Chapter Three

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

CONTENTS

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.

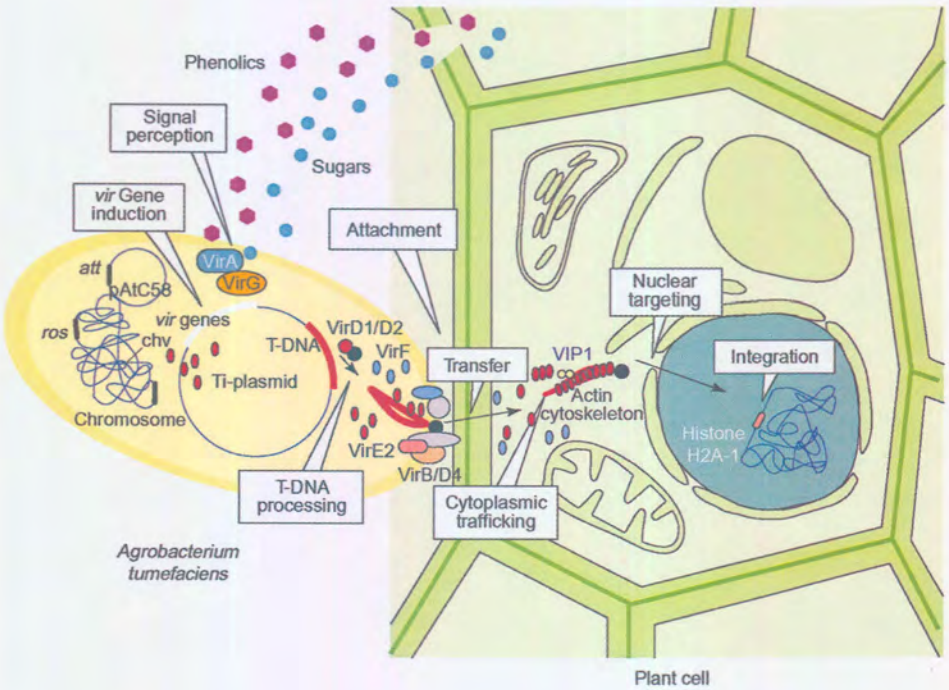

Figure 3.1 Schematic representation of the *Agrobacterium*-mediated transformation process taken from Gelvin (2003).

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.

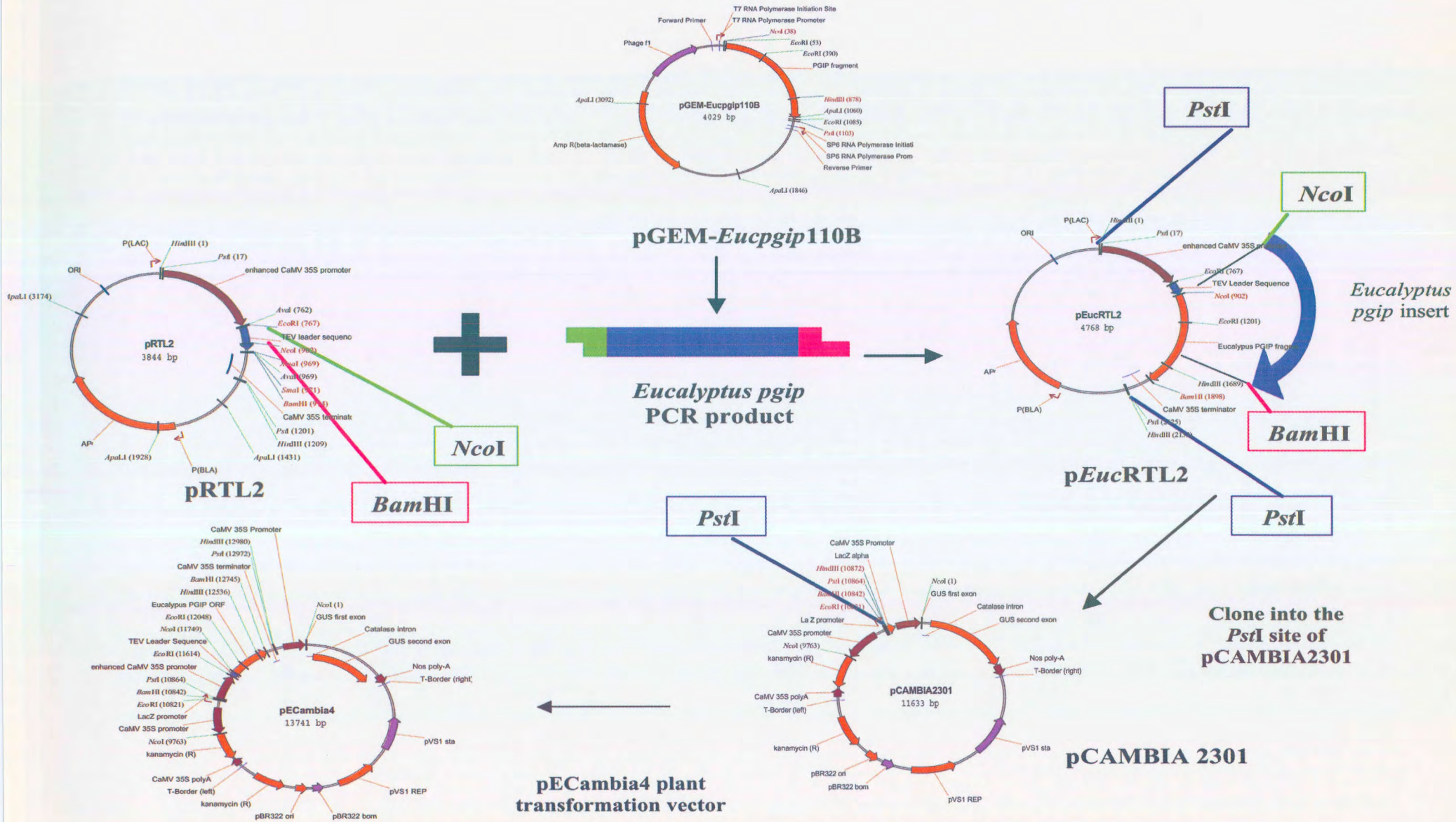

Figure 3.2 Schematic representation of the construction of the pECambia4 plant transformation vector.

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
```

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.

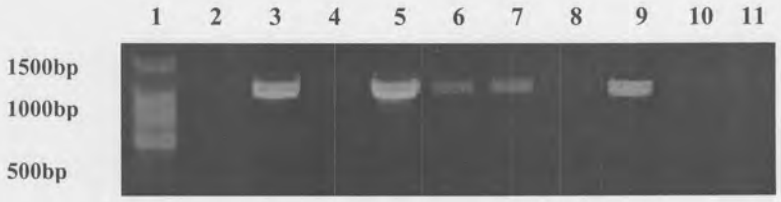

Figure 3.4 PCR analysis of the *Eucalyptus pgip* gene amplified from the pGEM-*Eucpgip* clones. The amplified products are approximately 1000bp in length. Lane 1 represents the 100bp DNA ladder (Promega) and lane 11 represents the negative control in which the template DNA was substituted with water.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

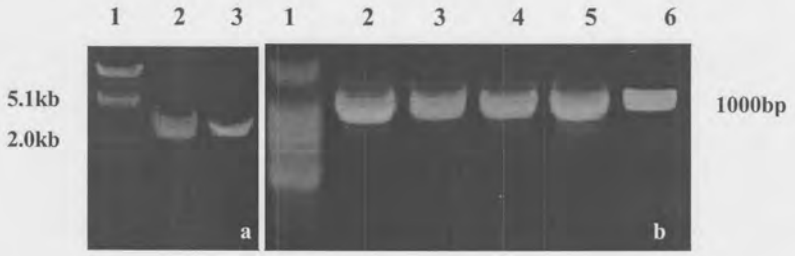

Figure 3.5 a. Restriction digestion of the intermediate pRTL2 cloning vector. Lane 1 contains the λ III marker, lane 2 represents the undigested plasmid (3844bp) and in lane 3, the pRTL2 vector has been digested with *Nco*I and *Bam*HI (3772bp). **b.** Restriction enzyme digestion of the PCR generated *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.

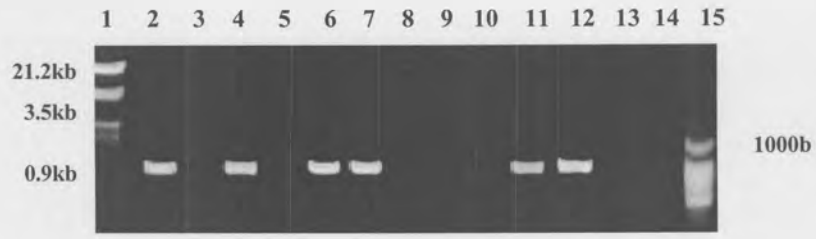

Figure 3.6 PCR analysis of p*Euc*RTL2 recombinants using the EN-PGIP-FWD and REV-PB-PGIP primers. Lane 1 contains the λ III marker and lane 15 contains the 100 bp DNA ladder (Promega). Lane 2 shows amplification of the *Eucalyptus pgip* gene from the pGEM-*Eucpgip*110B clone as a positive control and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

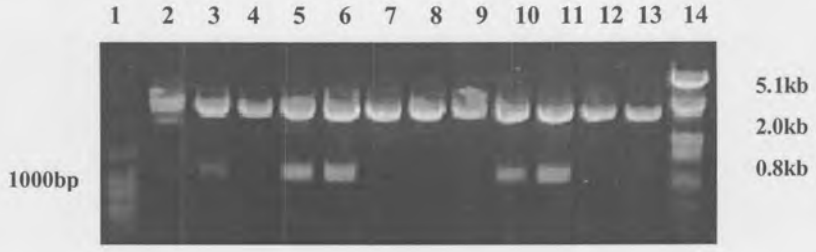

Figure 3.7 Restriction digestion analysis of p*Euc*RTL2 recombinants. Lanes 1 and 14 contain the 100bp ladder (Promega) and λ *Hind*III/*Eco*RI marker, respectively. Lane 2 contains an undigested p*Euc*RTL2 vector and lanes 3-13 represent putative recombinants p*Euc*RTL2-1 to pEucRTL2-11 digested with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

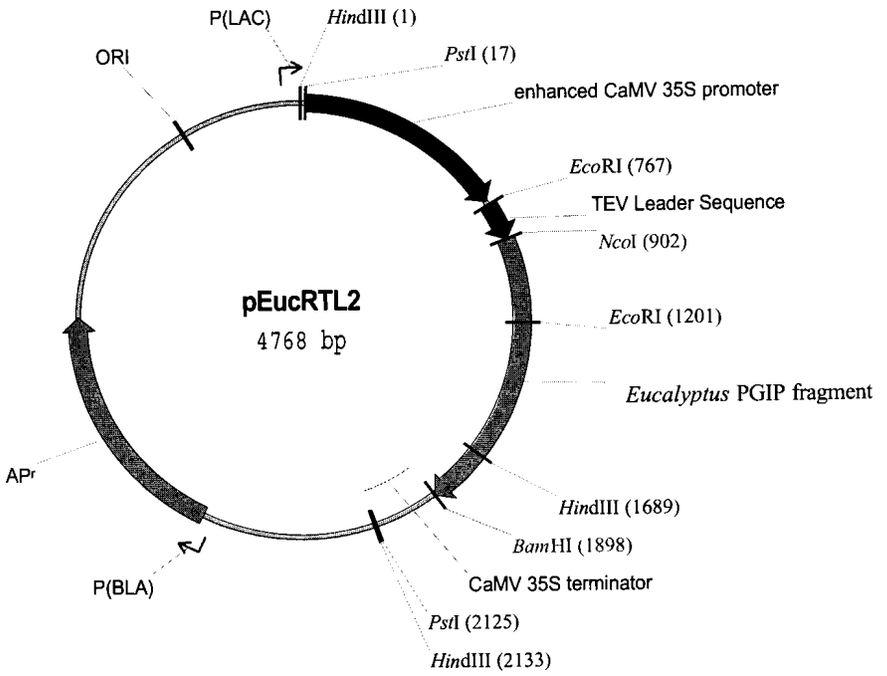

Figure 3.9 Plasmid map of the constructed p*Euc*RTL2 vector that contains the *Eucalyptus pgip* gene under control of an enhanced CaMV 35S promoter.

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.

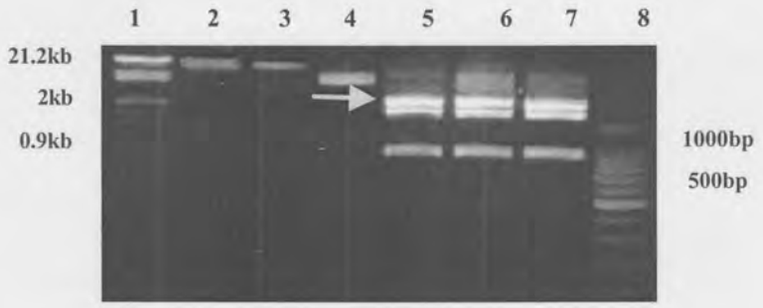

Figure 3.10 Restriction analysis of p*Euc*RTL2 with enzymes *Pst*I and *Sca*I. Lane1 contains the λIII marker and lane 8 contains the 100bp DNA ladder (Promega). Lane2 contains the undigested pCAMBIA2301 vector, while in lane 3, *Pst*I digested pCAMBIA2301 is represented. Lane 4 contains the undigested 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.

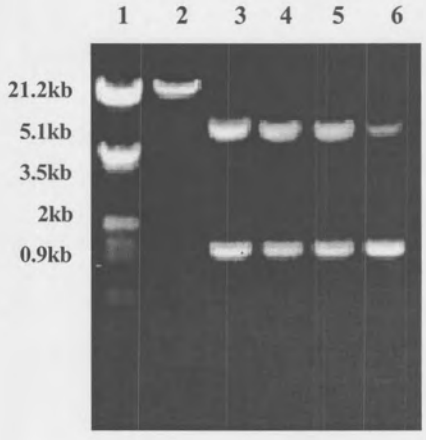

Figure 3.11 Restriction analysis of the pECambia putative recombinant plasmids with *Nco*I. Lane1 contains the λIII marker. Lane 2 represents the undigested pECambia plasmid DNA and lanes 3 – 6 represent the digested plasmids pECambia1-pECambia4, respectively.

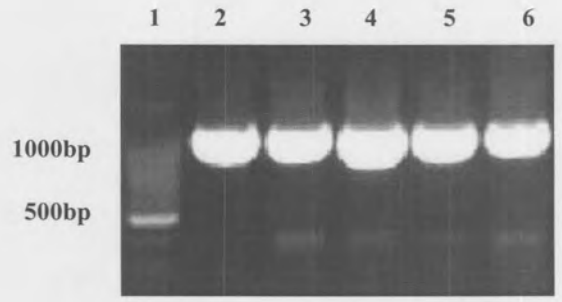

Figure 3.12 Amplification of the *Eucalyptus pgip* gene from the recombinant pECambia plant transformation constructs. Lane 1 contains the 100bp DNA ladder (Promega). Lane 2 represents the amplification product obtained from the p*Euc*RTL2 plasmid. Lanes 3-6 represent products obtained from the recombinant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

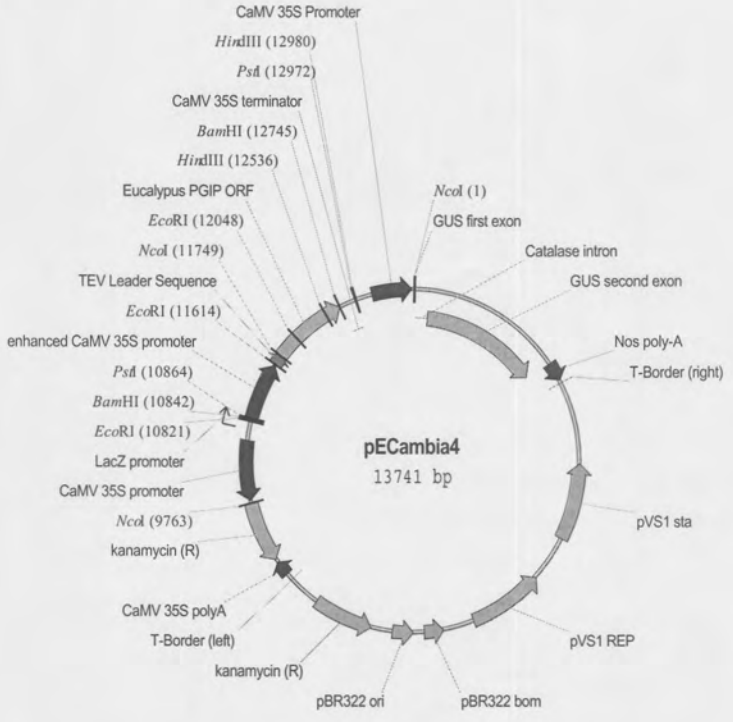

Figure 3.13 Plasmid map of the constructed pECambia4 *Agrobacterium*-based plant transformation vector.

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.

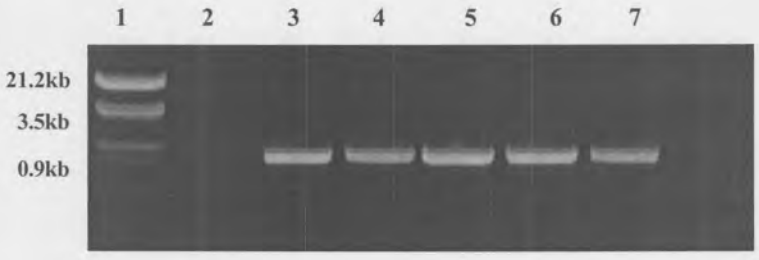

Figure 3.14 PCR analysis of plasmid DNA isolated from *A. tumefaciens* LBA4404 transconjugants using EN-FWD-PGIP and REV-PB-PGIP primers. Lane 1 contains the λIII marker, lane 2 shows a PCR reaction in which ddH_2O replaced the template DNA, lane 3-6 shows amplification using the *A. tumefaciens* LBA4404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

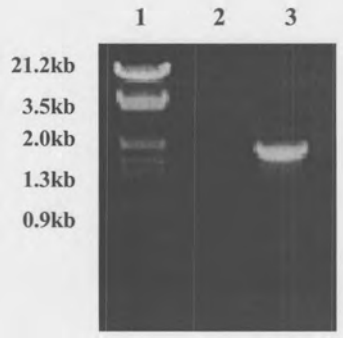

Figure 3.15 PCR analysis of the C58/pCAMBIA 2301 construct using GUSforward and GUSreverse primers. Lane 1 contains the λIII marker. Lane 2 represents the negative water control and lane 3 shows the amplified GUS product from the isolated C58/pCAMBIA2301 plasmid.

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.

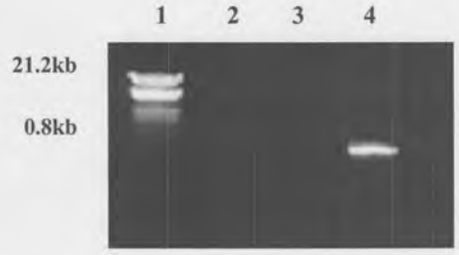

Figure 3.16 PCR screening of *N. tabacum* cv. LA burley plants using NPTII primers. Lane1, λII marker, lanes 2 and 3 represent amplification reactions in which the genomic DNA from LA burley was used as a template. Lane 4 contains a PCR product obtained from genomic DNA extracted from transgenic event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

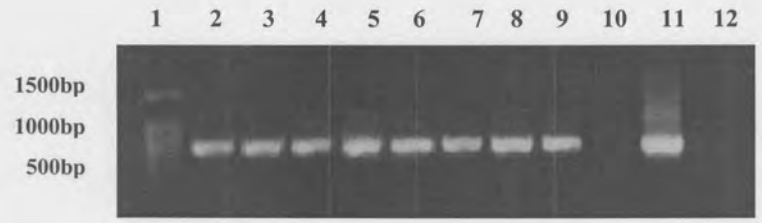

Figure 3.19 PCR analysis of transgenic tobacco lines with kanamycin primers $NPTII_L$ and $NPTII_R$. Lane 1 contains a 100bp DNA ladder (Promega). Lanes 2-9 represent amplification from transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 respectively. Lane 10 contains a PCR reaction 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.

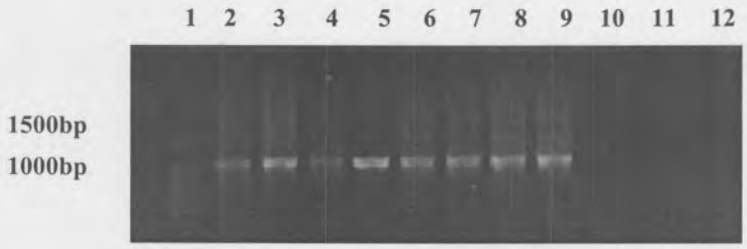

Figure 3.20 PCR analysis of transgenic tobacco lines using the *pgip* gene-specific primers EN-FWD-PGIP and REV-PB-PGIP. A 100bp DNA ladder is represented in lane 1. Lanes 2 - 9 contain the transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 respectively. Lane 10 contains an untransformed 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.

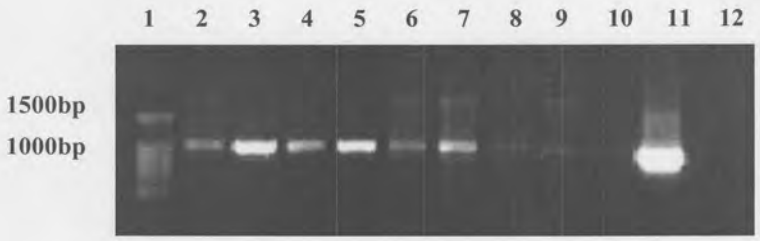

Figure 3.21 PCR analysis of transgenic tobacco lines using the GUS-intron primers. The 100bp DNA ladder is indicated in lane 1. Lanes 2 - 9 contain amplification products from the transgenic lines LAT8, LAT17, LAT18, LAT21, LAT26, LAT33, LAT36, and LAT43 respectively. Lane 10 represents an untransformed 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.

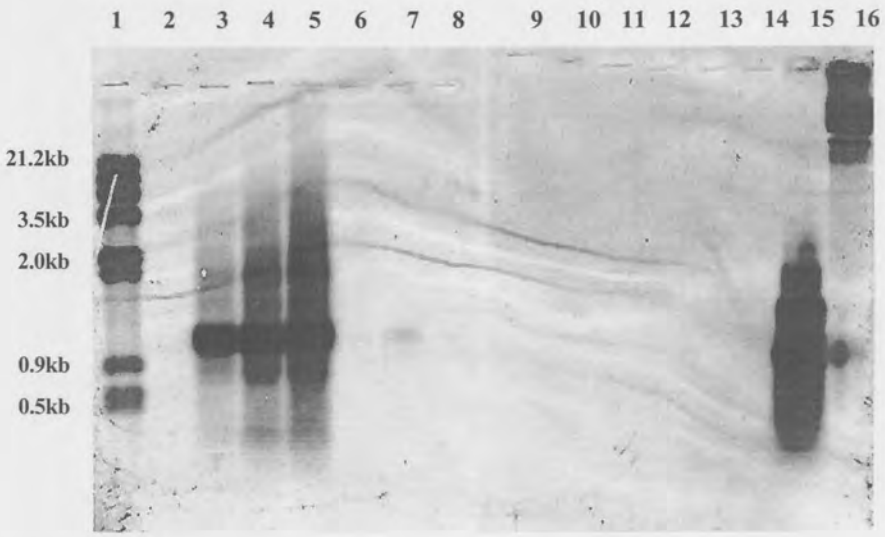

Figure 3.22 Southern blot analysis of PGIP in tobacco genomic DNA. DNA was digested with *Eco*RI and hybridised with a digoxygenin-labeled *Eucalyptus* pgip gene. A DIG-labelled λ *Hind*III/*Eco*RI marker is indicated in lane 1. Lane 2 contains the untransformed control. In lanes 3-5 genomic DNA from 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).

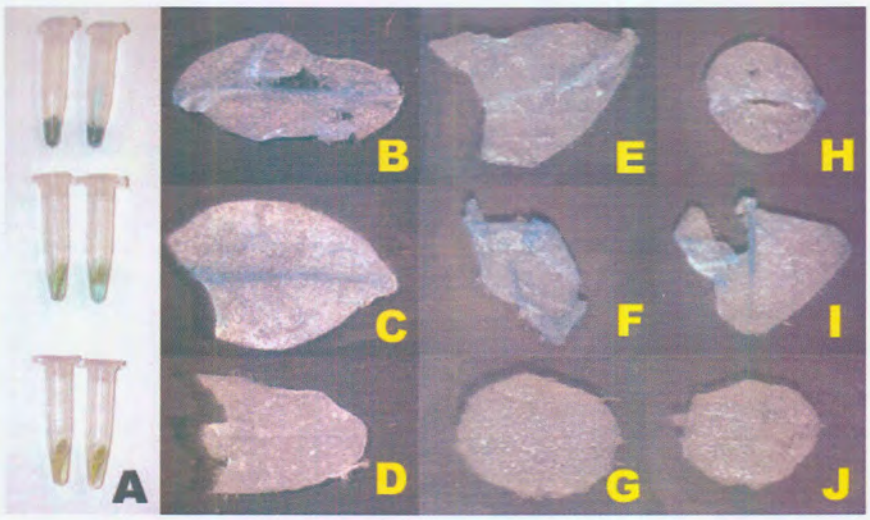

Figure 3.23 Histochemical localisation of GUS activity obtained in tobacco plants transformed with (B, E, H) *A. tumefaciens* C58/pCAMBIA2301 (C2), (C, F, I) *A.tumefaciens* LBA4404/pECambia4 (LAT18) and in (D, G, J) untransformed tobacco leaves (U6).

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.

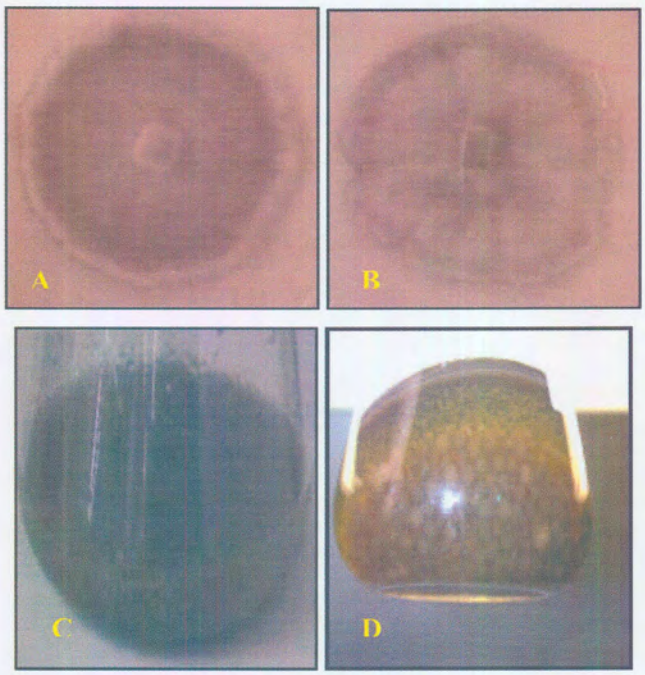

Figure 3.24 Growth of the *C. zuluense* fungal isolates **(A)** CRY1001 and **(B)** CRY1047 on malt-extract agar. Growth of isolates **(C)** CRY1001 and **(D)** CRY1047 on minimal salts medium containing citrus pectin for the production of polygalacturonases.

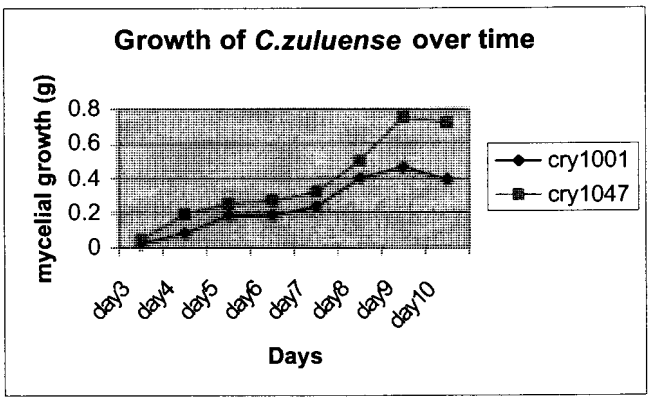

Figure 3.25 The growth of *C. zuluense* isolates determined by measuring the dry weight over time (days).

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.

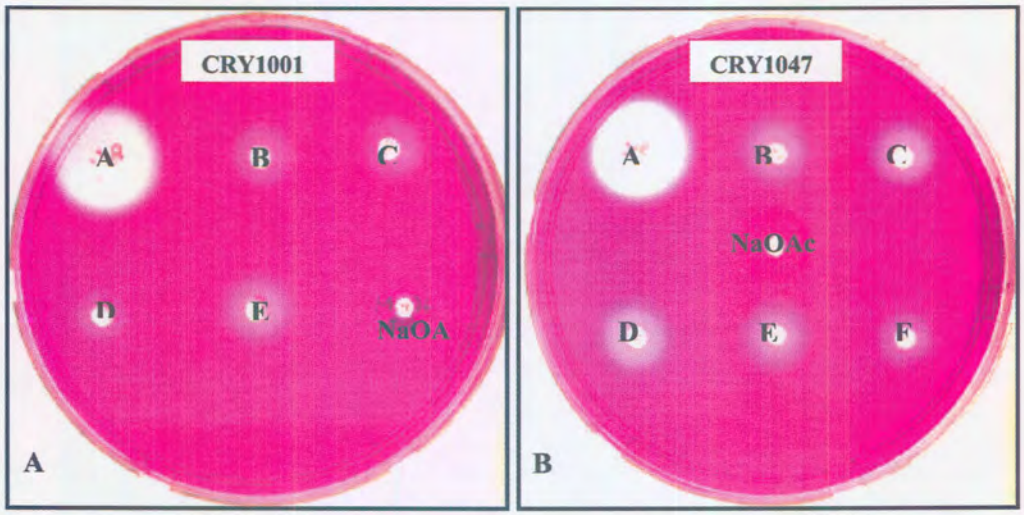

Figure 3.26 A. Agarose diffusion assay of ammonium sulphate precipitated culture filtrates from CRY1001. Zones B-E represent filtrates collected from days 3 to 6, respectively. Zone (A) represents PG activity from *Botrytis* PG and NaOAc represents a negative control in which 40mM NaOAc was added. **B.**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

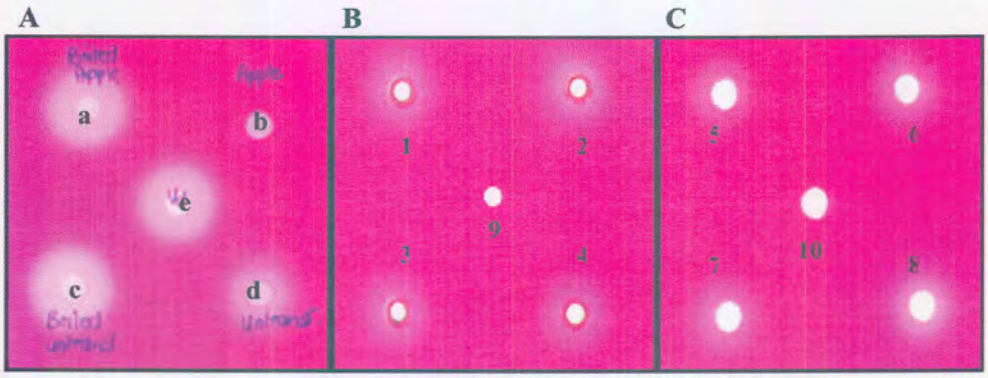

Figure 3.27 Agarose diffusion assay to test inhibition of *C. zuluense* (CRY1047) polygalacturonases by protein extracts from transgenic and non-transgenic tobacco plants. **(A)** Positive control reaction showing inhibition of *V. dahliae* (Vd) PGs by apple PGIP extracts. Zones of activity are as follows: **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).

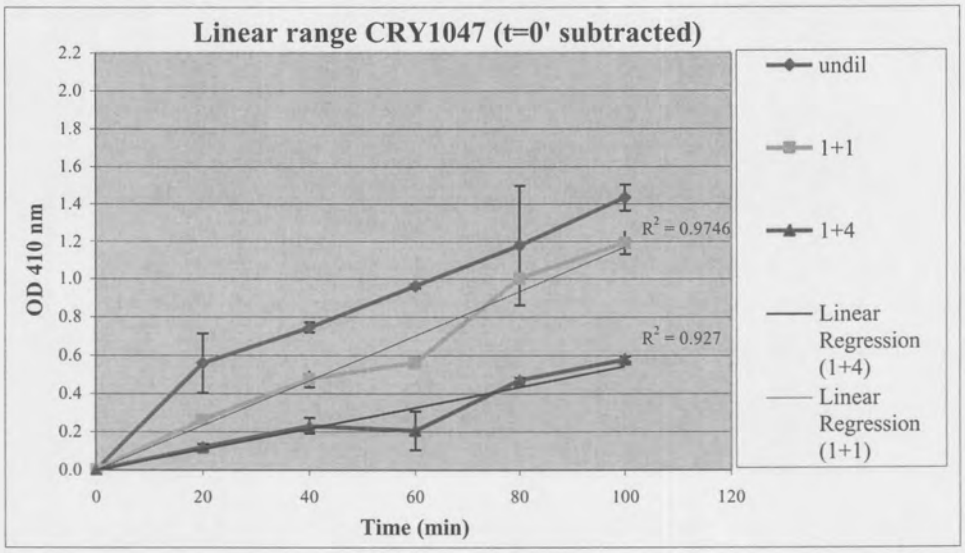

Figure 3.28 Linear trend for *C. zuluense* (CRY1047) polygalacturonase activity determined over a period of 100 min. The undiluted, 1+1 dilution and 1+4 dilution reaction results of CRY1047 PGs are represented.

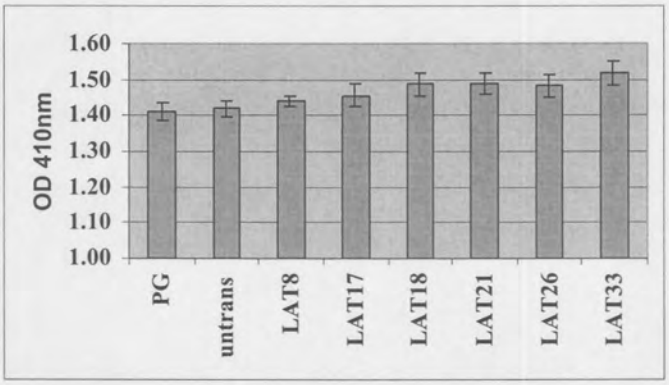

Figure 3.29 *C. zuluense* (CRY1047) PG activity in the presence of PGIP extracts from transgenic (LAT8, LAT17, LAT18, LAT21, LAT26, LAT33) and non-transformed (untrans) tobacco lines.

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.