1 min at 94°C, 2 min at 55°C, 1 min at 72°C). The reaction had an initial denaturation step of 3 min at 94°C and a final elongation step of 7 min at 72°C. A control PCR amplification reaction was performed using 50ng of the p*Euc*RTL2 plasmid DNA.

Restriction enzyme digestion was performed to determine the presence and orientation of the expression cassette in the pCAMBIA 2301 vector. Fifty nanograms of plasmid DNA was digested at 37°C with 5U *Nco*I in a final volume of 20µl. The samples were separated on a 1% (w/v) agarose gel in 1 X TAE buffer (pH 8.0).

Production of transconjugant *Agrobacterium tumefaciens* LBA4404

Growth and maintenance of the *Agrobacterium* strains

The *Agrobacterium tumefaciens* strains used in this study include the recipient LBA4404 strain that shows resistance to the antibiotic rifampicin and C58, which harbours a pCAMBIA 2301 vector and shows resistance to kanamycin and rifampicin. The C58 *Agrobacterium* strain was included in this study as a positive plant transformation control and was obtained from Professor AM Oberholster of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria. The LBA4404 strain was obtained from Professor K Kunert, also of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria. Both strains were maintained at -80°C in 50% (v/v) sterile glycerol. The LBA4404 bacterial culture was streaked onto YEP agar plates supplemented with 50µg/ml rifampicin (Rf), while the C58 culture containing the pCAMBIA 2301 plasmid was streaked out onto medium supplemented with both kanamycin and rifampicin. The plates were incubated at 28°C for 2 days to allow single bacterial colonies to develop.

Chemically based direct transformation of *A. tumefaciens*

Direct transformation is a two-stage process that requires firstly the production of competent *Agrobacterium* cells and secondly the 'freeze-thaw' transformation of these cells with the plant construct. The process usually takes 3 days to complete and is based on a method by An *et al.* (1988). Both the LBA4404 as well as the C58 *Agrobacterium* strains were subjected to direct transformation. The LBA4404 strain was transformed with the constructed pECambia4 plant transformation vector containing the *Eucalyptus pgip* expression cassette and the C58 *Agrobaterium* strain was transformed with an unmodified pCAMBIA 2301 plant transformation vector containing a GUS-intron gene.

Preparation of $CaCl_2$ competent *Agrobacterium* cells

Seed cultures of LBA4404 and C58 were prepared by inoculating 5ml of YEP medium supplemented with 50µg/ml rifampicin (Rf). The cultures were incubated at 28°C for 2 days when they reached the mid-log phase. Two millilitres of each seed culture was used to independently inoculate a larger volume of 50ml YEP medium containing 50µg/ml rifampicin (Rf). The cultures were incubated at 28°C with continuous shaking until an OD_{600} reading of 1 was reached. The samples were then centrifuged at 3000 x g for 15 min and the resulting bacterial pellets were gently resuspended in 1ml of ice-cold 10mM $CaCl_2$. The resuspensions were aliquoted into 100µl volumes in sterile microcentrifuge tube tubes and subjected to quick freezing in liquid nitrogen.

Transformation of competent *Agrobacterium* cells

Five microlitres of the mini-prep pECambia4 plasmid DNA was layered on top of 100µl frozen competent LBA4404 *Agrobacterium* cells. The same procedure was followed for the C58 *Agrobacterium* cells with the isolated pCAMBIA 2301 plasmid DNA. The DNA-bacteria mixtures were incubated in a 37°C water bath for 5 min, followed by the addition of 1ml YEP medium containing 50µg/ml rifampicin (Rf) as well as 25µg/ml kanamycin (Km). For antibiotic resistance gene expression the mixtures were incubated for 4 h at 28°C with continuous shaking. The cultures were centrifuged for 2 min at 12000 x g and the resulting pellets resuspended in 100µl YEP medium. YEP agar plates supplemented with both rifampicin and kanamycin were prepared and 50µl of the suspension was spread onto the plates, which were subsequently incubated at 28°C for 2 days when single colonies could be observed.

Verification of transconjugants

In order to verify the presence of the pECambia4 and pCAMBIA 2301 vectors in the transformed *Agrobacterium,* plasmid DNA was isolated from the *Agrobacterium* colonies using the plasmid 'quick screen' method described by An *et al.* (1988). PCR analysis of the isolated plasmid DNA was subsequently performed.

Plasmid DNA 'quick screen'

The *A. tumefaciens* cells were grown overnight at 28°C in 1ml YEP medium containing 50µg/ml rifampicin and 25µg/ml kanamycin. The overnight culture was centrifuged for 30 sec followed by resuspending the pelleted cells in 100µl of ice-cold Solution 1 (25mM Tris-HCl pH 8.0; 10mM EDTA; 50mM glucose). The resuspension was incubated for 10 min at room temperature before the addition of 200µl of Solution 2 (0.2N NaOH; 1% SDS). Following a second 10 min incubation at room temperature, 30µl of a 1:2 phenol-Solution 2 mixture was added and the sample was briefly vortexed. To this solution, 150µl 3M NaOAc (pH 4.8) was added and the sample was incubated at -20°C for 15 min. The sample was centrifuged for 3 min and the supernatant was transferred to a clean microcentrifuge tube, which was then filled with ice-cold 96% ethanol. The DNA was allowed to precipitate for 15 min at -80°C after which the sedimented pellet was collected by centrifugation and resuspended in 500µl of 0.3M NaOAc, pH 7.0. Absolute ethanol was added and the sample was incubated for a further 15 min at -80°C. The precipitated DNA was sedimented and the resulting pellet was washed with 1ml of ice-cold 70% ethanol. The pellet was air-dried and resuspended in 50µl of TE buffer.

PCR screening

The plasmid DNA isolated from the LBA4404 and C58 *Agrobacterium* colonies were used as template DNA in subsequent PCR reactions in order to assess the presence of the integrated construct. Oligonucleotide primers EN-PGIP-FWD and PB-REV-PGIP were used in PCR reactions for amplification of the *Eucalyptus pgip* gene in the

pECambia4 plant transformation vector. A 20µl PCR reaction was set up containing 50ng plasmid DNA as template, 0.25mM dNTPs, 10 X reaction buffer and 0,2µM of each primer. The cycle conditions included a denaturation step at 94°C for 30 sec, annealing at 55°C for 1 min and elongation at 72°C for 30 sec. The reaction contained an initial denaturation at 94°C for 3 min and a final elongation step at 72°C for 7 min. Oligonucleotide primers GUSforward and GUSreverse (Table 3.1) were used in a PCR reaction with plasmid DNA isolated from the C58/pCAMBIA 2301. The cycle parameters included denaturation at 94°C for 1 min followed by annealing at 67°C for 1 min and elongation at 72°C for 1 min. Thirty-five cycles were carried out with an initial denaturation step at 94°C for 2 min and a final elongation step at 72°C for 7 min. Amplified products were analysed by agarose gel electrophoresis.

Transformation of *Nicotiana tabacum*

Growth of bacterial strains for transformation

The *A. tumefaciens* strain carrying the plant expression vector pECambia4 was grown in liquid YEP medium containing 50µg/ml rifampicin and 25µg/ml kanamycin at 28°C on an orbital shaker until an OD_{600} reading of 1 was reached. Five millilitres of the bacterial culture was centrifuged at 3000 x g for 30 min and the pellet was washed in 500µl of 0.01M $MgSO_4$. Five hundred microlitres of the bacterial cells were diluted in 9.5ml of 0.01 M $MgSO_4$ to give an optical density reading of 0.1 and thus a cell density reading between 10^6 and 10^8 cfu/ml. The C58 bacterial strain was subjected to the same growth conditions as the *A. tumefaciens* carrying the pECambia4 plant expression vector.

Leaf disc transformation

Nicotiana tabacum cv LA Burley obtained from Dr. PM Chimwamurombe, were grown *in vitro* in a modified Murashige and Skoog (MS) medium containing 0.1g/L myo-inositol. Leaf discs were cut from sterile shoots and placed onto solid MS medium for a day prior to incubation with the bacterial co-cultivation medium

(Appendix B) for 20 min. The leaf discs were then removed from the co-cultivation medium and placed back onto the MS plates, which were then incubated for a further 2 days at 25°C in the dark to allow the bacterial DNA to transform the leaf cells. Untransformed controls were prepared by incubating the leaf discs on MS plates. Following the two-day incubation period, the leaf discs were transferred to shoot-inducing medium (Appendix B) supplemented with 250µg/ml cefotaxime to kill the *Agrobacterium* and 300µg/ml kanamycin to select for transformants. One half of the untransformed controls were incubated onto shoot-inducing medium containing both cefotaxime as well as kanamycin whereas the other half was placed onto medium lacking both antibiotics. This was done in order to test the efficiency of the antibiotics. The explants were incubated at 25°C with a 16 h light: 8 h dark cycle for approximately 30 days, transferring to fresh medium every two weeks until callus developed. Once callus had developed, they were cut out and transferred to shoot-inducing medium containing 250µg/ml cefotaxime and 300µg/ml kanamycin and incubated at 25°C with a 16 h light: 8 h dark cycle until shoots developed from the calli. The shoots were then removed and transferred to regeneration medium (Appendix B) in order to induce rooting. The plantlets were incubated at 25°C with a 16 h light: 8 h dark cycle for about four weeks until roots developed and they were then subcultured every three weeks.

Hardening off of transgenic plants

The transgenic tobacco plants were removed from *in vitro* culture and the agar was washed off the roots. The plants were placed in Hygromix (Hygrotech Seed (Pty) Ltd, Silvertondale, South Africa), which contains a 1:1 vermiculite: potting soil mixture. The plants were covered in a plastic bag and incubated at 25°C for 7 days to enable them to acclimatise after which the bags were completely removed and the plants were watered once a week with 0.5g/L Mulifeed® (Plaaskem Ltd.) solution. Plants started to produce flowers after a three-month incubation period in the glasshouse. The flowers were covered with brown paper bags and allowed to produce seed. Seed were harvested from the pods and stored for future use.

Analysis of gene integration

Genomic DNA isolation

Genomic DNA was isolated from the transgenic plants using the adapted method of Lin *et al* (2001). Approximately 100mg of young leaves placed in a 1.5ml microcentrifuge tube tube were exposed to liquid nitrogen for 10 sec followed by homogenisation to a fine powder with a pipette tip. The sample was resuspended in 600µl of a DNA extraction buffer, containing 2% SDS (w/v) and 2% β-mercaptoethanol (v/v). Following an incubation of 15 min at 65°C the samples were centrifuged at 12000 x g for 10 min at 4°C. The supernatant was transferred to a clean tube and 2µl of RNaseA (10mg/ml) was added. The sample was incubated for 30 min at 37°C. An equal volume of a phenol:chloroform:isoamyl alcohol (v/v/v) mixture was added and the samples were gently inverted several times. This was followed by centrifugation at 12000 x g for 3 min. The aqueous phase was transferred to a clean microcentrifuge tube and the phenol:chloroform:isoamyl alcohol extraction was repeated several times until the aqueous phase was clear. The DNA was precipitated by adding 0.6 vol of ice-cold isopropanol to the sample and incubating at -20°C for 10 min. The DNA was pelleted by centrifuging at 12000 x g for 10 min and the resulting pellets were washed twice with 1ml of ice-cold 70% ethanol. The pellets were allowed to dry and then resuspended in 50µl of TE buffer (pH 8.0). The DNA was quantified using the Hoefer® DyNa Quant® 200 fluorometer (Hoefer, Germany) according to the manufacturer's instructions. The fluorometer was calibrated with 1 x TNE buffer, pH 7.4 containing 1µg/ml Hoechst 33258 DNA binding buffer (Boehringer Manheim) as a DNA standard.

PCR analysis

Touchdown-PCR was conducted in 0.2ml thin-walled tubes in the GeneAmp PCR system 9700 (Applied Biosystems). Approximately 50ng of genomic DNA was used in a 25µl PCR reaction containing 10 X PCR buffer, 0.38mM dNTPs, 1.5mM $MgCl_2$, 0.3µM oligonucleotide primer and 1 U of *Taq* polymerase (Roche Diagnostics, Germany). The touchdown-PCR program was an initial denaturation at 94°C for 4

min followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec (-1°C) and elongation at 72°C for 1 min. This was followed by 20 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 1 min and a final elongation step at 72°C for 10 min.

The primer conditions used for screening of the transgenes were as follows:

1. EN-PGIP-FWD and REV-PB-PGIP for amplification of the *Eucalyptus pgip* gene.
2. $NPTII_L$ and $NPTII_R$ for *nptII* amplification and
3. GUS-$intron_L$ and GUS-$intron_R$ for amplification of the GUS gene.

The characteristics of all the primers used in this study are indicated in table 3.1.

Table 3.1 Characteristics of oligonucleotide primers used in this study

Primer	Sequence 5' – 3'	Tm (°C)	%GC
EN-PGIP-FWD	CCCAAAACCATGGAGCTCAAGTTCTTCAC	66	48
REV-PB-PGIP	CGGATCCTTTACTTGCAGCTTGGGAGGGGTGCACCACACAG	75	58
$NPTII_L$	GAGGCTATTCGGCTATGACTG	64	52
$NPTII_R$	ATCGGGAGCGGCGATACCGTA	68	62
GUS-$intron_L$	AATTGATCAGCGTTGGTGGGAAAGC	74	48
GUS-$intron_R$	GAGGTTAAAGCCGACAGCAGCAGTT	76	52
GUSforward	GAAACCCCAACCCGTGAAAT	57	50
GUSreverse	CTGCGGTTTTTCACCGAAGT	57	50

Genomic DNA extracted from tissue culture C58/pCAMBIA 2301 transformed plants was included as a positive control. Negative water controls were also included to test for any contamination. Amplification products were separated on a 1% (w/v) agarose gel stained with ethidium bromide and visualised under UV light.

Southern blot analysis

Genomic DNA was extracted from greenhouse plants using the extraction method of Lin *et al.* (2001) as described previously in this chapter. Two micrograms of genomic

DNA extracted from the transgenic tobacco lines (LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36 and LAT43), a non-transformed control and *Eucalyptus grandis* (TAG5) were restricted with *Eco*RI (Roche). The digested samples were electrophoresed on a 1% (w/v) agarose gel at 50Volts for an hour and 10 min. Approximately 50ng of the *Eucalyptus pgip* fragment and plant transformation vector pECambia4, were included on the 1% (w/v) agarose gel as controls.

Following electrophoresis, the gel was prepared for Southern alkaline transfer. Exposure of the gel briefly to UV-light enabled nicking of the DNA contained within it. The DNA was depurinated by incubating the gel in 100ml of 0.25N HCl for 10 min at room temperature with gentle shaking. The gel was rinsed in dH_2O and then incubated for 30 min at room temperature in denaturation solution (0.5M NaOH; 1.5M NaCl). After rinsing the gel in dH_2O, it was incubated in neutralising solution (1M Tris-HCl pH 8.0; 1.5M NaCl; 10mM EDTA) for a further 30 min.

The upward capillary transfer method was used to transfer the DNA within the gel to the positively charged nylon membrane (Roche Diagnostics, Germany) using the 20 x SSC (0.3M Na Citrate; 1.5M NaCl pH 7.0) transfer buffer. An overnight transfer period was allowed after which the transfer apparatus was dismantled and the membrane was briefly rinsed in 2 x SSC. The DNA was fixed to the membrane by UV-crosslinking at 0.15 Joules for approximately 1 min. The membrane was rinsed and ready for pre-hybridization.

Probe selection and labelling

The *Eucalyptus pgip* fragment amplified with oligonucleotide primers EN-PGIP-FWD and REV-PB-PGIP was selected as a probe and thus labelled using the DIG High Prime DNA labelling and Detection Starter Kit 1 (Roche Applied Science, Germany) according to the manufacturer's protocols. The λ III marker was included in the labelling reaction. The reactions were incubated overnight at 37°C. The labelling efficiency for each of the labelled probes were determined as set out in the

DIG High Prime DNA labelling and Detection Starter Kit 1 Instruction manual (Roche Applied Science, Germany).

DIG Hybridization

Pre-hybridization and hybridisation reactions were performed in the TECHNE Hybridiser HB-1D hybridisation chamber at 37°C. The membrane was pre-hybridized in 10ml of of DIG Easy Hyb solution (Roche Applied Science, Germany) for 30 min. Twenty nanograms per millilitre of DIG-labelled PGIP probe and λIII marker was denatured by boiling for 5 min and then added to the pre-heated hybridisation solution. The pre-hybridization solution was removed and the probe/hybridisation solution was added to the membrane. Following an overnight hybridisation at 37°C, the membrane was washed twice in 2 x SSC, 0.1% SDS for 5 min and twice in 0.5% SSC, 0.1% SDS for 15 min at 40°C and with constant agitation. Immunological detection was performed using a colour-substrate solution containing NBT/BCIP (Roche Applied Science, Germany). After the hybridisation and stringency washes, the membrane was incubated in a washing buffer for 5 min at room temperature. This was followed by incubation for 30 min in a Blocking Solution. The anti-Digoxigenin –AP antibody (Roche Applied Science, Germany) was diluted 1: 50 000 in blocking solution and the membrane was incubated for a further 30 min in this antibody solution. The substrate-substrate solution was prepared by diluting 200µl of NBT/BCIP stock in 10ml of detection buffer. The membrane was equilibrated for 2 min in detection buffer before being placed between two sheets of plastic to which the colour-substrate solution was then added. The plastic bag was sealed and placed in the dark at room temperature to allow colour development. The reaction was stopped after approximately 16 h by washing the membrane in 50 ml sterile ddH_2O.

Analysis of gene expression

Histochemical GUS Assay

Transient expression of β-glucuronidase was investigated by means of a histochemical assay (Jefferson *et al.*, 1987). Leaf samples from non-transformed plants as well as from putatively transformed tobacco plants containing a GUS-intron gene were collected and incubated in 1ml X-Gluc buffer at 37°C. Following an overnight incubation the leaf samples were de-stained in 70% ethanol.

PGIP inhibition Assays

The transformed tobacco plants were tested for the expression of PGIP. Crude protein extracts made from transformed and untransformed control plants were used to test for inhibitory activity towards the endo-polygalacturonases of the *Eucalyptus* pathogen *C. zuluense*. Inhibition experiments are discussed in the section that follows.

Analysis of PGIP activity on *Coniothyrium zuluense* PGs

Fungal Isolate and Growth Conditions

A large number of *C. zuluense* isolates were collected from severely infected *Eucalyptus* species, clones and hybrids in the Zululand forestry region of KwaZulu-Natal (Van Zyl, 1999). Cultures were maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), at the University of Pretoria.

Growth of *Coniothyrium* isolates for polygalacturonase production

The virulent *C. zuluense* isolates CRY1001 and CRY1047 were selected from the culture collection and used for subsequent inhibition assays. Prior to the inhibition assays the isolates were grown on 2% malt extract agar (2g malt extract, 2g agar, 100ml distilled water) at 25°C for two weeks. Five mycelium plugs from each of the

two isolates were inoculated in two separate flasks containing 10ml Czapex Dox broth supplemented with 1mg/ml ampicillin. The flasks were incubated at 25°C on an orbital shaker for seven days when the *Coniothyrium* mycelium plugs grew as individual white fluffy masses. The white *Coniothyrium* masses from each flask were removed from the Czapex Dox and crushed with a sterile glass rod in 2ml fresh Czapex Dox broth containing sterile glass pieces. Polygalacturonase production was induced by inoculating 25ml of a sterile liquid minimal salts medium (2.0g NH_4NO_3; 1.0g KH_2PO_4; 0.1g $MgSO_4$; 0.5g yeast extract; 1.0g NaOH; 3.0g $_{DL}$-malic acid; 1liter distilled water) supplemented with 0.5% w/v washed citrus pectin (obtained from D. Oelofse, ARC Rooderplaat) as a carbon source, with the 2ml of crushed mycelium *Coniothyrium* culture. Ampicillin was added to a final concentration of 200µg/ml. The cultures were further incubated at 25°C with continuous shaking for 10 days. Mycelium from the individual flasks were harvested from each of the isolates from day 3 to day 10 by suction filtration through Whatmann no. 4 filter paper, using a Buchner funnel. The filter paper was dried at 37°C overnight and the dry-weight, which represented the growth of the fungal cultures, was recorded. The culture-filtrate was filter-sterilized twice through a 0.45µ disposable syringe filter and stored at 4°C.

Agarose Diffusion Assay to determine polygalacturonase activity

Polygalacturonase activity was visualised using an agarose diffusion assay described by Taylor and Secor (1988). Assay medium containing 1% Type II agarose, 0.01% polygalacturonic acid and 0.5% ammonium oxalate dissolved in 100ml of buffer containing 100mM citrate, 200mM Na_2HPO_4, pH 5.3 was prepared. The medium was heated to dissolve the PGA and agarose. Ten millilitres of assay medium was poured into 65mm diameter petri dishes and holes were then punched in the solidified medium using a no.1 cork borer. Each well was filled with 30µl of the culture filtrates collected at different days, a standard and a control solution. The plates were incubated overnight at 27°C. To visualize the endopolygalacturonase activity the plates were flooded with 10ml of 0.05% ruthenium red (Sigma) and incubated at 37°C for 1 h. Excess dye was removed by washing the plates several times with ddH2O.

The plates were incubated overnight at room temperature before the clear zones could be visualised and measured

Ammonium sulphate precipitation of *C. zuluense* culture filtrates

The culture filtrates of CRY1001 and CRY1047 isolates from day3 to day7 were individually subjected to ammonium sulphate precipitation, which removes the medium-derived pectin and also concentrates the endopolygalacturonases. Ammonium sulphate was added at a final concentration of 80% to the culture filtrates (5.16g ammonium sulphate per 10ml filtrate). The samples were mixed gently to allow the ammonium sulphate to dissolve completely before being incubated at 4°C with gentle agitation for 5 h. The endopolygalacturonase precipitate was collected by centrifuging the samples at 7000 x g for 30 min at 4°C. The pellets were dried and re-suspended in 40mM sodium acetate, pH 5.0 at a tenth of the original volume. To determine whether the endopolygalacturonase activity had been retained, the agarose-diffusion assay was repeated using aliquots of the ammonium sulphate precipitated isolates. The samples were stored at 4°C.

Preparation of crude protein extracts from transgenic tobacco plants expressing the *Eucalyptus* PGIP

Transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 were selected and crude proteins extracts from each were prepared and tested for PGIP activity. Approximately 1g of leaf material from each transgenic line was homogenised to a fine powder in liquid nitrogen with a mortar and pestle. The homogenised leaf material was transferred to a larger tube to which cold 20mM NaOAc, pH 5.0 containing 1M NaCl (2ml buffer per gram of leaf tissue) was added. The samples were gently mixed at 4°C for 3 h after which they were centrifuged at 13000 x g for 30 min at 4°C. The supernatant containing the PGIP extracts were retained and stored at 4°C. Crude protein extractions were also made from an untransformed control plant.

Protein concentration determination of PGIP extracts using the BioRAD assay

The concentration of the PGIP proteins extracted from the transgenic and non-transgenic lines as well as protein concentrations from the ammonium sulphate precipitated endopolygalacturonases were determined using the microassay procedure as outlined in the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Germany). The procedure is based on the Bradford method where bovine serum albumin is used as a standard. The optical density readings were done at a wavelength of 595nm.

Inhibition of *C. zuluense* PGs by PGIP extracts from transgenic tobacco plants

The agarose diffusion assay was conducted to determine whether the PG culture filtrates from the *C. zuluense* isolates could be inhibited by the PGIP extracts from the transgenic tobacco lines. The agarose diffusion assay medium was prepared as described in a previous section of this chapter. *Verticillium dahliae* PG and the apple PGIP proteins extracted from transgenic and non-transgenic lines were used in this study as a positive control reaction (Obtained from I. Maritz, ARC, Rooderplaat) (I. Maritz; 2002). All reactions were carried out in a final volume of 50µl. A 25µl aliquot of PG (28µg/ml) was mixed with an equal volume of the PGIP extract (25µg/ml) from a transgenic line. The mixture was incubated for 20 min at 25°C after which a 40µl aliquot was placed in a well in the agarose diffusion assay medium. Each plate contained a control reaction in which the PGIP extract from either transformed or non-transformed tobacco was boiled prior to the addition of the fungal PG. The plates were incubated overnight at 27°C and stained with 10ml of 0.05% ruthenium red dye to visualise the clear zones.

Determining the linear trend of the *C. zuluense* PG activity

The ammonium sulphate precipitated PG activity from *C zuluense* isolate CRY1047 was determined by reducing end-group analysis using the PAHBAH colour reagent.

Reactions were run in triplicate and over a time course of 100 min. In order to determine the activity range of the PGs produced, samples were taken at six time points (t = 0; t = 20; t = 40; t = 60; t = 80 and t = 100 min). In this experiment reactions were carried out with undiluted, 1+2 dilution, 1+5 dilution, 1+10 dilution, 1+15 dilution and 1+20 dilution volumes of the *C. zuluense* PGs. Forty microlitres of each dilution series was mixed with 40µl of 20mM NaOAc (pH 4.7) and the mixture was incubated at 25°C for 20 min prior to the addition of the substrate. A 72µl aliquot of the PG mix was then added on ice to 108µl of 0.42% polygalacturonic acid (PGA) to give a final PGA concentration of 0.25%. At t = 0, a 25µl aliquot was removed from the PG:PGA mix and immediately boiled for 10 min, while the rest of the reaction mixture was placed at 30°C for the total time course of 100min. At each of the six time points, a 25µl aliquot was removed, placed in a safe-lock tube and boiled for 10 min to stop the reaction. The samples were cooled on ice followed by a quick spin to sediment the condensate. The reaction volumes were then increased by the addition of 225µl ddH_2O and 750µl of a freshly prepared 1% PAHBAH reagent (p-4-amino-2-hydroxybenzoic acid hydrazide, Sigma). The samples were boiled for a further 10 min and allowed to cool before determining the absorbance values at 410nm in a spectrophotometer. The average of each triplicate sample at each of the six time points were determined and subtracted from the t = 0' value.

Reducing sugar assay to determine inhibitory activity of PGIP

In addition to the agarose diffusion assay, transgenic tobacco PGIP extracts were also tested for inhibitory activity using the reducing sugar assay. A 1:1 dilution of the PG extract in 20mM NaOAc (pH 4.7) was used in each reaction. Several reactions were included to test efficiency of the assay. These included a control reaction containing an equal volume of the PG extract and 20mM NaOAc (pH 4.7); a control reaction containing equal volumes of *C. zuluense* PG and PGIP extract from non-transgenic tobacco and the test reactions containing equal volumes of PG and the respective PGIP extracts from each of the transgenic tobacco lines. All reactions were done in triplicate and samples were collected at two time points, t = 0 and t = 40 min.

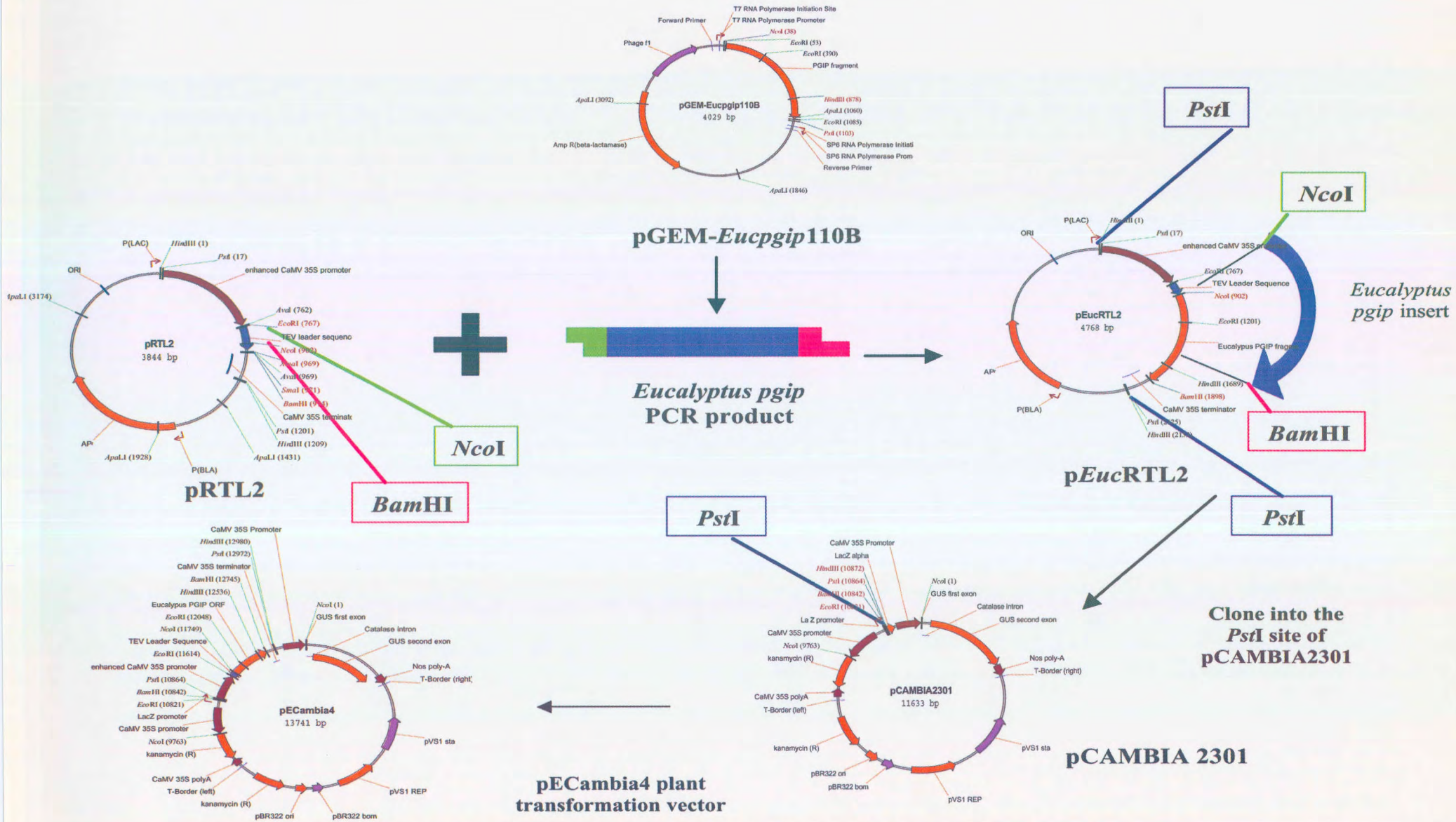

Figure 3.2 Schematic representation of the construction of the pECambia4 plant transformation vector.

Results

Construction of the *Eucalyptus pgip* expression cassette

Preparation of the *Eucalyptus pgip* gene

The gene-specific primer EN-FWD-PGIP was designed from the nucleotide sequence of the partial *Eucalyptus pgip* gene cloned in the pGEM-T-Easy commercial cloning vector (Promega) (pGEM-*Eucpgip*110B) (Appendix C). The nucleotide region used for synthesising the EN-FWD-PGIP primer is indicated in figure 3.3a. The reverse primer, REV-PB-PGIP, was designed from the C-terminal nucleotide sequence of the pear *pgip* gene (figure 3.3b). At the time of construction, the genome walking PCR results discussed in Chapter 2 were not available and thus the pear *pgip* gene sequence was used in the design of the REV-PB-PGIP primer.

The oligonucleotide primer, EN-PGIP-FWD was designed based on the 5' end of the *Eucalyptus pgip* gene and contains an *Nco*I restriction site. Based on its 98% identity to the partial *Eucalyptus pgip* gene, the 3' end of the pear *pgip* gene was used to design the REV-PB-PGIP primer. The REV-PB-PGIP primer has been designed to contain a 15bp region representative of the 3' end of the pear *pgip* gene and a *Bam*HI restriction site for cloning into the pRTL2 vector.

a.

```
ATG GCG GCC GCG GGA ATT CGA TTA CAT CTC TCA GGC TCT CAA CCA   45

                          EN-FWD-PGIP ⇒
  5' CCC AAA ACC ATG GAG CTC AAG TTC TTC AC 3'
AAA CCC AAA ACA ATG GAG CTC AAG TTC TTC ACC TTC CTC TCC CTA   90
                  M   E   L   K   F   F   T   F   L   S   L   11

ACC CTA CTC TTC TCC TCC GTC CTA AAC CCC GCT CTC TCC GAT CTC   135
 T   L   L   F   S   S   V   L   N   P   A   L   S   D   L   26

TGC AAC CCC GAC GAC AAA AAA GTC CTC CTA CAA ATC AAG AAA GCC   180
 C   N   P   D   D   K   K   V   L   L   Q   I   K   K   A   4
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b.

```
 L   N   V   S   Y   N   R   L   C   G   Q   I   P   V   G    315
CTG AAC GTG AGC TAC AAC AGG CTG TGT GGT CAG ATT CCT GTG GGT    945

 G   K   L   Q   S   F   D   E   Y   S   Y   F   H   N   R    330
GGA AAG TTG CAG AGC TTC GAC GAG TAT TCT TAT TTC CAT AAC CGA    990

   ⇐ REV-PB-PGIP
 C   L   C   G   A   P   L   P   S   C   K   *
TGC TTG TGC GGT GCT CCA CTC CCA AGC TGC AAG TAA AGG CCA   1032
  3'GAC ACA CCA CGT GGG GAG GGT TGC ACG TTC ATT TCC TAG GC 5'
    CTG TGT GGT GCA CCC CTC CAC ACT GC ← 3'end Eucalyptus pgip
     L   C   G   A   P   L   H   T
```

Figure 3.3 (a) Nucleotide sequence of the 5' region of the partial *Eucalyptus pgip* gene obtained from sequence analysis of the pGEM-*Eucpgip* 110B clone using T7 and SP6 primers (Chapter2). Regions used for the synthesis of the gene-specific EN-FWD-PGIP primer is indicated in blue. The primer was designed to contain an *Nco*I site, which is highlighted in yellow. **(b)** Nucleotide sequence of the downstream region of the pear *pgip* gene. The REV-PB-PGIP primer is indicated in green. The primer shows a region of 18bp (blue) that is homologous to the pear *pgip* gene sequence. The 15bp region indicated in green is homologous to the C-terminal region of the partial *Eucalyptus pgip* gene sequence and the *Bam*HI site is highlighted in yellow. The sequence highlighted in blue represents the 3'-end of the pGEM-*Eucpgip*110B clone and shows the region of homology to the REV-PB-PGIP primer.

The amino acid sequence of the *Eucalyptus* PGIP is indicated below the nucleotide sequence and in red.

The pGEM-*Eucpgip* clones were used in a PCR reaction with the gene-specific primers EN-FWD-PGIP and REV-PB-PGIP. The amplified products are indicated in figure 3.4. PCR products can be observed in lanes 3,5,6,7 and 9, which represent amplification from plasmids pGEM-*Eucpgip*110B, pGEM-*Eucpgip*110D, pGEM-*Eucpgip*110E, pGEM-*Eucpgip*110F and pGEM-*Eucpgip*110H, respectively.

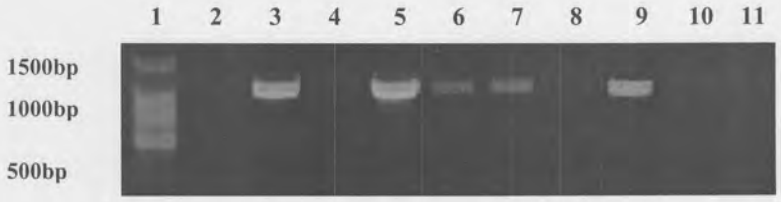


Figure 3.4 PCR analysis of the *Eucalyptus pgip* gene amplified from the pGEM-*Eucpgip* clones. The amplified products are approximately 1000bp in length. Lane 1 represents the 100bp DNA ladder (Promega) and lane 11 represents the negative control in which the template DNA was substituted with water. Lanes 2- 10 represent reactions containing plasmids pGEM-*Eucpgip*110A- pGEM-*Eucpgip*110I.

Construction of the p*Euc*RTL2 cloning vector

The PCR generated *Eucalyptus pgip* gene from the pGEM-*Eucpgip*110B, pGEM-*Eucpgip*110D, pGEM-*Eucpgip*110E and pGEM-*Eucpgip*110H clones were digested with restriction enzymes *Nco*I and *Bam*HI (Figure 3.5a). The *Eucalyptus pgip* gene does not contain any *Nco*I and *Bam*HI restriction sites within the nucleotide sequence and therefore digestion with these enzymes results in the production of a fragment of approximately 990bp with sticky ends for cloning into the pRTL2 vector (Figure 3.5b). Similarly, digestion of the pRTL2 plasmid resulted in the production of two bands, a 3772bp fragment and a smaller fragment of 72bp.

The PCR amplified and restriction digested *Eucalyptus pgip* gene from the pGEM-*Eucpgip* 110B clone was selected and subsequently placed under control of the CAMV dual 35S promoter in the pRTL2 intermediate cloning vector. PCR analysis with the EN-FWD-PGIP and REV-PB-PGIP primers produced an expected fragment of approximately 1000bp in recombinant clones p*Euc*RTL2-2, p*Euc*RTL2-4, p*Euc*RTL2-5, p*Euc*RTL2-9 and p*Euc*RTL2-10 (Figure 3.6). Similarly, restriction digestion with *Nco*I and *Bam*HI lead to the release of the 1kb *Eucalyptus pgip* gene from the same recombinant p*Euc*RTL2 clones (Figure 3.7), thus verifying the presence of the *pgip* gene in the intermediated pRTL2 cloning vector.

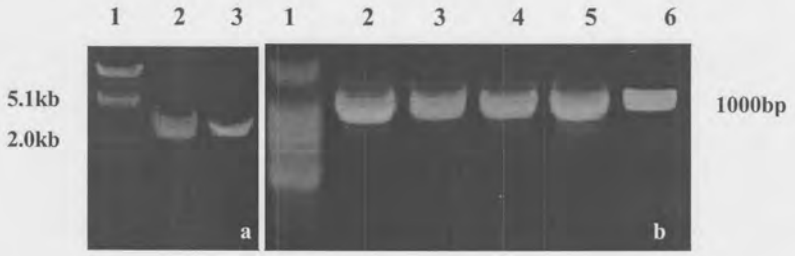


Figure 3.5 a. Restriction digestion of the intermediate pRTL2 cloning vector. Lane 1 contains the λ III marker, lane 2 represents the undigested plasmid (3844bp) and in lane 3, the pRTL2 vector has been digested with *Nco*I and *Bam*HI (3772bp). **b.** Restriction enzyme digestion of the PCR generated *Eucalyptus pgip* fragment with *Nco*I and *Bam*HI. Lane 1 contains the 100bp DNA ladder (Promega), lane 2 contains the undigested PCR product and lanes 3-6 represent PCR products that have been subjected to restriction digestion.

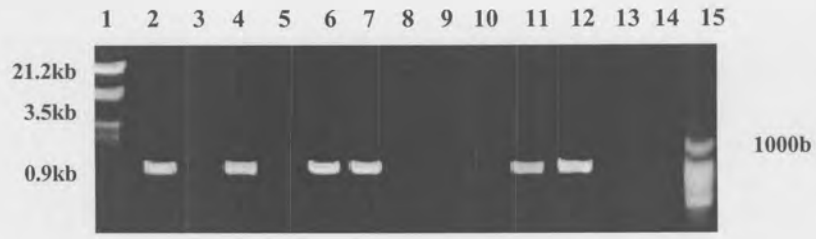


Figure 3.6 PCR analysis of p*Euc*RTL2 recombinants using the EN-PGIP-FWD and REV-PB-PGIP primers. Lane 1 contains the λ III marker and lane 15 contains the 100 bp DNA ladder (Promega). Lane 2 shows amplification of the *Eucalyptus pgip* gene from the pGEM-*Eucpgip*110B clone as a positive control and lane 14 represents a negative water control. Lanes 3-13 represents PCR reactions with putative p*Euc*RTL2-1 to pEucRTL2-11 recombinants, respectively.

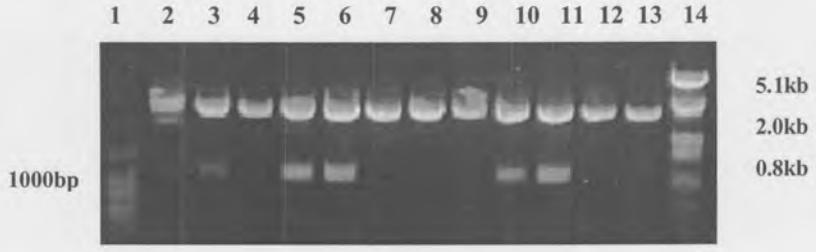


Figure 3.7 Restriction digestion analysis of p*Euc*RTL2 recombinants. Lanes 1 and 14 contain the 100bp ladder (Promega) and λ *Hind*III/*Eco*RI marker, respectively. Lane 2 contains an undigested p*Euc*RTL2 vector and lanes 3-13 represent putative recombinants p*Euc*RTL2-1 to pEucRTL2-11 digested with *Nco*I and *Bam*HI.

Sequence analysis of the p*Euc*RTL2 expression cassette

The recombinant p*Euc*RTL2-2 plasmid was selected and sequenced using the pBI121 sequencing primer #2 in order to determine whether the 5'-junction point between the pRTL2 vector and the *Eucalyptus pgip* gene were in frame. The nucleotide sequence of the 5'-junction point is indicated in figure 3.8. Sequence analysis confirmed that

the 5'-junction between the pRTL2 vector and the *Eucalyptus pgip* gene were in frame thus indicating that the correct translation product would be obtained.

```
                    +1          EcoRI
CAT TTG GAG AGG ACC TCG AGA ATT CTC ATC ACA ACA TAT ACA AAA   45
                   XhoI▲

CAA ACG AAT CTC AAG CAA TCA AGC ATT CTA CTT CTA TTG CAG CAA   90

TTT AAA TCA TTT CTT TTA AAG CAA AAG CAA TTT TCT GAA AAT TTT   135

                                  ▼          EN-FWD-PGIP ⇒
CAC CAT TTA CGA ACG ATA GCC ATG GAG CTC AAG TTC TTC ACC TTC   180
                        M   E   L   K   F   F   T   F     8

CTC TCC CTA ACC CTA CTC TTC TCC TCC GTC CTA AAC CCC GCT CTC   225
 L   S   L   T   L   L   F   S   S   V   L   N   P   A   L    23

TCC GAT CTC TGC AAC CCC GAC GAC AAA AAA GTC CTC CTA CAA ATC   270
 S   D   L   C   N   P   D   D   K   K   V   L   L   Q   I    38

AAG AAA GCC TTC GGC GAC CCC TAT GTC TTG GCC TCA TGG AAA TCA   315
 K   K   A   F   G   D   P   Y   V   L   A   S   W   K   S    53

GAC ACC GAC TGT TGT GAT TGG TAC TGC GTC ACC TGT GAC TCA ACC   360
 D   T   D   C   C   D   W   Y   C   V   T   C   D   S   T    68

ACA AAC CGC ATC AAC TCC CTC ACC ATC TTT GCC GGC CAG GTA TCC   405
 T   N   R   I   N   S   L   T   I   F   A   G   Q   V   S    83

GGC CAA ATC CCC GCC CTA GTT GGA GAC TTG CCG TAC CTT GAA ACC   450
 G   Q   I   P   A   L   V   G   D   L   P   Y   L   E   T    98

CTT GAA TTC CAT AAG CAA CCC AAT CTC ACT GGC CCA ATC CAA CCC   495
 L   E   F   H   K   Q   P   N   L   T   G   P   I   Q   P   113

GCC ATT GCC AAG CTC AAA GGA CTC AAG TCT CTC AGG CTC AGC TGG   540
 A   I   A   K   L   K   G   L   K   S   L   R   L   S   W   128

ACC AAC CTC TCA GGC TCT GTC CCT GAC TTC CTC AGC CAA CTC AAG   585
 T   N   L   S   G   S   V   P   D   F   L   S   Q   L   K   143

AAC CTC ACA TTC CTC GAC CTC TCC TTC AAC AAC CTC ACC GGT GCC   630
 N   L   T   F   L   D   L   S   F   N   N   L   T   G   A   158

ATC CCC AGC TCG CTT TCT GAG CTC CCA AAC CTC GGC GCT CTT CGT   675
 I   P   S   S   L   S   E   L   P   N   L   G   A   L   R   173

CTA GAC CGC AAT AAG CTC ACA GGT CAT ATT CCG ATA TCG TTT GGG   720
 L   D   R   N   K   L   T   G   H   I   P   I   S   F   G   188

CAG TTC ATT GGC AAC GTT CCA GAC TGT
 Q   F   I   G   N   V   P   D
```

Figure 3.8 Nucleotide and amino acid sequence of the p*Euc*RTL2-2 plasmid. The positions of the *Xho*I and *Eco*RI sites of the pRTL2 vector are indicated in red and green, respectively. The *Nco*I site of the pRTL2 vector into which the *Eucalyptus*

pgip gene was cloned is indicated and underlined in blue. The position and nucleotide sequence of the EN-FWD-PGIP primer is highlighted in blue. The transcription start point (+1) is indicated in purple and the translation start codon (ATG) is highlighted in yellow. The first black arrow below the *Eco*RI site indicates the start position of the TEV leader sequence, while the second arrow above and including the *Nco*I site indicates the end of the TEV leader sequence.

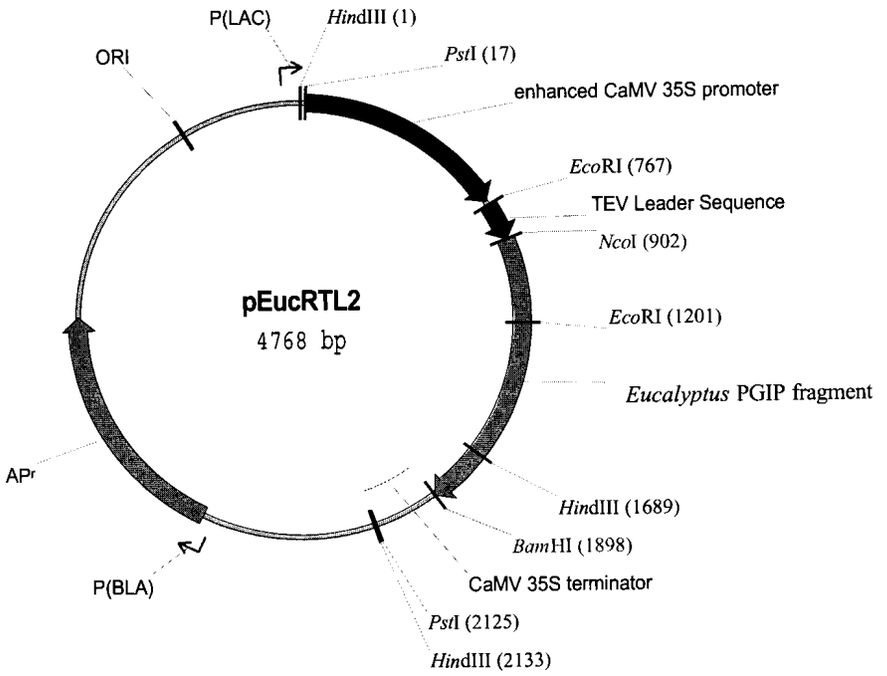


Figure 3.9 Plasmid map of the constructed p*Euc*RTL2 vector that contains the *Eucalyptus pgip* gene under control of an enhanced CaMV 35S promoter.

Construction of the pECambia4 plant transformation vector

The 2108bp *Eucalyptus pgip* expression cassette was excised from the p*Euc*RTL2-2 vector and cloned into the pCAMBIA 2301 plant transformation vector. The p*Euc*RTL2 vector contains two *Pst*I and two *Hind*III restriction sites at either side of the expression cassette. Nucleotide inspection of the *Eucalyptus pgip* gene revealed

that the sequence contained a *Hind*III restriction site within the gene, but not a *Pst*I site. Digestion with *Pst*I, should therefore lead to the release of the complete *Eucalyptus pgip* expression cassette, however, several attempts to digest the plasmid with *Pst*I alone were unsuccessful. The 4768bp p*Euc*RTL2 vector contains two *Pst*I sites that releases the expression cassette and one *Sca*I site located within the ampicillin resistance gene. A double-digest with restriction enzymes *Sca*I and *Pst*I lead to the production of three fragments (Figure 3.10). The 2108bp *Eucalyptus* expression cassette excised from the vector by *Pst*I digestion, a 918bp fragment generated by the *Sca*I-*Pst*I digestion and the remaining 1742bp of the p*Euc*RTL2 could thus be observed.

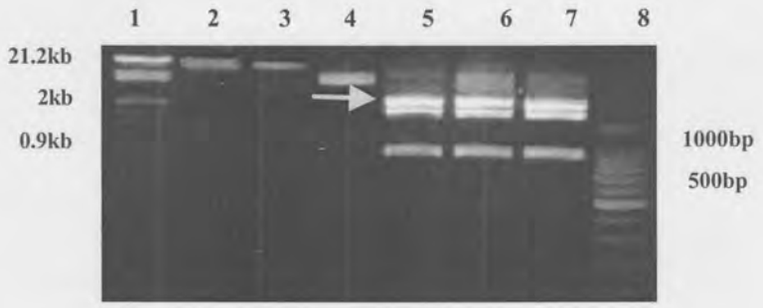


Figure 3.10 Restriction analysis of p*Euc*RTL2 with enzymes *Pst*I and *Sca*I. Lane1 contains the λIII marker and lane 8 contains the 100bp DNA ladder (Promega). Lane2 contains the undigested pCAMBIA2301 vector, while in lane 3, *Pst*I digested pCAMBIA2301 is represented. Lane 4 contains the undigested p*Euc*RTL2 vector and lanes 5-7 represent the *Pst*I- *Sca*I digested plasmids. The yellow arrow indicates the 2108bp *Eucalyptus pgip* expression cassette.

Putative pECambia plant transformation recombinants were subjected to restriction analysis with *Nco*I in order to determine the orientation of the inserted *Eucalyptus pgip* expression cassette. Digestion with *Nco*I should result in the production of three bands. The *Eucalyptus pgip* expression cassette contains a single *Nco*I site and the pECambia vector backbone contains two sites for *Nco*I activity. Following digestion, the expression cassette is therefore divided into two fragments of 1986bp and 1992bp, which are present on the gel as a doublet and the 9763bp fragment represents the

remaining portion of the pECambia4 plant transformation vector (Figure 3.11). PCR analysis of the putative pECambia recombinants using gene-specfic primers EN-FWD-PGIP and REV-PB-PGIP, lead to the amplification of a 1kb *Eucalyptus pgip* gene, thereby further verifying the presence of the gene in the plant transformation construct (Figure 3.12). The p*Euc*RTL2-2 construct was used as a positive control in the PCR analysis.

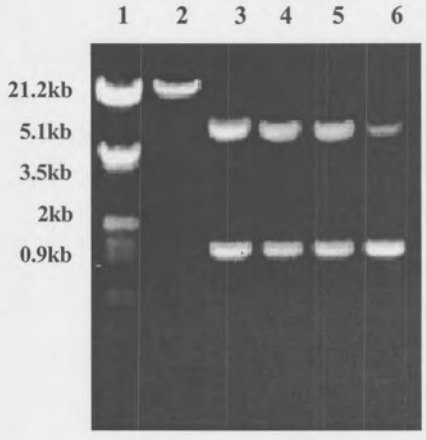


Figure 3.11 Restriction analysis of the pECambia putative recombinant plasmids with *Nco*I. Lane1 contains the λIII marker. Lane 2 represents the undigested pECambia plasmid DNA and lanes 3 – 6 represent the digested plasmids pECambia1-pECambia4, respectively.

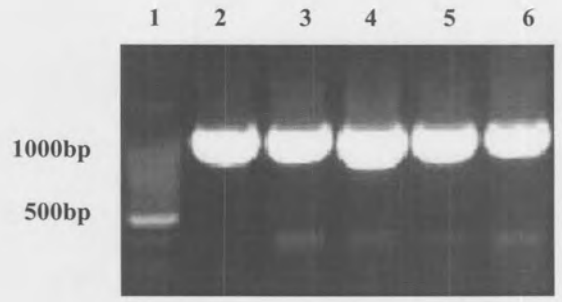


Figure 3.12 Amplification of the *Eucalyptus pgip* gene from the recombinant pECambia plant transformation constructs. Lane 1 contains the 100bp DNA ladder (Promega). Lane 2 represents the amplification product obtained from the p*Euc*RTL2 plasmid. Lanes 3-6 represent products obtained from the recombinant pECambia1 – pECambia4 vectors, respectively.

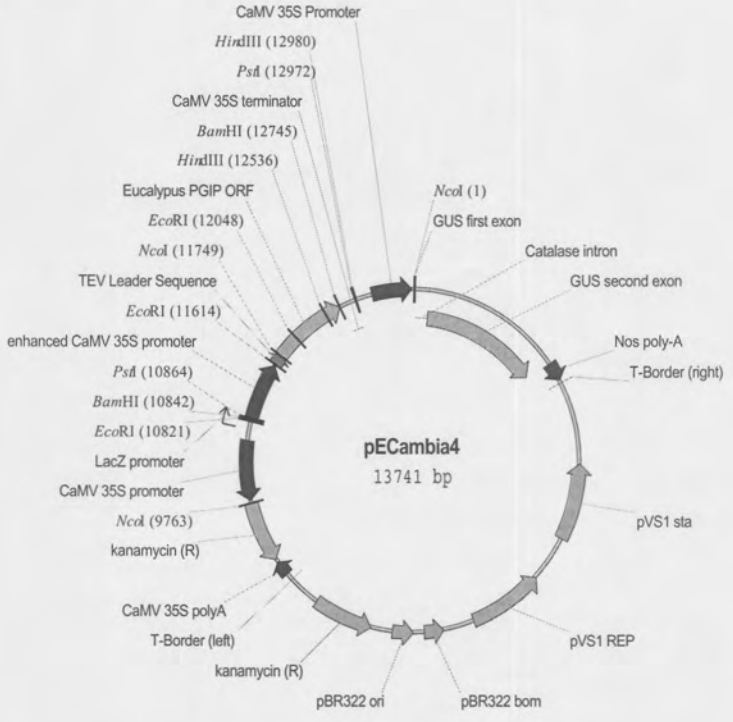


Figure 3.13 Plasmid map of the constructed pECambia4 *Agrobacterium*-based plant transformation vector.

Direct transformation of *A. tumefaciens* LBA4404

The recombinant plant transformation construct designated as pECambia4 was selected and used for the direct transformation of $CaCl_2$ competent *Agrobacterium* (LBA4404) cells. The presence of the vector in the *Agrobacterium* strain was verified by selecting the transconjugants on YEP medium supplemented with kanamycin and rifampicin. Isolation of the plasmid DNA from the *Agrobacterium* strain and using it in a PCR reaction with EN-FWD-PGIP and REV-PB-PGIP primers further verified the presence and structure of the pECambia4 vector. Amplification products are presented in figure 3.14. A negative water control was included to test for any PCR contaminants.

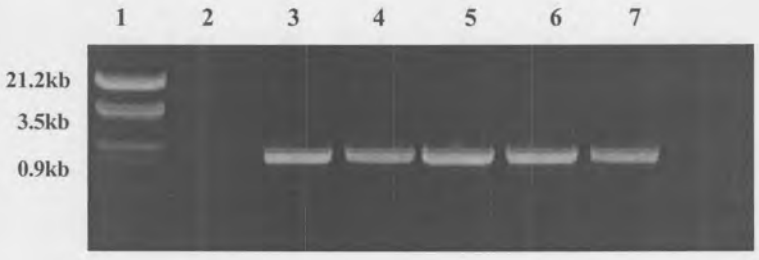


Figure 3.14 PCR analysis of plasmid DNA isolated from *A. tumefaciens* LBA4404 transconjugants using EN-FWD-PGIP and REV-PB-PGIP primers. Lane 1 contains the λIII marker, lane 2 shows a PCR reaction in which ddH_2O replaced the template DNA, lane 3-6 shows amplification using the *A. tumefaciens* LBA4404 (pECambia4) constructs and lane 7 represents amplification of the *Eucalyptus pgip* gene from pEucRTL2 plasmid DNA.

The isolated plasmid DNA from the C58/pCAMBIA 2301 *Agrobacterium* strain was tested by PCR analysis for the presence of the pCAMBIA 2301 vector. PCR analysis with GUSforward and GUSreverse primers lead to the amplification of a fragment of approximately 1700bp thus verifying the presence of the pCAMBIA 2301 vector in the C58 *Agrobacterium* strain (Figure 3.15).

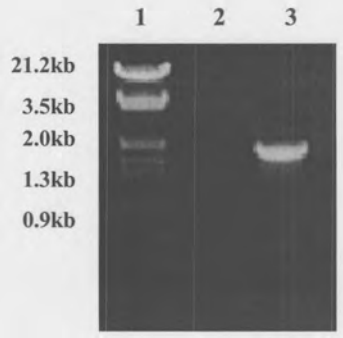


Figure 3.15 PCR analysis of the C58/pCAMBIA 2301 construct using GUSforward and GUSreverse primers. Lane 1 contains the λIII marker. Lane 2 represents the negative water control and lane 3 shows the amplified GUS product from the isolated C58/pCAMBIA2301 plasmid.

Production of transgenic tobacco plants

N. tabacum cv. LA Burley explants were transformed with the *A. tumefaciens* LBA4404-pECambia4 strain using the leaf-disc transformation method. The C58 *Agrobacterium* strain, which harbours a pCAMBIA 2301 plant transformation vector carrying a GUS-intron gene was used to transform 20 leaf-discs and represented the positive control reaction. To test the efficiency of the antibiotics, untransformed controls included 10 leaf discs placed on medium containing the antibiotics kanamycin and rifampicin and 10 discs placed on medium lacking antibiotics.

Table 3.2 Summary of the regeneration of *Nicotiana tabacum* cv. LA Burley plantlets produced from the different transformation reactions performed.

Bacterial strain	Transformation Reaction	Antibiotic	No. leaf-discs	No. Shoots	No. Roots	%plants produced
LBA4404	LBA4404/pECambia4	Km^+Cef^+	60	43	43	72
C58	C58/pCAMBIA2301	Km^+Cef^+	20	8	8	40
-	Control + antibiotics	Km^+Cef^+	10	10	0	0
-	Control - antibiotics	Km^-Cef^-	10	10	6	60

Km – kanamycin

Cef – cefotaxime

A summary of the various transformation reactions performed in this study is provided in table 3.2. From the 60 leaf-discs transformed with the pECambia4 construct, callus proliferation could be observed on the edges of 43 discs and shoots were regenerated from each of the 43 discs. A single shoot from each leaf-disc was sub-cultured onto rooting medium and all 43 shoots produced roots. Eight leaf-discs transformed with the C58/pCAMBIA 2301 bacterial strain produced callus and subsequently shoots. The control reaction to which antibiotics kanamycin (300µg/ml) and cefotaxime (250µg/ml), which kills off residual *Agrobacterium*, were added produced unexpected results in that the plants were able to survive. In normal reactions, these antibiotics should lead to the death of the leaf-discs. However, the leaf-discs were stably maintained on the medium and eventually produced shoots.

The original *N. tabacum* cv LA Burley plant used for these transformation experiments was tested to determine whether it may have contained a kanamycin resistance gene and therefore could possibly have been a previously transformed plant. Genomic DNA was isolated and used in a PCR reaction with $NPTII_L$ and $NPTII_R$ primers. The amplification products are indicated in figure 3.15. Genomic DNA isolated from a plant (LAT17) transformed with the *A.tumefaciens* LBA4404/pECambia4 stain was included in a PCR reaction with the *nptII* primers as

a positive control. Amplification with the $NPTII_L$ and $NPTII_R$ primers thus resulted in the generation of an approximately 0.8kb *nptII* gene. The *nptII* gene could however not be amplified from the genomic DNA of the original LA Burley plant (lanes 2 and 3, Figure 3.16) therefore indicating that the original LA Burley plant was indeed untransformed.

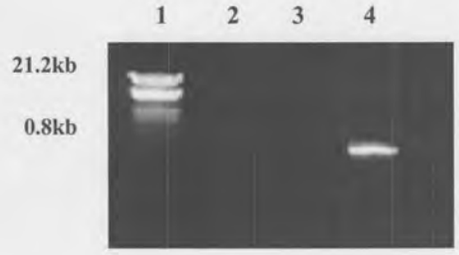


Figure 3.16 PCR screening of *N. tabacum* cv. LA burley plants using NPTII primers. Lane1, λII marker, lanes 2 and 3 represent amplification reactions in which the genomic DNA from LA burley was used as a template. Lane 4 contains a PCR product obtained from genomic DNA extracted from transgenic event LAT17.

In order to verify these results, a separate experiment was set up to determine the kanamycin concentration at which the untransformed plants would not be able to survive. MS-rooting medium containing various concentrations of kanamycin ranging from 0µg/ml to 400µg/ml was prepared and the shoots developed from the callus-forming leaf-discs were sub-cultured onto the medium. After approximately a week, untransformed shoots maintained on medium supplemented with 80µg/ml started showing signs of deterioration and at two weeks, plants maintained on medium containing kanamycin ranging in concentration from 80µg/ml-400µg/ml died.

In total 43 LA Burley plants transformed with the LBA4404/pECambia4 construct were fully regenerated, hardened off and transferred to the greenhouse. These plants were designated as LAT1 to LAT43. Eight plants transformed with the C58/pCAMBIA 2301construct designated as C1 to C8 were regenerated *in vitro* but due to some unforeseen problems with the plant incubator they were never hardened off. Six untransformed control plants designated as U1 to U6 were regenerated *in*

vitro. Only four of these plants (U2, U3, U5 and U6) were subsequently hardened off and transferred to the greenhouse.

Figure 3.17 Tobacco plantlets producing roots on MS-rooting medium supplemented with 100µg/ml kanamycin and 250µg/ml cefotaxime.

Figure 3.18 Regenerated control and transgenic plants that display indistinguishable phenotypic characteristics.

Verification of gene integration

PCR analysis of putatively transformed plants

The 43 transgenic lines transformed with the pECambia4 construct were able to form a stable root system in vitro. The ability to generate the transgenic lines on medium containing the antibiotic kanamycin indicated that the expression cassette within the T-DNA borders of the plant transformation vector was effectively transferred into the genome of the tobacco plantlets. To further verify the transgenic state of the plants, PCR analysis using *nptII*, GUS and *Eucalyptus pgip* specific primers were performed.

Genomic DNA extracted from the 20 randomly selected transgenic lines and 6 untransformed control lines were used as templates in a touchdown PCR reaction with the various primer combinations. The six untransformed control plants were negative for each of the primer combinations tested. From the 20 randomly selected transgenic plants, only eight were PCR positive. These included transgenic events LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36 and LAT43. The eight PCR positive samples were selected and are represented in figures 3.19-3.21. Plants that did not result in the production of a PCR fragment were discarded.

A PCR reaction containing genomic DNA extracted from an *in vitro* tobacco plant (C2) transformed with the C58/pCAMBIA2301 strain was included as a positive control in all reactions and a PCR reaction containing ddH_2O was included as a negative control. Amplification with the *nptII* primers gave an expected fragment size of approximately 600bp as represented in figure 3.17, while the *pgip* gene-specific primers EN-FWD-PGIP and REV-PB-PGIP as well as the GUS-intron primers resulted in fragments of approximately 1000bp (Figure 3.20 and Figure 3.21).

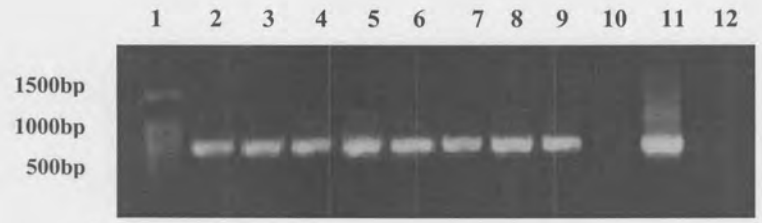


Figure 3.19 PCR analysis of transgenic tobacco lines with kanamycin primers $NPTII_L$ and $NPTII_R$. Lane 1 contains a 100bp DNA ladder (Promega). Lanes 2-9 represent amplification from transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 respectively. Lane 10 contains a PCR reaction of genomic DNA from an untransformed control plant (U6). Lane11 represents a reaction with genomic DNA extracted from a positive control plant (C2) transformed with the C58/pCAMBIA 2301 strain. Lane 12 shows the negative water control, which confirms that there are no PCR contaminants in the reactions.

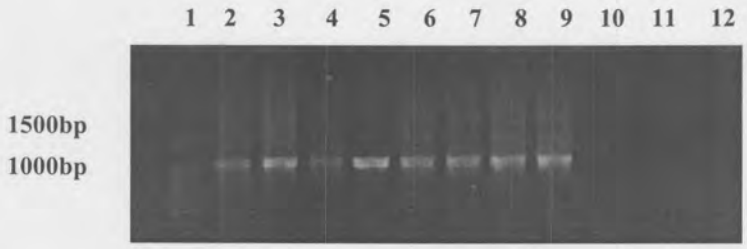


Figure 3.20 PCR analysis of transgenic tobacco lines using the *pgip* gene-specific primers EN-FWD-PGIP and REV-PB-PGIP. A 100bp DNA ladder is represented in lane 1. Lanes 2 - 9 contain the transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 respectively. Lane 10 contains an untransformed control (U6) and lane 11 represents amplification from the C58/pCAMBIA 2301 transformed positive control plant (C2). A negative water control is included in lane 12.

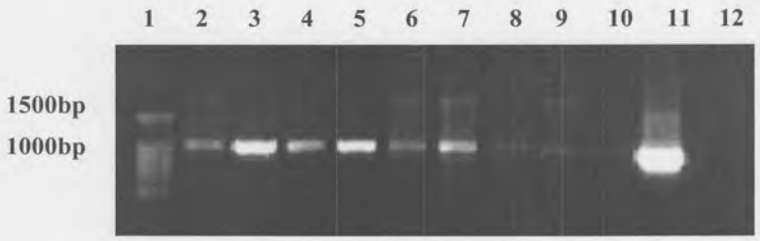


Figure 3.21 PCR analysis of transgenic tobacco lines using the GUS-intron primers. The 100bp DNA ladder is indicated in lane 1. Lanes 2 - 9 contain amplification products from the transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 respectively. Lane 10 represents an untransformed control reaction (U6) and lane 11 represents amplification from a positive control plant (C2) transformed with the C58/pCAMBIA 2301 strain. A PCR reaction with ddH_2O is indicated in lane 12.

Southern blot analysis

To verify transgene insertion into the tobacco genome and to determine the number of copies integrated, a Southern blot analysis on genomic DNA extracted from the eight PCR positive transgenic lines, was performed. Genomic DNA extracted from the *Eucalyptus grandis* TAG5 clone together with DNA extracted from an untransformed control tobacco plant was included in the southern analysis. All DNA samples included in the analysis were digested with *Eco*RI and probed with a DIG-labelled *Eucalyptus pgip* gene probe. The *Eco*RI digested untransformed control line was spiked with 10ng, 25ng and 50ng of the *Eucalyptus pgip* gene. The enzyme cuts the *Eucalyptus pgip* gene once, but within the T-DNA border, there are three sites for *Eco*RI. The PGIP probe was thus expected to produce at least two hybridisation signals for each of the transgenic lines tested (Figure 3.22). No hybridisation signals were however observed for any of the transgenic lines tested (lanes 6-14). The DIG-labeled *pgip* probe however did hybridise efficiently to the spiked untransformed controls (lanes 3, 4 and 5) as well as to the *Eucalyptus pgip* fragment (lane 15) and the pECambia4 construct (lane 16). A faint band of approximately 1kb could be observed

with transgenic line LAT17 (lane 7), but this could possibly be due to overflow from the untransformed tobacco spiked with the *pgip* gene.

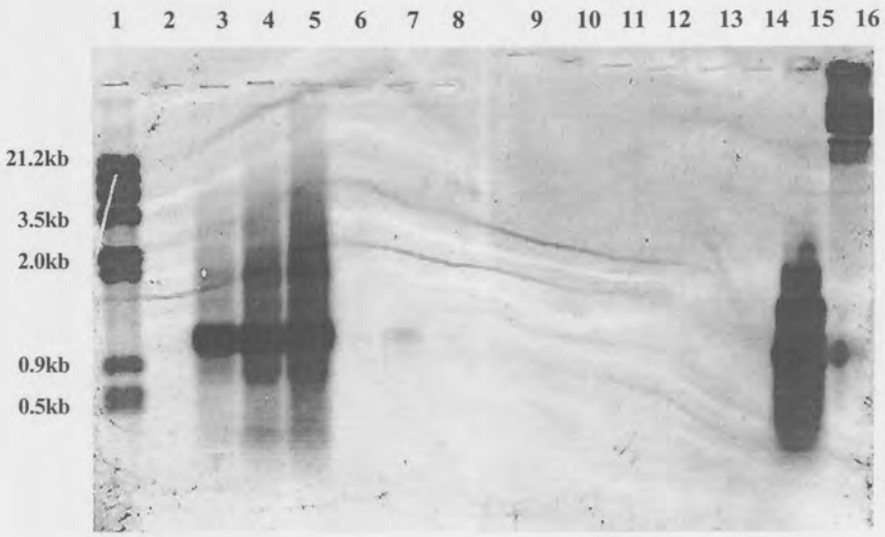


Figure 3.22 Southern blot analysis of PGIP in tobacco genomic DNA. DNA was digested with *Eco*RI and hybridised with a digoxygenin-labeled *Eucalyptus* pgip gene. A DIG-labelled λ *Hind*III/*Eco*RI marker is indicated in lane 1. Lane 2 contains the untransformed control. In lanes 3-5 genomic DNA from the untransformed control were spiked with 10, 25 and 50ng of the *Eucalyptus pgip* gene. Lane 6 – 14 contains transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 respectively. The *Eucalyptus pgip* gene was loaded in lane 15 and the plant transformation construct, pECambia4, is represented in lane 16.

The number of copies of the *Eucalyptus pgip* gene represented in the tobacco genome was calculated as follows:

Nicotiana tabacum:

Diploid genome (2C) = 7.8pg (Arumuganathan and Earle, 1991)

1. Number of copies in a diploid genome

   = total amount of DNA loaded per lane (µg)/mass of the 2C genome

   = 10×10^6pg / 7.8pg

$= 1.3 \times 10^6$ copies

Thus 10µg = 1.3×10^6molecules

And 2µg = 2.6×10^5 molecules

2. Mass of 1 Mole of 1bp = 660Da (ie 6×10^{23} molecules of 1bp = 660g)

Mass of 1 molecule of 1bp = 1.096×10^{-9}pg

$\therefore$ 1pg = Mass of 0.912×10^9 molecules of 1bp in size

1µg = Mass of 9.12×10^{11} molecules of 1Kb in size

$\therefore$ Mass of 1 molecule of 1Kb = 1Kb / Mass of 9.12×10^{11} molecules of 1Kb in size

$= 1.096 \times 10^{-12}$µg

$= 1.096 \times 10^{-6}$pg

3. *Eucalyptus pgip* = 1000bp

Mass of 1 molecule of *Eucalyptus pgip* = (1Kb)(1.096×10^{-6})pg

$= 1.096 \times 10^{-6}$pg

4. Mass of 2.6×10^5 molecules of *Eucalyptus pgip* in 2µg of genomic DNA

$= (2.6 \times 10^5)(1.096 \times 10^{-6})$

= 0.28pg

Equivalent of 1 copy of *Eucalyptus pgip* in 2µg genomic DNA = 0.28pg

The untransformed line was spiked with 10ng, 25ng and 50ng of *Eucalyptus pgip* PCR product as indicated in figure 3.21 lanes 3-5, respectively. The number of copies represented by the 10ng pgip spike was calculated to be 35 714 copies (10 x 10^3pg/0.28pg/copy). Similarly the 25ng spike represented 89 285 copies (25 x 10^3pg/0.28pg/copy) and the 50ng spike, 178 571 copies (50 x 10^3pg/0.28pg/copy) of the gene in the tobacco genome.

Verification of gene expression

Analysis of GUS activity

The histochemical assay was used to determine whether the transgenic lines displayed any GUS activity. The plant transformation construct pECambia4 contains a GUS intron gene within the T-DNA border indicating that together with the gene of interest, the GUS intron gene would also be incorporated into the tobacco genome (Figure 3.13). GUS activity was detected in 14 of the 43 LBA4404/pECambia4 transgenic events and in each of the eight C58/pCAMBIA 2301 transformed tissue-culture tobacco plants. The six untransformed plants showed no blue staining following incubation in the X-Gluc buffer, confirming the absence of the GUS-intron gene (Figure 3.23).

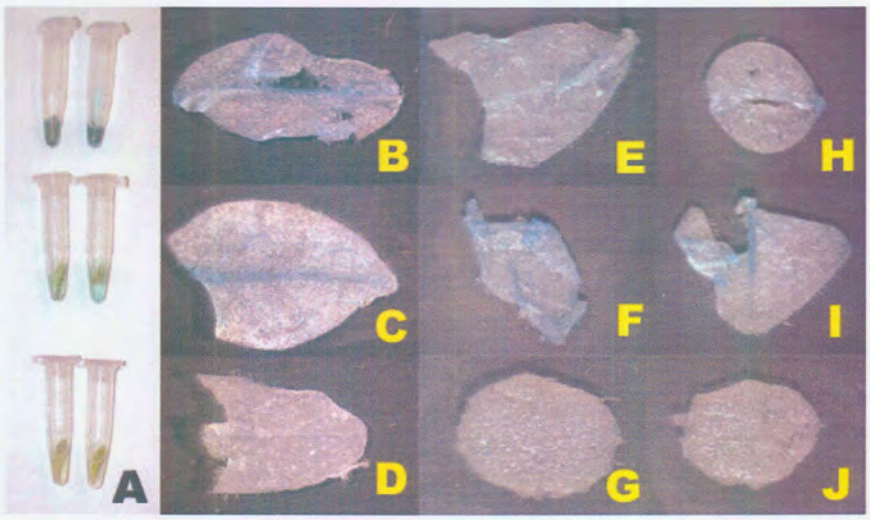


Figure 3.23 Histochemical localisation of GUS activity obtained in tobacco plants transformed with (B, E, H) *A. tumefaciens* C58/pCAMBIA2301 (C2), (C, F, I) *A.tumefaciens* LBA4404/pECambia4 (LAT18) and in (D, G, J) untransformed tobacco leaves (U6).

PGIP inhibition Assays

Induction of PG activity from *C. zuluense* fungal isolates

Virulent *C. zuluense* isolates CRY1001 and CRY1047 initially collected from severely infected *Eucalyptus* species, were obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute. Both isolates were grown on a minimal salts medium containing 0.5% citrus pectin as the carbon source. The growth of the fungal cultures is indicated in figure 3.24.

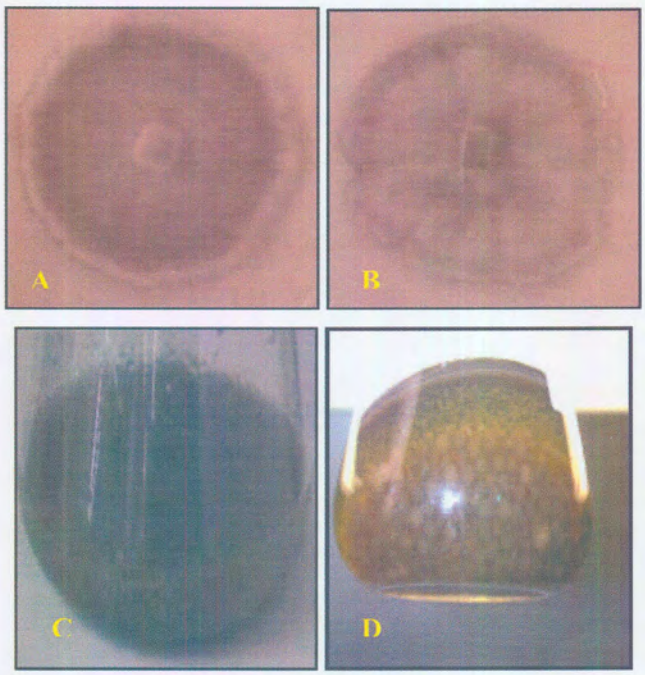


Figure 3.24 Growth of the *C. zuluense* fungal isolates **(A)** CRY1001 and **(B)** CRY1047 on malt-extract agar. Growth of isolates **(C)** CRY1001 and **(D)** CRY1047 on minimal salts medium containing citrus pectin for the production of polygalacturonases.

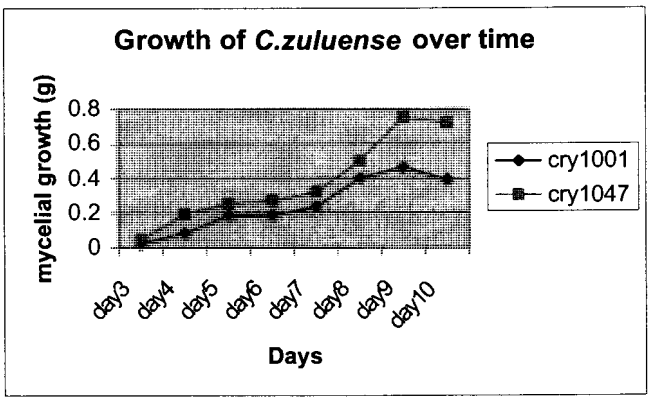


Figure 3.25 The growth of *C. zuluense* isolates determined by measuring the dry weight over time (days).

Polygalacturonases were produced in *C. zuluense* isolates CRY1001 and CRY1047 grown on pectin as a sole carbon source. The cultures grew with an increase in dry weight over the 10 day incubation period (Figure 3.25). The mycelial growth obtained for the CRY1047 isolate was slightly greater than that of the CRY1001 isolate, although the growth rate for each plateaued at day 9.

Agarose-diffusion assay to determine PG activity

The agarose diffusion assay was used to determine the polygalacturonase activity of the filtrates from both the CRY1001 and CRY1047 isolates. *Botrytis* PG obtained from L. Matsaunyane of the Agricultural Research Council (ARC Roodeplaat), was included in the assay as a positive control, while 40mM NaOAc represented a negative control. Zones of polygalacturonase activity could be observed for both isolates but the activity was much lower than that observed for the *Botrytis* PG. The *C. zuluense* PG isolates were concentrated by ammonium sulphate precipitation, which lead to an improvement in activity and an increase in the zone sizes. Plates displaying cleared zones of activity are indicated in figures 3.26 a and b. PG filtrates from each of the isolates showing the highest activity were selected and used to determine the inhibitory activity of the PGIP extracts.

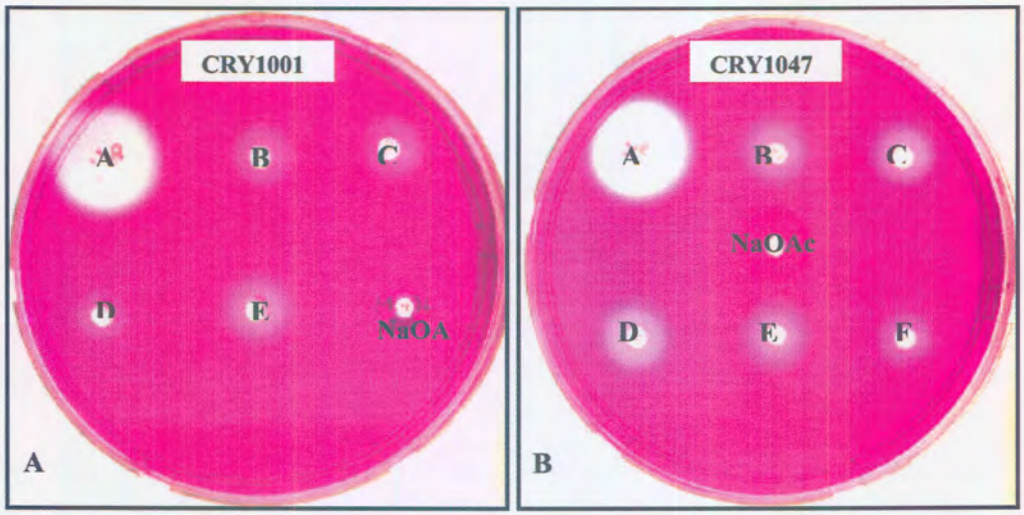


Figure 3.26 A. Agarose diffusion assay of ammonium sulphate precipitated culture filtrates from CRY1001. Zones B-E represent filtrates collected from days 3 to 6, respectively. Zone (A) represents PG activity from *Botrytis* PG and NaOAc represents a negative control in which 40mM NaOAc was added. **B.** Agarose diffusion assay of ammonium sulphate precipitated culture filtrates from CRY1047. PG activity of culture filtrates from days 3 to 7 is represented by zones b-f respectively. Zone (A) contains the *Botrytis* PG and NaOAc represents the negative control.

Agarose-diffusion assay to determine the inhibition of *C. zuluense* PGs by *Eucalyptus* PGIP extracts from transgenic tobacco

The PG filtrate for the *C. zuluense* isolate CRY1001 showed the largest zone size at day 6 indicating the highest PG activity at that time point. Similarly, the highest PG activity for the CRY1047 isolate was observed at day 5. PGIP extracts from the various transgenic lines were thus tested for inhibitory activity against the ammonium sulphate precipitated PGs from the CRY1001 and CRY1047 isolates. A positive control reaction containing *V. dahliae* PG and apple PGIP extracts from transgenic and non- transgenic tobacco was included in this assay. The *V. dahliae* PG was completely inhibited by the apple PGIP extracts from transgenic tobacco (Figure 3.27

A, well b). In contrast, *Eucalyptus* protein extracts from the eight PCR positive transgenic lines were unable to inhibit PGs produced by *C. zuluense*. No significant difference in zone sizes could be observed when both CRY1047 and CRY1001 isolates were used. Clear zones of polygalacturonase activity observed around wells containing the PGIP extracts indicated the absence of the inhibitor in the extracts from these transgenic tobacco lines (Figure 3.27).

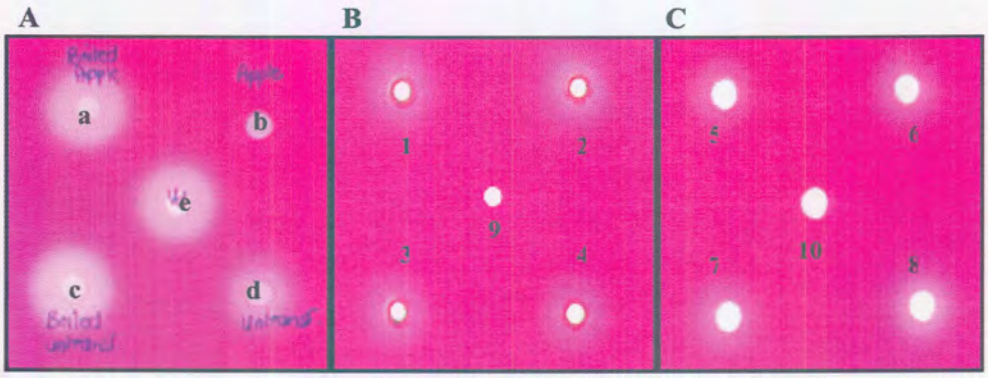


Figure 3.27 Agarose diffusion assay to test inhibition of *C. zuluense* (CRY1047) polygalacturonases by protein extracts from transgenic and non-transgenic tobacco plants. **(A)** Positive control reaction showing inhibition of *V. dahliae* (Vd) PGs by apple PGIP extracts. Zones of activity are as follows: **a)** Vd: boiled apple PGIP; **b)** Vd:apple PGIP; **c)** Vd: boiled PGIP(non-transformed); **d)** Vd: PGIP(non-transformed) **e)** Vd:NaOAc. **(B and C)** Test reactions using protein extracts from transgenic tobacco lines LAT17 and LAT18 respectively. Zones represent : **1)** Cz:NaOAc, **2)** Cz:PGIP(non-transformed), **3)** Cz:PGIP(LAT17), **4)** Cz:boiled PGIP(LAT17), **5)** Cz:NaOAc, **6)** Cz:PGIP(non-transformed), **7)** Cz:PGIP(LAT18), **8)** Cz:boiled PGIP(LAT18), **9)** and **10)** NaOAc.

Reducing sugar assay to determine the inhibition of *C. zuluense* PGs by *Eucalyptus* PGIP extracts from transgenic tobacco

A reducing sugar assay was also performed to test the inhibitory activity of protein extracts from the transgenic tobacco plants. The linear trend of ammonium sulphate precipitated *C. zuluense* PG activity was determined over a period of 100 min (Figure 3.28). Optimal enzyme activity could be observed for the 1:1 dilution of PG in 20mM

NaOAc (pH 4.7) at t = 40min. A 1:1 dilution of polygalacturonase activity from the CRY1047 isolate was tested against the proteins extracted from the selected transgenic lines. The proteins extracted from transgenic lines showed no inhibition towards polygalcturonases produced by *C. zuluense* (Figure 3.29).

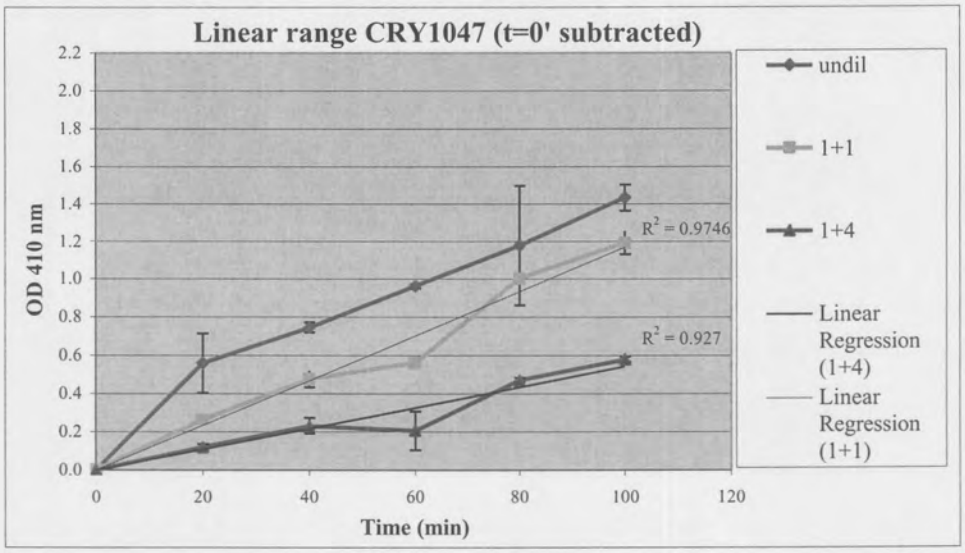


Figure 3.28 Linear trend for *C. zuluense* (CRY1047) polygalacturonase activity determined over a period of 100 min. The undiluted, 1+1 dilution and 1+4 dilution reaction results of CRY1047 PGs are represented.

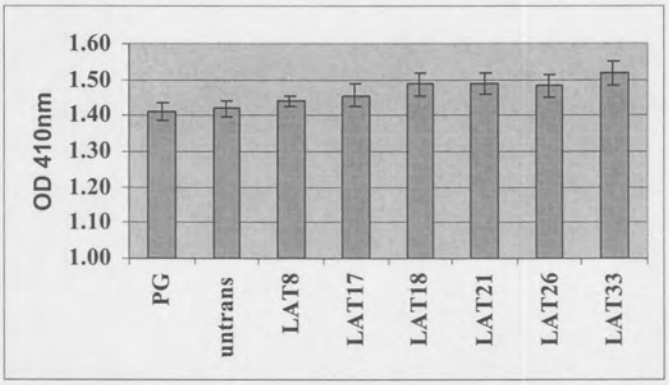


Figure 3.29 *C. zuluense* (CRY1047) PG activity in the presence of PGIP extracts from transgenic (LAT8, LAT17, LAT18, LAT21, LAT26, LAT33) and non-transformed (untrans) tobacco lines.

Discussion

Fungal plant pathogens secrete an arsenal of cell-wall degrading enzymes that enable the penetration of their hosts and subsequent establishment of infection (Hahn *et al.*, 1994; Schäfer, 1994). Pectin degrading enzymes, especially the polygalacturonases (PGs) are of particular interest in the field of host-pathogen interactions. Proteins that inhibit the activity of polygalacturonases on plant cell walls have been isolated from a wide range of plant species (De Lorenzo and Cervone, 1997). These polygalacturonase-inhibiting proteins (PGIPs) have been proposed to have an important function in the resistance of plants to pathogenic fungi (Cervone *et al.*, 1989; De Lorenzo and Cervone, 1997). In order to determine the functional characteristics of PGIPs, cloned genes were expressed in plants either stably through genetic transformation or transiently through infection of *Nicotiana benthamiana* with a modified potato virus X (PVX) (Desiderio *et al.*, 1997; Leckie *et al.*, 1999; Berger *et al.*, 2000; Powell *et al.*, 2000; De Lorenzo *et al.*, 2001).

This chapter demonstrates the construction of a viable plant transformation vector that carries a *Eucalyptus pgip* gene and the subsequent *Agrobacterium*-mediated transformation of *N. tabacum* cv LA Burley plants. Transgenic plants exhibited phenotypic characteristics indistinguishable from non-transgenic plants. Gene integration and expression studies were thus conducted to determine the inhibitory activity of *Eucalyptus pgip* from transgenic tobacco against polygalacturonases extracted from the *Eucalyptus* fungal pathogen, *C. zulunse.*

In the construction of an expression cassette, a partial *Eucalyptus pgip* gene was amplified from TAG5 genomic DNA using gene-specific primers PC6 and SPPGIP2 (chapter2). Due to the unavailability of the genome walking 5' upstream and 3' downstream nucleotide sequence of the *Eucalyptus pgip* gene, additional primers EN-FWD-PGIP and REV-PB-PGIP were designed from the nucleotide sequences of the

partial *Eucalyptus pgip* gene and the published pear *pgip* gene, respectively and used to amplify a complete *Eucalyptus pgip* gene containing the translation termination signal derived from the pear *pgip* sequence (Figure 3.3). Amplification with these primers enabled the incorporation of restriction sites *Nco*I and *Bam*HI at the 5' and 3' ends respectively for the efficient cloning of the complete *pgip* gene into the *Nco*I/*Bam*HI site of the intermediate pRTL2 vector. Nucleotide sequencing of the p*Euc*RTL2 expression cassette confirmed that the orientation of the insert was correct and that the 5' junction point between the *pgip* gene and the pRTL2 were in-frame (figure 3.8). The orientation of the *Eucalyptus pgip* expression cassette subsequently cloned into the *Pst*I site of the pCAMBIA 2301 was determined by *Nco*I digestion, which resulted in the production of a 9763bp fragment and a doublet containing a 1986bp and a 1992bp fragment. This profile indicates that the enhanced CaMV 35S promoter that drives expression of the *Eucalyptus pgip* gene lies next to the CaMV 35S promoter that drives the expression of the *nptII* gene. All pECambia4 recombinants tested showed the same restriction profile and therefore contained the *Pst*I expression cassette in the same orientation.

Transgenic plants were selected on medium supplemented with the antibiotics kanamycin and cefotaxime. The ability of plants to regenerate on this medium indicated stable gene integration. PCR analysis of tobacco plants transformed with the *Eucalyptus pgip* expression cassette verified the presence of the *nptII* gene, the *Eucalyptus pgip* gene and the GUSintron gene in eight transgenic plants. The positive control reaction in which tobacco plants were transformed with the C58/pCAMBIA2301 strain was selected on medium containing kanamycin and contained both the *nptII* as well as the GUSintron gene as indicated by PCR analysis. These plants were however lost during subsequent sub-culturing processes due to technical difficulties experienced with the plant incubator. Regenerated C58/pCAMBIA 2301 transformed plants were thus not available for subsequent screening.

Despite the fact that PCR results provided positive verification of gene integration, Southern blot analysis showed differently. The eight PCR positive transgenic lines probed with a DIG-labelled *pgip* fragment did not produce any hybridisation signals.

As a control, an untransformed line spiked with different concentrations of the *pgip* fragment was included and each produced intense hybridisation signals representative of a minimum of 35 714 copies of the gene (Figure 3.22). A faint hybridisation signal observed in lane 7 of figure 3.22 representing transgenic line LAT17 was initially thought to be a positive result indicating gene integration. The occurrence of this signal is however due to an overflow of the untransformed line spiked with 50ng of the *Eucalyptus pgip* fragment, which was calculated to represent as much as 178 571 copies of the gene. The *Eucalyptus pgip* fragment as well as the plant transformation construct pECambia4 was included in the hybridisation reaction as controls and positive results could be obtained for both, indicating that the reaction was carried out optimally. As expected, the non-transformed line showed no hybridisation signals.

The observed discrepancy between the PCR and Southern blot results can be explained by the fact that only 2µg of genomic DNA from each transgenic line tested was used for Southern blot analysis. For optimal results a minimum concentration of 10µg should be used. However, due to time constraints genomic DNA was extracted from fairly young plants with few leaves thus resulting in a lower concentration. From these results it is impossible to determine with certainty whether the *Eucalyptus pgip* gene had firstly, been inserted into the tobacco genome and secondly, if it were inserted, how many copies would that represent. A Southern blot experiment using a higher DNA concentration would possibly provide the relevant information.

The histochemical assay is a qualitative means of determining GUS gene expression. For quantitative results, which determine the level of GUS expression a fluorometric assay, using MUG (4-methylumbelliferyl-β-D-glucuronide) as a substrate can be performed (Jefferson *et al.,* 1987). Histochemical GUS assays conducted on the 43 transgenic lines indicated the expression of the GUS-intron gene in 14 of these plants by the observed blue staining of leaf pieces. The *Eucalyptus pgip* expression cassette was constructed to contain both the GUS-intron gene as well as the *pgip* gene within the T-DNA border, each gene being controlled by a CaMV dual 35S promoter and terminator sequence. The expression of the GUS-intron gene is thus a further indication of functional plant expression signals and therefore positive *Eucalyptus pgip* gene integration.

The activity of the *Eucalyptus pgip* gene in transgenic tobacco plants was investigated by PGIP inhibition assays. Polygalacturonase activity appeared higher for CRY1047 isolate when compared to the CRY1001 activity. The PGs in samples from day 3 to day 6 for isolate CRY1001 and from day 3 to day 7 for CRY1047 were collected separately by ammonium sulphate precipitation. Agarose diffusion assay of ammonium sulphate precipitated samples showed highest PG activity at day 5 for CRY1001 and day 6 for CRY1047 (Figure 3.26).

PGIP proteins were extracted from transgenic tobacco lines and used in an inhibition assay against PGs from *C. zuluense* isolates CRY1001 and CRY1047. Neither the CRY1001 nor the CRY1047 PG activities were inhibited by any of the PGIP extracts from the selected transgenic lines. To test the efficiency of the assay medium, inhibition of *V. dahliae* PGs by apple PGIP extracts from transgenic tobacco plants were included as a positive control reaction. In these reactions, *V. dahliae* PG was completely inhibited by the apple PGIP extracts. Similar results were expected for the *C. zuluense* isolates, however several repetitions of the assay confirmed that the *Eucalyptus* PGIPs from the transgenic tobacco plants were unable to inhibit the polygalacturonase activity.

The linear trend of *C. zuluense* PG activity revealed that at t = 40 min, PG activity increased in a linear range, which was optimal for conducting the assay. Reducing sugar assays conducted at 40 min with a 1+1 dilution of CRY1047 PG tested against inhibition by PGIP extracts from transgenic tobacco lines were thus expected to show some level of inhibition. However, optical density readings for PGIPs from transgenic lines were somewhat higher than the non-transformed and PG control reactions. This indicates that a greater amount of PG activity is present in the transgenic lines as compared to the controls and thus no inhibition is observed. In order to obtain meaningful results, exact PG and PGIP protein concentrations need to be taken into consideration and a uniform concentration should be used for each assay. The *Eucalyptus* PGIP protein concentrations determined by the Biorad protein assay was between 16 and 20µg/ml. These concentrations were much lower than those determined for the *C. zuluense* PGs, which were determined to be 28µg/ml for

the CRY1047 isolate and 31µg/ml for the CRY1001 isolate. Equal volumes of PG and PGIP were used for the assay, clearly indicating that the higher concentration of the fungal PGs present in the assay reaction could not efficiently be inhibited by the *Eucalyptus* PGIPs.

The fact that results from both the reducing sugar assay and the agarose diffusion assay show that *Eucalyptus* PGIP from transgenic lines cannot inhibit *C. zuluense* PGs can be explained in several ways:

- The amount of *Eucalyptus pgip* that accumulates in transgenic tobacco is insufficient to maximally inhibit endo-PGs from *C. zuluense*
- The fungal isolate produces many isozymes of PG, all of which cannot be inhibited by the *Eucalyptus pgip.*
- PGs produced by the fungal isolates CRY1047 and CRY1001 cannot be inhibited by the *Eucalyptus* PGIP although these are the most virulent *Eucalyptus* isolates available and thus selected for use in this study. Chimwamurombe (2001) used the *C. zuluense* isolate CMW2100 and was able to show that PGs produced by this isolate can be inhibited by PGIPs extracted from *Eucalyptus grandis* clones.
- In order to determine whether *C. zuluense* PGs were produced and able to inhibit *Eucalyptus* PGIPs as determined previously by Chimwamurombe (2001), a control reaction containing PGIPs extracted from the *Eucalyptus grandis* TAG5 clone should have been included in the inhibition assays.
- Only eight transgenic plants were included in the inhibition assays and a possibility does exist that some of the other plants would have shown some degree of inhibition.
- Mutations in the *Eucalyptus pgip* construct could also result in the absence of a translatable product although the presence of the gene was confirmed by PCR and restriction analysis. However, sequencing with the pBI121 primer verified the 5' junction region between the pRTL2 vector and the *Eucalyptus pgip* gene only. The complete *Eucalyptus pgip* sequence was not obtained using this primer and therefore the possibility of errors incorporated by the EN-FWD-PGIP and REV-PB-PGIP primers cannot be excluded.

- Variable or no expression of transgenes could be explained by 'position-effects', which refers to the position of the genome in which the T-DNA integrates (Gelvin, 1998).
- Rearrangements or multiple copy integration of T-DNA in direct or inverted repeat conformation leads to transgene silencing.

This chapter reports on the production of transgenic tobacco plants. Positive results of transgene expression could only be obtained from PCR and histochemical GUS assays. Further expression studies are required to positively confirm that the plants generated do indeed contain a translatable mRNA product. Ideally Northern blot analysis can be performed to determine whether a translatable product is present in each of the transgenic lines. PGIP inhibition assays with a range of other *C. zuluense* fungal isolates, in particular CMW2100 could potentially provide valuable information regarding the specificity of *Eucalyptus* PGIP for the various fungal PGs.

SUMMARY

The genetic engineering of plants for increased resistance to fungal pathogens has been extensively employed since the identification and characterization of several plant disease resistance (*R*) genes at the beginning of the molecular biology era. Much focus has been placed on the polygalacturonase-inhibiting proteins (PGIPs) since they are the only LLR proteins for which a ligand (PG) has been demonstrated. Prior to the development of transgenic plants with increased disease resistance, the isolation and molecular characterization of these disease resistance genes are of great importance.

In a previous study conducted by Chimwamurombe (2001), a partial *Eucalyptus pgip* gene was isolated from five *Eucalyptus* species and used for sequence comparisons. PGIP inhibition assays were conducted to determine the interaction between *Eucalyptus* PGIPs and endopolygalacturonases from different fungal pathogens. Chimwamurombe (2001) was able to show that the *E. grandis* TAG5 clone produced PGIPs that were able to inhibit PGs produced by the *Eucalyptus* fungal pathogen, *Coniothyrium zuluense.*

The complete *Eucalyptus pgip* gene was obtained in this study by genome walking PCR. A 397bp fragment, which shared an overlap of 91bp with the partial *Eucalyptus* sequence at the 5' end of the gene, represented the upstream fragment. Similarly, a 639bp downstream fragment was obtained. The downstream fragment shared an overlap of 39bp with the partial *Eucalyptus pgip.* Results presented in Chapter 2 indicate that the complete *E. grandis* PGIP showed 99% amino acid identity and similarity to the published pear PGIP sequence, with 3 amino acid differences and 3 nucleotide differences. This indicates a possible divergence of these PGIPs from a common ancestral PGIP. Additionally, differences between the PGIP polypeptide sequences of the published partial *E. grandis* PGIP and the complete PGIP presented in this study were observed. Several reasons have been attributed to the observed differences, the most obvious being the use of different *Eucalyptus* clones in each study.

The functionality of the *E. grandis pgip* gene was determined by producing transgenic tobacco plants via *Agrobacterium*-mediated transformation. The construction of the plant transformation vector used in subsequent transformation experiments entailed the cloning of the *Eucalyptus pgip* gene into an intermediate pRTL2 vector. The *Eucalyptus* expression cassette contained within the p*Euc*RTL2 recombinant contained a dual CaMV35S promoter as well as a TEV leader sequence for enhanced translation. The *Eucalyptus pgip* expression cassette was cloned into the commercially available pCAMBIA 2301 plant transformation vector, which contains a GUS-intron gene as well as kanamycin resistance gene. The resulting recombinant pECambia4 construct was used for the *Agrobacterium*-mediated transformation of tobacco plants.

A total of 43 tobacco plants transformed with the pECambia4 construct were fully regenerated, hardened off and transferred to the greenhouse. PCR analysis showed that only eight of the 20 randomly selected transgenic lines, were transformed with the pECambia4 construct. Histochemical GUS localization revealed that 14 of the 43 transgenic lines contained the GUS-intron gene and therefore, presumably the *Eucalyptus pgip* gene. Southern blot analysis conducted to determine the gene copy number was however not successful.

PGIP extracts from the transgenic tobacco lines were assayed for inhibitory activity towards PGs produced by *C. zuluense.* No inhibition of PG extracts could be observed. Lack of PGIP activity could possibly be due to the presence of multiple copies of the gene, which could result in transgene silencing or due to the incorporation of mutations within the *Eucalyptus pgip* construct.

Results pertaining to the inhibition of *C. zuluense* PGs by *Eucalyptus* PGIP extracts in this study are inconclusive. This report however, includes the characterization of the composite *Eucalyptus pgip* gene, the construction of a viable plant transformation vector and the successful transformation of *N. tabacum* plants with the construct. The establishment of disease resistance in transgenic tobacco plants is the first step towards the generation of transgenic *Eucalypts* expressing high levels of PGIPs.

OPSOMMING

Sedert die identifikasie en karakterisering van verskeie plant weerstandsgene vroeër in die molekulêre era, is die genetiese modifikasie van plante vir verhoogde weerstand teen fungale patogene wydverspreid aangewend. Daar is grootliks gefokus op the poligalakturonase-inhiberings proteïene (PGIPe), aangesien hulle die enigste LLR proteiene is waarvoor daar 'n ligand (PG) bekend is. Voor transgeneniese plante met verhoogde siekte weerstand egter ontwikkel kan word, is die isolasie en molekulêre karakterisering van hierdie weerstandsgene van primêre belang.

In 'n vorige studie deur Chimwamurombe (2001) is 'n gedeelte van die *Eucalyptus pgip* geen uit vyf *Eucalyptus* spesies geïsoleer en gebruik in die vergelyking van basispaar volgorder. PGIP inhibisie proewe is uitgevoer om die interaksie tussen *Eucalyptus* PGIPs en endopoligalaktorunase van verskillende fungale patogene te bepaal. Chimwamurombe (2001) het verder ook bewys dat die *E. grandis* TAG5 kloon PGIPe produseer wat die PGs van die *Eucalyptus* fungus patogeen, *Coniothyrium zuluense* inhibeer.

Tydens hierdie studie is die volledige *Eucalyptus pgip* geen verkry deur "genome walking" PCR. 'n 397bp fragment, wat 91bp oorvleuel met die gedeeltelike *Eucalyptus* basispaar volgorder by the 5' gedeelte verteenwoordig the stroomop fragment. Soortgelyk is die 639bp stroomaf fragment verkry. Die stroomaf fragment oorvleuel 39bp met die gedeeltelike *Eucalyptus pgip* geen Resultate in Hoofstuk 2 dui aan dat die volledige *E. grandis pgip* 99% verwant is aan die gepubliseerde peer PGIP basispaar volgorder, met 3 aminosuur en 3 nukleotied verskille. Dit dui op 'n moontlike wegbeweeg van hierdie PGIPe van die gemeenskaplike voorouer PGIP. Verskille tussen die PGIP polipeptied van die gepubliseerde gedeeltelike *Eucalyptus* PGIP en die volledige PGIP van hierdie studie is ook waargeneem. Verskeie oorsake kon bydrae tot hierdie verskille, en die mees ooglopend is die gebruik van verskillede *Eucalyptus* klone in die onderskeie studies.

Die funksie van die *E. grandis pgip* geen is ondersoek deur transgeniese tabakplante te maak deur middel van die *Agrobacterium*-bemiddelde transformasie. Die ontwerp van die plant transformasie vektor wat in die opeenvolgende transformasie eksperimente gebruik is, het die klonering van die *Eucalyptus pgip* gene in die tussenganger pRTL2 vektor behels. Die *Eucalyptus* uitdrukkingskasset het 'n dubbelle CaMV35S promotor sowel as 'n TEV leier binne die p*Euc*RTL2 rekombinant. Die *Eucalyptus pgip* uitdrukkingskasset is in die kommersiële pCAMBIA 2301 plant transformasie vektor, wat 'n GUS-intron geen sowel as 'n kanamisien weerstandsgeen bevat, gekloneer. Die daaropeenvolgende rekombinante pECambia4 is gebruik vir die *Agrobacterium*-bemiddelde transformasie van die tabakplante.

'n Totaal van 43 tabakplante, wat met die pECambia4 getransformeer is, is suksesvol getransformeer, verhard en oorgedra na die glashuis. PCR analise het aangedui dat slegs agt van die 20 lukraak verkose getransformeerde lyne met die pECambia4 getransformeer is. Histochemiese GUS lokalisasie het aangedui dat 14 van die 43 transgeniese lyne die GUS-intron geen bevat en dus ook die *Eucalyptus pgip* geen. Southern klad analise om die aantal geen kopieë te bepaal was egter onsuksesvol.

PGIP ekstraksies uit die transgeniese tabaklyne is geanaliseer vir moontlike inhibisie van PGs wat deur *C. zuluense* geproduseer word. Geen inhibisie van die PG ekstrakte kon waargeneem word nie. Die afwesigheid van PGIP aktiwiteit is waarskynlik as gevolg van die teenwoordigheid van veelvoudige kopieë van die geen wat kan lei tot die afskakeling van die geen. Dit kan ook wees as gevolg van die insluiting van mutasies in die *Eucalyptus pgip*.

Resultate van die inhibisie eksperimente van *C. zuluense* PGe deur die *Eucalyptus* PGIP ekstrakte was onbeduidend. Die verhandeling sluit egter die karakterisering van die volledige *Eucalyptus pgip* geen, die konstruksie van 'n lewensvatbare plant transformasie vektor en die suksesvolle transformasies van *N. tabacum* plante met die gesintetiseerde plasmied. Die ontwikkeling van siekte weerstand in transgeniese tabak plante is die

eerste stap in die ontwikkeling van transgeniese *Eucalyptus* wat hoë vlakke van PGIPe produseer.

Appendices

APPENDIX A

List of Amino Acids and Their Abbreviations

Nonpolar Amino Acids (hydrophobic)

amino acid	three letter code	single letter code
glycine	Gly	G
alanine	Ala	A
valine	Val	V
leucine	Leu	L
isoleucine	Ile	I
methionine	Met	M
phenylalanine	Phe	F
tryptophan	Trp	W
proline	Pro	P

Polar (hydrophilic)

serine	Ser	S
threonine	Thr	T
cysteine	Cys	C
tyrosine	Tyr	Y
asparagine	Asn	N
glutamine	Gln	Q

Electrically Charged (negative and hydrophilic)

aspartic acid	Asp	D
glutamic acid	Glu	E

Electrically Charged (positive and hydrophilic)

lysine	Lys	K
arginine	Arg	R
histidine	His	H

The Standard Genetic Code

First Position (5' end)	Second Position				Third Position (3' end)
	U	C	A	G	
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met Start	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Start Codon
Stop Codon
Nonpolar Side Chain
Uncharged Polar Side Chain
Charged Polar Side Chain

From: http://www.cbs.umn.edu/~amundsen/chlamy/code.html

Nucleotide Base Codes
IUPAC

Symbol	Nucleotide
A	adenine
C	cytosine
G	guanine
T	Thymine - DNA Uracil - RNA

Single letter amino acids and wobble predictions for designing degenerate oligonucleotide primers

AA	Predicted 3' codon	AA	Predicted 3' codon
B	C, G, T	M	A, C
D	A, G, T	N	A, C, G, T
H	A, C, T	R	A, G
K	G, T	S	C, G
V	A, C, G	W	A, T
Y	C, T		

APPENDIX B

Composition of buffers and solutions

- **Agarose Diffussion Assay medium**

  100mM Citrate

  200mM Na_2HPO_4

  0.5% (w/v) Ammonium oxalate

  0.01% (w/v) Polygalcturonic acid (PGA)

  1% (w/v) TypeII agarose (Sigma)

  pH 5.3

- **Antibiotic Stock Solutions**

  Ampicillin

  Cefotaxime

  Kanamycin

  Rifampicin

- **Blocking Solution**

  1 x working solution : 1% (w/v) dissolved in Maleic acid buffer

- **Citrate/Phosphate Buffer**

  0.1M Citric acid

  0.2M Na_2HPO_4

  pH 5.3

- **Co-cultivation Medium**

  Composition per liter:

  4.42g MS salts (Highveld Biological)

  1ml 1000 X vitamin stock

  0.1mg NAA

  1mg BAP (6-Benzylaminopurine)

30g Sucrose

8g Agar

pH 5.8

- **Denaturation Solution**

  0.5M NaOH

  1.5M NaCl

- **Detection Buffer**

  0.1M Tris-HCl

  0.1M NaCl

  pH 9.5

- **0.5M EDTA**

  Dissolve 186.1g disodium-EDTA in 1 liter ddH_2O

  Adjust pH to 8.0

- **GUS Assay Solution**

  0.1% (w/v) X-Gluc

  0.01% (v/v) Tween 20

  10mM EDTA (pH8.0)

  50mM NaH_2PO_4

- **0.1M IPTG Stock**

  Dissolve 1.2 g isopropyl-β-D-thiogalactopyranoside in 50ml dH_2O. Filter sterilize and store at 4°C.

- **Luria Bertani (LB) Medium**

  Composition per litre:

  10g Bacto®-tryptone

  5g Bacto®-yeast extract

  5g NaCl

  Adjust pH to 7.0

- **LB Agar**

  Add 15g Bacto®agar to 1 liter of LB medium.

- **2M Mg^{2+} Stock**

  20.33g $MgCl_2.6H_2O$

  24.65g $MgSO_4.7H_2O$

  Add distilled water to 100ml.

  Filter-sterilize.

- **Maleic Acid Buffer**

  0.1M Maleic acid

  0.15M NaCl

  pH 7.5

- **2% Malt Extract Agar (MEA)**

  Composition per liter:

  20g Malt extract

  15g Bacto®Agar

- **Minimal Salts Medium**

  Composition per liter:

  2g NH_4NO_3

  1g KH_2PO_4

  0.1g $MgSO_4$

  0.5g yeast extract

  1g NaOH

  3g $_{DL}$-Malic acid

  Supplement with 0.5% sodium polygalacturonic acid (PGA)

- **MS (Murashige and Skoog) Medium for Tobacco**

  Composition per liter:

  4.42g MS Salts (Highveld Biological)

1ml 1000 x Vitamin stock
30g Sucrose
0.1g Myo-inositol
7g Agar
pH 5.8

- **Neutralising Solution**

  1M Tris-HCl pH 8.0
  1.5M NaCl
  10mM EDTA

- **PAHBAH**

  1% ρ-4-amino-2-hydroxybenzoic acid hydrazide
  0.4M NaOH
  0.1M HCl

- **RNase A (10mg/ml)**

  Dissolve 10mg RNase A in 1ml ddH_2O.
  Heat to 100°C for 10min. Allow cooling to room temperature.
  Store at -20°C.

- **Regeneration Medium**

  Composition per liter:
  4.42g MS salts (Highveld Biological)
  1ml 1000 x Vitamin stock
  1mg BAP (6-Benzylaminopurine)
  0.5mg IAA (Indole-3-Acetic Acid)
  30g Sucrose
  7g Agar
  pH 5.8

- **Ruthenium Red Solution**

  0.05% (w/v) in ddH_2O

- **10% SDS**

  Dissolve 100g electrophoresis grade SDS in 1 liter water.

  Heat to 68°C. Adjust pH to 7.2.

- **Solution 1**

  25mM Tris-HCl p H 8.0

  10mM EDTA

  50mM Glucose

- **Solution 2**

  0.2N NaOH

  1% SDS

- **Solution 3**

  3M Potassium Acetate p H4.8

- **SOC Medium**

  Composition per 100ml:

  2g Bacto®-tryptone

  0.5g Bacto®-yeast extract

  1ml 1M NaCl

  0.25ml 1M KCl

  1ml 2M Mg^{2+} stock, filter-sterilized

  1ml 2M Glucose, filter-sterilized

- **20 x SSC**

  0.3M NaCitrate

  1.5M NaCl

  pH 7

- **50 x TAE**

  Composition per liter:

  242g Tris

  57.1ml Glacial acetic acid

  100ml 0.5M EDTA pH 8.0

- **TE Buffer**

  10mM Tris-HCl pH 8.0

  1mM EDTA pH 8.0

- **1 x TNE Buffer**

  10mM Tris-HCl p H 8.0

  1mM EDTA pH 8.0

  0.2M NaCl

  pH 7.4

- **Washing Buffer**

  0.1M Maleic acid

  0.15M NaCl

  pH 7.5

  0.3% (v/v) Tween 20

- **2 x Washing Solution**

  2 x SSC

  0.1% SDS

- **0.5 x Washing Solution**

  0.5 x SSC

  0.1% SDS

- **X-gal (50mg/ml)**

  Dissolve 100mg 5-Bromo-4-chloro-3-indolyl-β-D-galactoside in 2ml N,N'-dimethylformamide. Cover with aluminum foil and store at -20°C.

- **YEP Medium**

  10g Bacto®-peptone

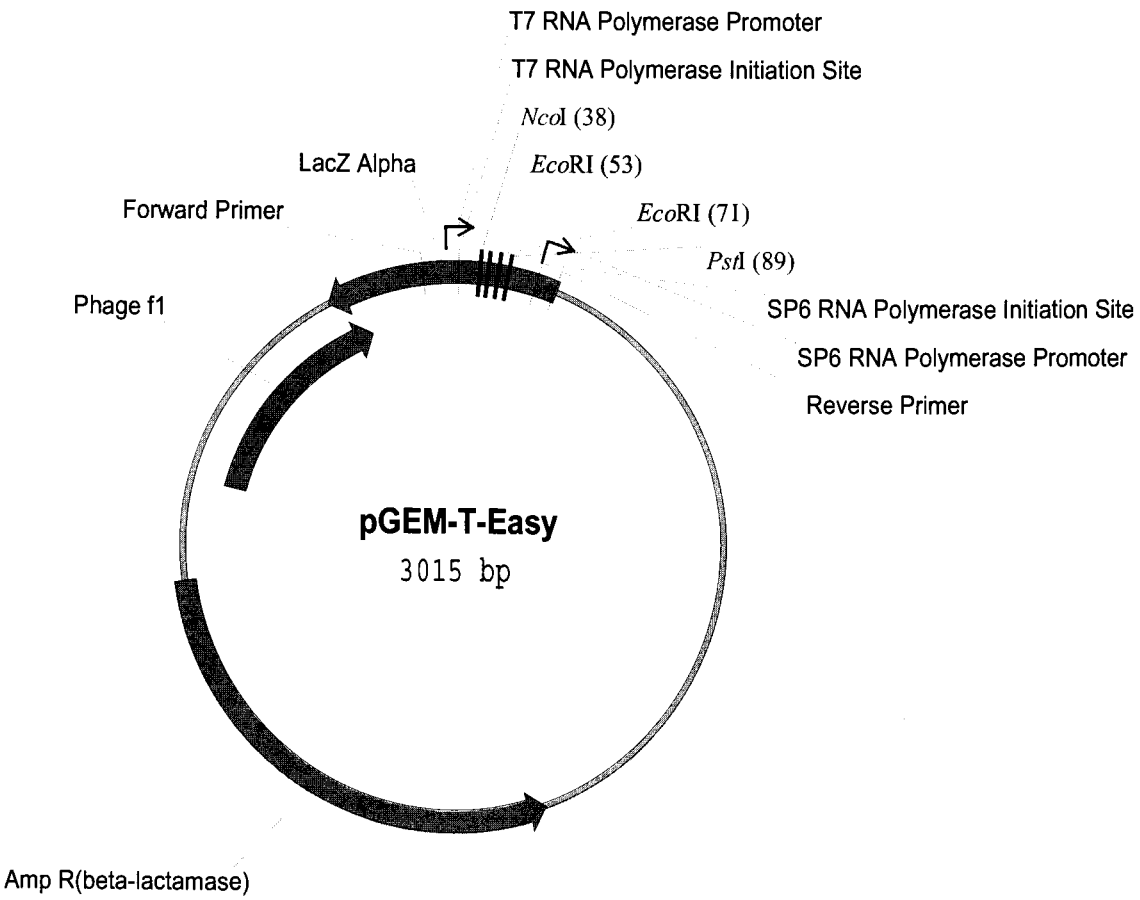
  10g Bacto®-yeast extract

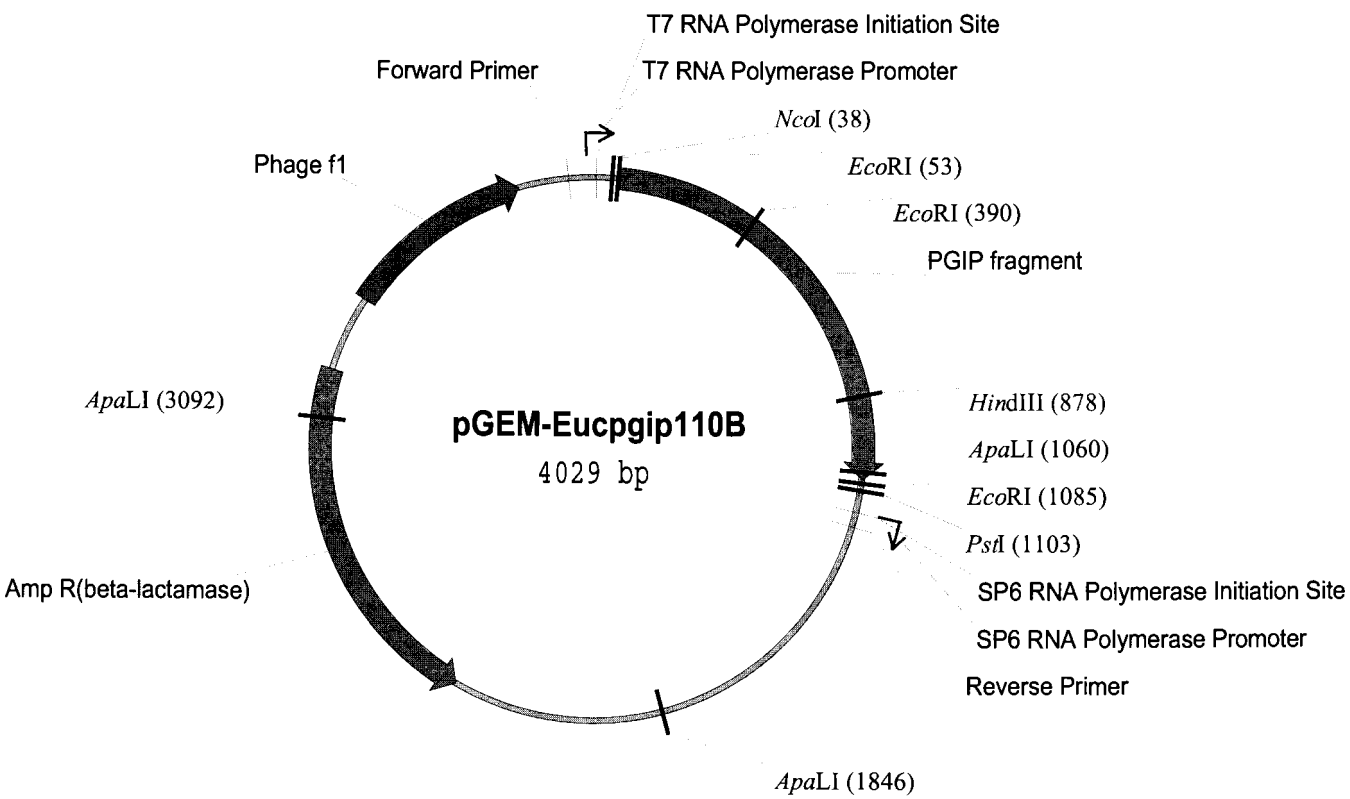
  5g NaCl

  Add 15g Bacto®-Agar for YEP-Agar

APPENDIX C

Plasmid Maps

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Forward Primer
T7 RNA Polymerase Promoter
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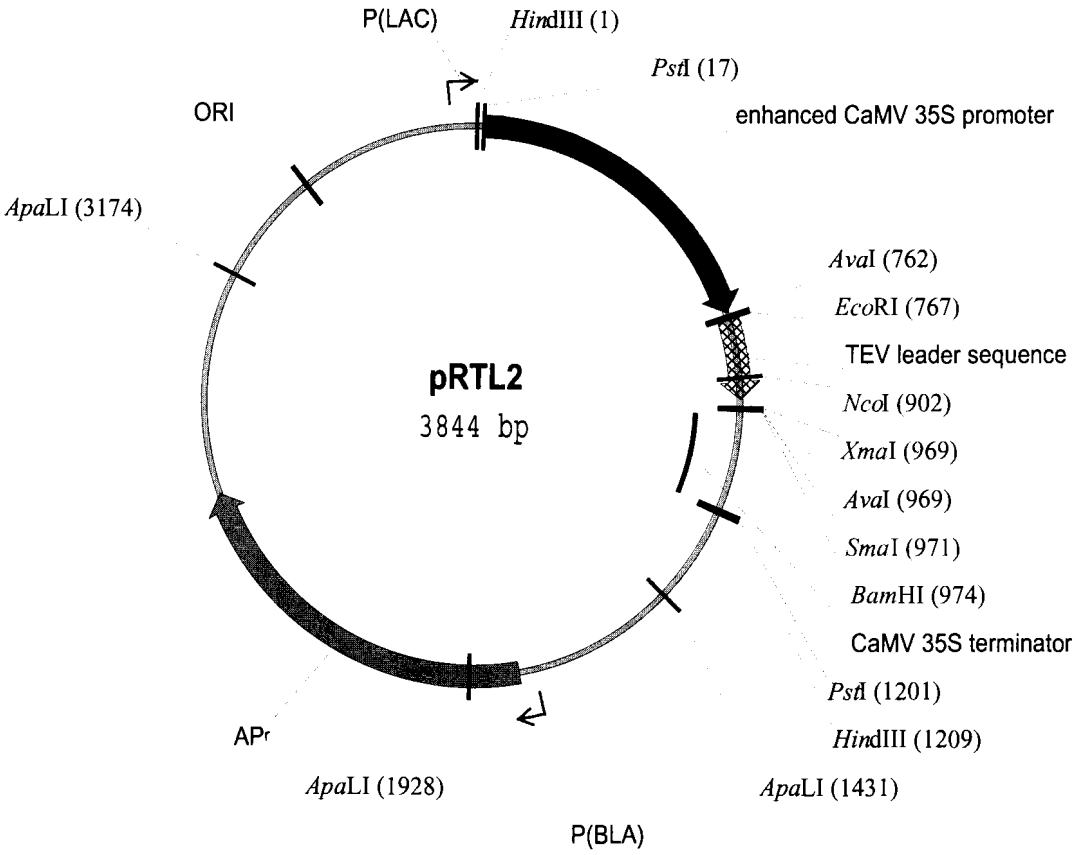
Phage f1
EcoRI (53)
EcoRI (390)
PGIP fragment
ApaLI (3092)
pGEM-Eucpgip110B
4029 bp
HindIII (878)
ApaLI (1060)
EcoRI (1085)
PstI (1103)
Amp R(beta-lactamase)
SP6 RNA Polymerase Initiation Site
SP6 RNA Polymerase Promoter
Reverse Primer
ApaLI (1846)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PstI (17)
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ORI
ApaLI (3174)
AvaI (762)
EcoRI (767)
TEV leader sequence
NcoI (902)
XmaI (969)
AvaI (969)
SmaI (971)
BamHI (974)
CaMV 35S terminator
PstI (1201)
HindIII (1209)
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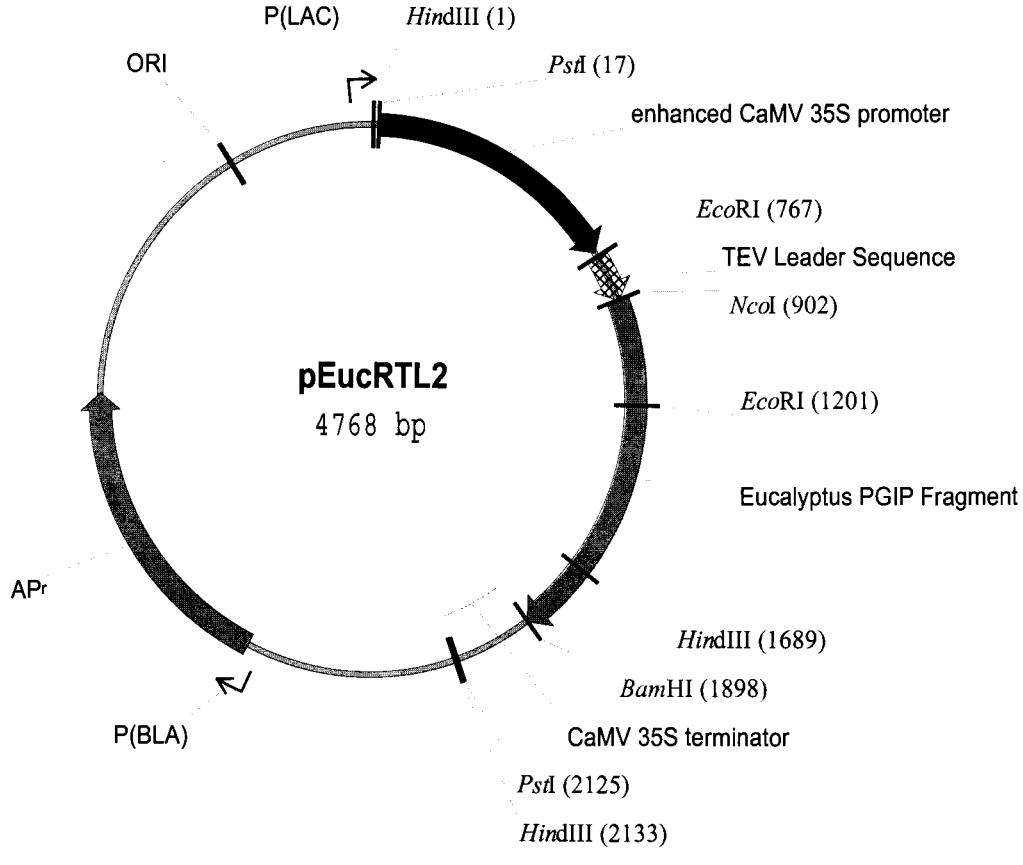
3844 bp
APr
ApaLI (1928)
ApaLI (1431)
P(BLA)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ORI
PstI (17)
enhanced CaMV 35S promoter
EcoRI (767)
TEV Leader Sequence
NcoI (902)
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4768 bp
EcoRI (1201)
Eucalyptus PGIP Fragment
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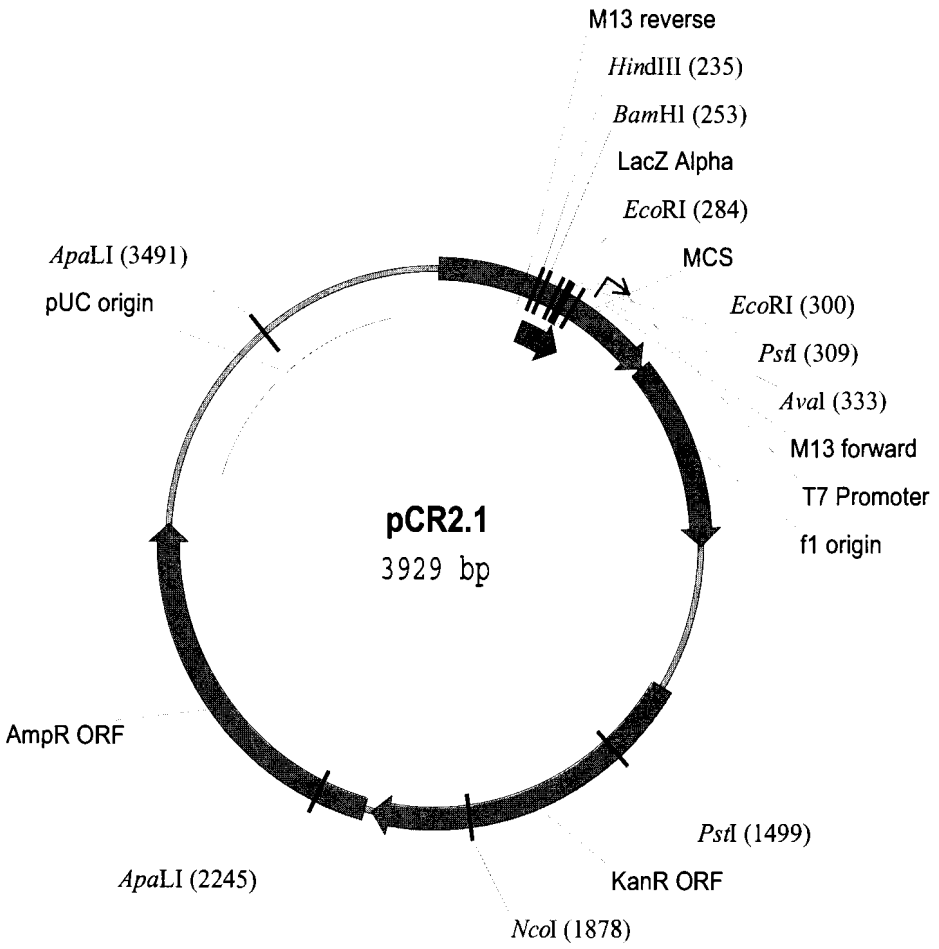
BamHI (1898)
P(BLA)
CaMV 35S terminator
PstI (2125)
HindIII (2133)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HindIII (235)
BamHI (253)
LacZ Alpha
EcoRI (284)
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PstI (309)
AvaI (333)
M13 forward
T7 Promoter
f1 origin
pCR2.1
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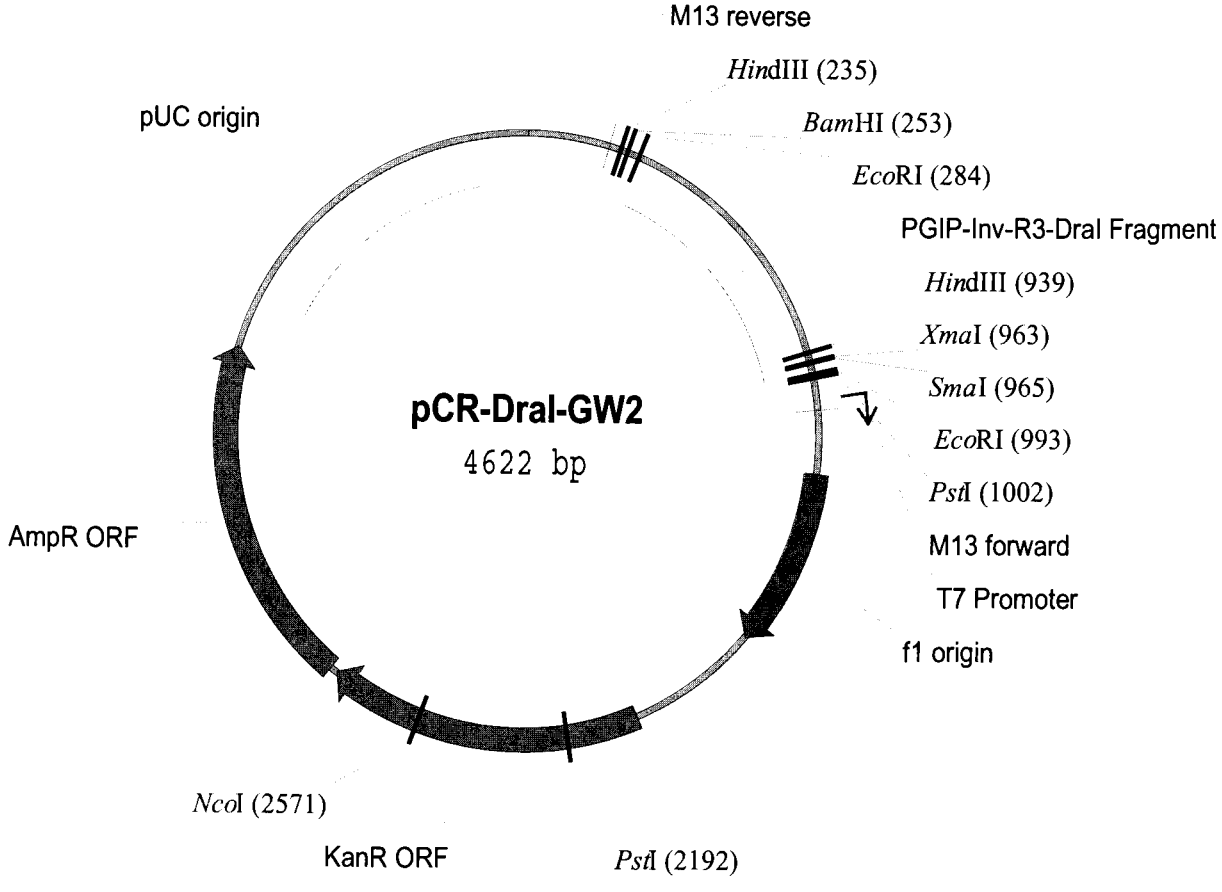
NcoI (1878)
KanR ORF
PstI (1499)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XmaI (963)
SmaI (965)
EcoRI (993)
PstI (1002)
M13 forward
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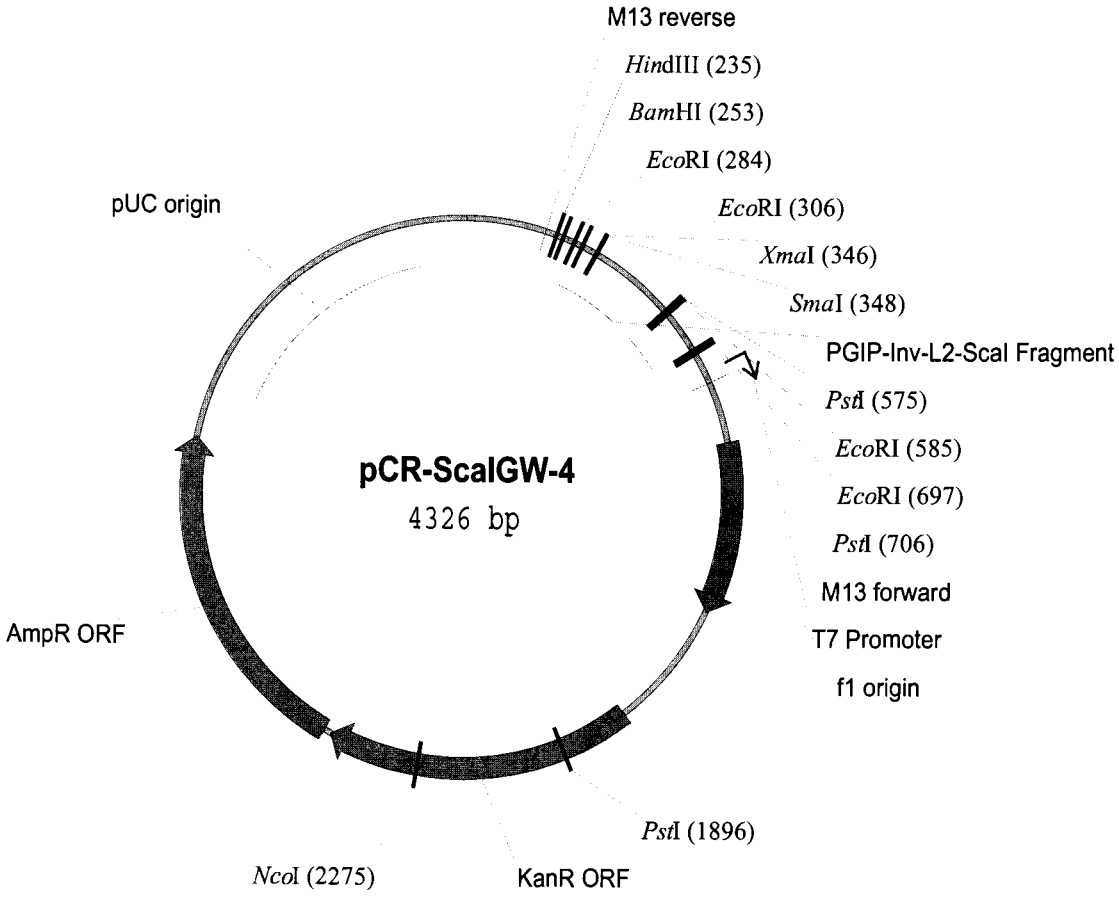
NcoI (2571)
KanR ORF
PstI (2192)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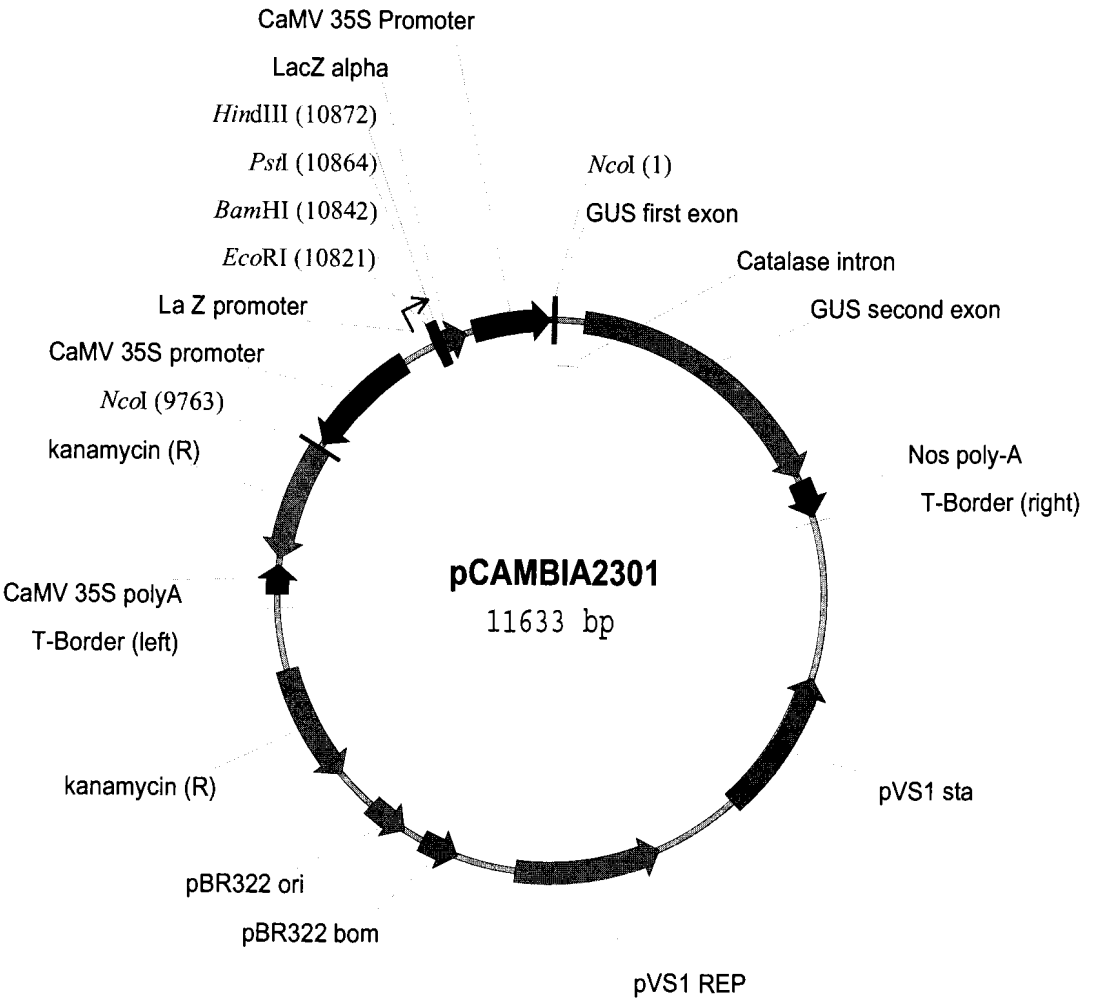
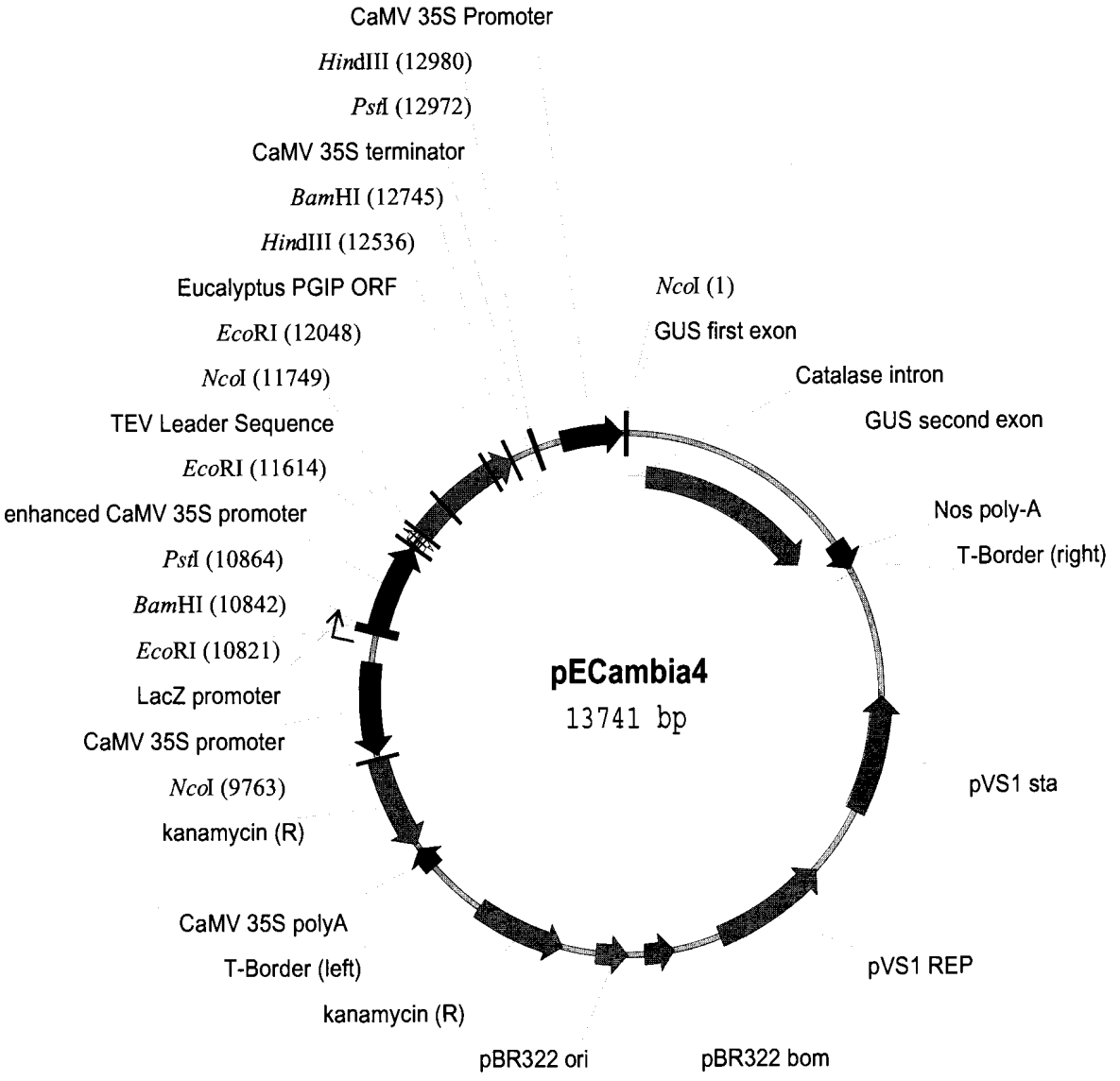
KanR ORF


CaMV 35S Promoter
LacZ alpha
HindIII (10872)
PstI (10864)
BamHI (10842)
EcoRI (10821)
La Z promoter
CaMV 35S promoter
NcoI (9763)
kanamycin (R)
CaMV 35S polyA
T-Border (left)
kanamycin (R)
pBR322 ori
pBR322 bom
pVS1 REP
pVS1 sta
NcoI (1)
GUS first exon
Catalase intron
GUS second exon
Nos poly-A
T-Border (right)
pCAMBIA2301
11633 bp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HindIII (12536)
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NcoI (11749)
TEV Leader Sequence
EcoRI (11614)
enhanced CaMV 35S promoter
PstI (10864)
BamHI (10842)
EcoRI (10821)
LacZ promoter
CaMV 35S promoter
NcoI (9763)
kanamycin (R)
CaMV 35S polyA
T-Border (left)
kanamycin (R)
pBR322 ori
pBR322 bom
pVS1 REP
pVS1 sta
Nos poly-A
T-Border (right)
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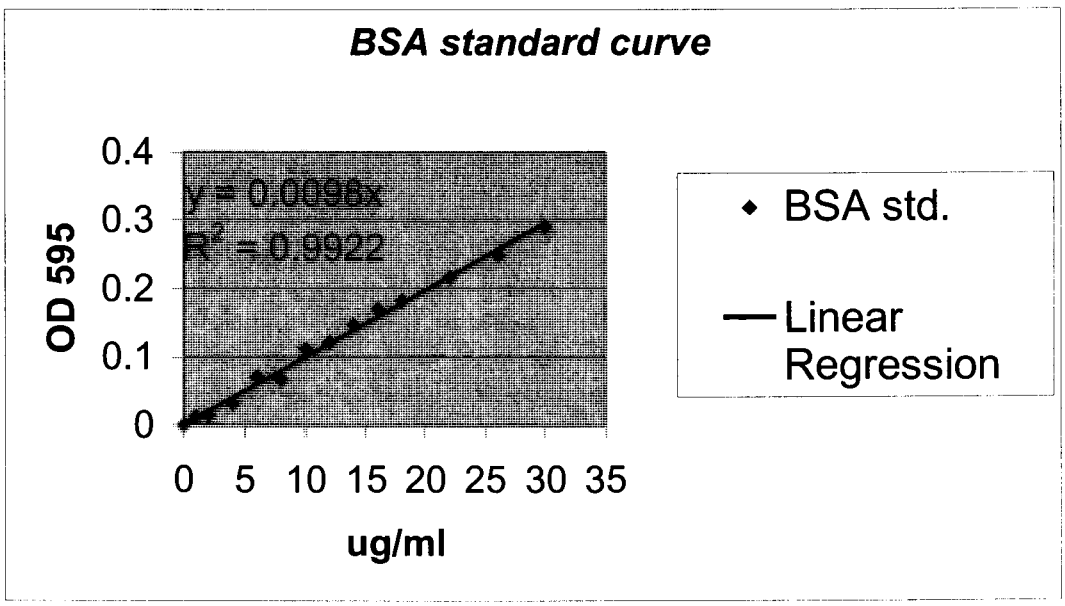
GUS first exon
Catalase intron
GUS second exon
pECambia4
13741 bp

APPENDIX D

BSA Standard Curve



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Angenon G, Dillen W, Van Montagu M. 1994. Antibiotic resistance markers for plant transformation. In: S.B. Gelvin, R.A. Schilperoort (eds), *Plant Molecular Biology Manual*. Kluwer Academic Publishers, Dordrecht. **C1**: 1-13.

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