Evaluation of polygalacturonase-inhibiting protein (PGIP)-mediated resistance against *Verticillium dahliae*, a fungal pathogen of potato

by

Inge Maritz

Dissertation submitted in fulfilment of the requirements for the degree
MASTER OF SCIENCE
in
PLANT BIOTECHNOLOGY

in the Faculty of Natural and Agricultural Science
School of Biological Science
Department of Botany
University of Pretoria
Pretoria

November 2002

TABLE OF CONTENTS

SUMMARY

Polygalacturonase-inhibiting proteins (PGIPs) are plant proteins believed to play a role in the defence against pathogenic fungi. In this study, it was hypothesized that apple PGIP1 could be used to confer enhanced resistance against *Verticillium*-wilt, a major disease of potato caused by the fungus *Verticillium dahliae*. Transgenic lines containing the apple *pgip1* gene under control of the enhanced CaMV 35S (e35S) promoter had been generated previously. Stable integration of the transgene into the potato genome was shown by the polymerase chain reaction (PCR) and Southern blot with a DIG-labelled apple *pgip1* fragment as probe.

Polygalacturonase (PG)-inhibiting assays (the agarose diffusion assay and reducing sugar assays) were employed to investigate the inhibiting activity of apple PGIP1 extracts, prepared from the transgenic potato lines, on the PGs secreted by *V. dahliae* grown on pectin medium. Inhibition was successful for all but one of the transgenic lines. Active PGIP1 was expressed in the leaves of *in vitro*- and glasshouse grown plants, as well as in roots of *in vitro*-grown plants. Due to the success of the *in vitro* inhibition results, it was anticipated that the apple *pgip1* transgene would protect the transgenic lines against *Verticillium*-wilt in a subsequent glasshouse trial.

The transgenic lines and untransformed BP1 potato control were planted in soil inoculated with *V. dahliae* microsclerotia and control soil. Assessments of the visual symptoms of yellowing and wilt were made on a scale of 1-5. Colonisation of stem sections was determined by plating onto potato dextrose agar plates. Disease index values were calculated from the symptom and colonisation data. Analysis of variance indicated six lines to be significantly different from the rest when grown in the inoculated soil, but five of them also showed significantly slower senescence symptoms when grown in the control soil. It is proposed that the physiological effect of an extended juvenile phase resulted in the apparent increased disease resistance. This could be caused by transformation or tissue culture-induced somaclonal variation of the potato plants. The hypothesis that transformation of the apple *pgip1* gene into potato would confer enhanced resistance against *Verticillium*-wilt was not supported by the data that was obtained.

Expression of antifungal genes by pathogen-inducible promoters is a valuable strategy in the development of disease resistant crops of importance. A construct containing the apple *pgip1* gene under control of the pathogen-inducible *gst1* promoter from *Arabidopsis thaliana* (L.) Heynh was generated. *Agrobacterium tumefaciens* GV3101(pMP90RK) was transformed with the plant transformation vector pCAMBIA2300 containing the *gst1* and e35S promoter-*pgip1* inserts. *A. thaliana* was transformed using the floral-dip method, and putative transgenic progeny were selected

by kanamycin selection of the seeds. PCR verified the insertion of the transgene into the genomes of T2 and T3 lines. Gene expression from the two promoters was compared by performing PGIP extractions and the agarose diffusion assay. The *gst1* promoter was active even without induction by methyl-salicylate. Both constructs led to the expression of active apple PGIP1 against *V. dahliae* PG in the heterologous plant *A. thaliana*.

OPSOMMING

Poligalakturonase-inhiberende proteïene is plant proteïene wat 'n rol speel in die beskerming teen patogeniese fungi. In hierdie studie word dit voorgestel dat appel PGIP1 gebruik kan word om verhoogde weerstand teen *Verticillium*-verwelk, 'n belangrike siekte van aartappel veroorsaak deur die fungus *V. dahliae*, te verleen. Transgeniese lyne wat die appel *pgip1* geen onder beheer van die verbeterde CaMV 35S (e35S) promoter bevat is voorheen gegenereer. Stabiele integrasie van die transgeen in die aartappel genoom is bewys deur die polimerase ketting reaksie (PKR) en Southern klad met 'n DIG-gemerkte appel *pgip1* fragment as 'n peiler.

Poligalakturonase (PG)-inhiberende analises (die agarose diffusie en reduserende suiker analises) is aangewend om die inhiberende aktiwiteit van appel PGIP1, berei van die transgeniese aartappel lyne, op die PG ensieme afgeskei deur *V. dahliae* in pektien medium te ondersoek. Inhibisie was suksesvol vir al die potensiële transgeniese lyne behalwe een. Aktiewe PGIP1 was uitgedruk in die blare van *in vitro*- en glashuis gekultiveerde plante, asook die wortels van *in vitro* plante. As gevolg van die sukses van die *in vitro* inhibisie resultate, was dit voorspel dat die appel *pgip1* transgeen die transgeniese lyne teen *Verticillium*-verwelk sal beskerm in die daaropvolgende glashuisproef.

Die transgeniese lyne en ongetransformeerde BP1 aartappel kontrole is geplant in *V. dahliae* mikrosklerotia-geïnokuleerde grond en kontrole grond. Evaluasies van die visuele simptome van vergeling en verwelk is gemaak op 'n skaal van 1-5. Kolonisasie van die stingels is bepaal deur dit uit te plaat op aartappel dekstrose agar plate. Siekte-indekse is bereken van die simptome en kolonisasie data. Analise van variansie het aangedui dat ses lyne betekenisvol verskil het van die res toe dit in die geïnokuleerde grond gegroei is, maar vyf van hulle het ook betekenisvol stadiger veroudering simptome getoon toe dit in die kontrole grond gegroei is. Dit word voorgestel dat die fisiologiese effek van vertraagde volwassenheid verantwoordelik is vir die skynbare verhoogde siekte-weerstand. Hierdie kon veroorsaak gewees het deur transformasie of somaklonale variase geinduseer deur weefselkultuur van die aartappelplante. Die hipotese dat die transformasie met die appel *pgip1* geen in aartappel verhoogde weerstandbiedendheid teen *Verticillium*-verwelk kon verleen was nie ondersteun deur die verkrygde data nie.

Uitdrukking van fungi-werende gene deur patogeen-stimuleerbare promoters is 'n waardevolle strategie in die ontwikkeling van siekte-bestande belangrike gewasse. 'n Konstruk wat die appel *pgip1* geen onder beheer van die patogeen-stimuleerbare *gst1* promoter van *Arabidopsis thaliana* (L.) Heynh bevat is gegenereer. *Agrobacterium tumefaciens* GV3101(pMP90RK) is getransformeer met die plant transformasie vektor pCAMBIA2300 wat die *gst1* en e35S promoter-*pgip1* insetsels bevat.

A. thaliana is getransformeer met die blom-doop metode, en potensiële transgeniese nageslag is geselekteer met kanamycin seleksie van die sade. Integrasie van die transgeen in die genome van T2 en T3 lyne is geverifieer met PKR. Geen-ekspressie van die twee promoters is vergelyk deur PGIP ekstraksies te toets met die agarose diffusie analise. Die *gst1* promoter was aktief selfs sonder induksie deur metiel-salisilaat. Beide konstrukte het gelei tot die ekspressie van aktiewe appel PGIP1 teen *V. dahliae* PG in die heteroloë plant *A. thaliana*.

ACKNOWLEDGEMENTS

My sincere gratitude and appreciation to:

My supervisor, Prof. Dave Berger, for his advice and input into this project.

My co-supervisor, Mr. Dean Oelofse, for guidance in the techniques.

The UK-SA bilateral fund, for funding this project.

Prof. Gary Loake at the University of Edinburgh, for valuable experience acquired in his laboratory.

The ARC-Roodeplaat, for the opportunity to further my studies.

My family and friends, for their interest and encouragement.

My fiancé, Albert, for his sustained support.

LIST OF ABBREVIATIONS

ADA	Agarose diffusion assay
ARC	Agricultural Research Council
AS	Ammonium sulphate
ATP	Adenosine triphosphate
Avr	Avirulence
bp	Basepair
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
CTAB	Hexadecyl trimethyl ammonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanidine triphosphate
dH_2O	Distilled water
DIG	Digoxygenin
DIG-11-dUTP	Digoxygenin-11-deoxyuridine triphosphate
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPE	Diphenyl ether
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine tetraacetic acid
endoPG	Endopolygalacturonase
ERE	Ethylene-responsive element
exoPG	Exopolygalacturonase
gDNA	Genomic DNA
GST	Glutathione *S*-transferase
GUS	β-glucuronidase
kb	Kilobasepair
lsd	Least significant difference
LB	Luria Bertani
LRR	Leucine-rich repeat

MCS	Multiple cloning site
Me-Sa	Methyl-salicylate
MS	Murashige and Skoog
NaAc	Sodium acetate
nptII	Neomycin phosphotransferase II
PAHBAH	*p*-hydroxybenzoic acid hydrazide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PG	Polygalacturonase
PGA	Polygalacturonic acid
PGIP	Polygalacturonase-inhibiting protein
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
TAE	Tris-acetate ethylenediamine tetraacetic acid
TE	Tris ethylenediamine tetraacetic acid
TEV	Tobacco etch virus
Tm	Melting temperature
TNE	Tris-sodium chloride ethylenediamine tetraacetic acid
Tris	Tris hydroxy methyl aminoethane
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

INDEX OF FIGURES

CHAPTER 1
Aim of study

Verticillium-wilt is a major disease limiting potato (*Solanum tuberosum* L.) yield under arid growing conditions. It is caused by the soil-borne fungal pathogen, *Verticillium dahliae*. Current control approaches include sanitation of fields and equipment, crop rotation, soil fumigation and chemical control. Since current control strategies are not effective in managing the disease, and chemical treatments may be deleterious to the environment, alternative strategies are required. Breeding for resistant potato lines faces many challenges, therefore the transgenic manipulation to confer resistance against this disease is proposed.

The literature review will reveal the importance of polygalacturonase inhibiting protein (PGIP), a plant defence gene, in resistance against fungal pathogens. It has been shown previously to be successful in conferring fungal resistance to a heterologous plant. Pear PGIP was able to confer resistance to tomato *in vivo* against the fungal pathogen *Botrytis cinerea*, whereas endogenous tomato PGIP was not effective (Powell *et al.*, 2000). Apple and pear PGIP was reported to be 97% identical in nucleotide sequence, and it was thus anticipated that apple PGIP would also be a successful inhibitor of *B. cinerea* infection. A crude total PGIP extract from apple, *Malus domestica* cv. Granny Smith, was indeed able to inhibit *B. cinerea* PGs (D.K. Berger, D. Oelofse and M. Arendse, personal communication). An apple *pgip1* gene was isolated using inverse polymerase chain reaction methods and cloned at ARC-Roodeplaat (Arendse *et al.*, 1999). A project was thus proposed to transform tomato with apple *pgip1* to confer resistance against *B. cinerea*. This fungal pathogen causes substantial losses of the crop during cold storage. Subsequent transformation of the apple *pgip1* gene into tobacco cv. LA Burley and transgenic expression of the protein showed it to be an active inhibitor of other fungal PGs, but not PGs from *B. cinerea*. It is thus possible that apple *pgip1* is not the gene coding for the PGIP in the crude apple extract that is responsible for the inhibitory activity against *B. cinerea* PGs. It has been shown that only one amino acid substitution can change the PG:PGIP interaction specificity (Leckie *et al.*, 1999). Apple contains more than one *pgip* gene (Yao *et al.*, 1999), so one of the other genes could code for the PGIP responsible for *B. cinerea* PG inhibition.

Apple PGIP1 expressed in transgenic tobacco was, however, successful in inhibiting PGs from *V. dahliae*, the fungus causing *Verticillium*-wilt of potato (see Chapter 6 for inhibition results). This protein may therefore be useful in the genetic engineering of potato for resistance against this fungal pathogen. The focus of this MSc study therefore changed from evaluating apple PGIP1 in transgenic tomato against *B. cinerea*, to evaluating apple PGIP1 in potato vs. *V. dahliae*. Transgenic potato cv.

BP1 plants, containing the apple *pgip1* gene under control of the constitutive enhanced CaMV 35S promoter, had been generated subsequently by A. Veale (ARC Roodeplaat) and maintained in tissue culture.

The aim of this study was the molecular characterisation of the transgenic potato lines containing the apple *pgip1* gene, and to assess whether it confers enhanced fungal resistance to *V. dahliae* during a glasshouse trial. In addition, the expression of resistance genes under control of a pathogen inducible plant promoter, the *gst1* promoter from *Arabidopsis thaliana* (L.) Heynh (Yang *et al.*, 1998), was evaluated by creating transgenic *A. thaliana* plants containing apple *pgip1* under control of this promoter.

The hypothesis is that the apple PGIP1 will be effective against pectin-degrading enzymes from the potato pathogen *V. dahliae*, and will confer enhanced resistance to the pathogen under glasshouse conditions. It is also hypothesised that the *gst1* promoter will drive pathogen-inducible expression of the apple *pgip1* gene in transgenic *A. thaliana*.

The literature review in Chapter 2 focuses on the role of polygalacturonase-inhibiting proteins in the defence against phytopathogenic fungi. It also reviews the specific interaction between a plant and a fungus, namely *Verticillium*-wilt of potato. Chapter 3 describes the cloning of the apple *pgip1* gene under control of a pathogen-inducible promoter, whereas Chapter 4 describes the transformation of *A. thaliana* with this construct and molecular analysis of the transgenics. Chapters 5 to 7 will deal with the main aim of this project, which is to evaluate whether PGIP confers enhanced resistance against *V. dahliae* to potato. The molecular characterisation of apple *pgip1* transgenic potato plants is covered in Chapter 5, and the inhibition of *V. dahliae* PGs by transgenically expressed apple PGIP1 in Chapter 6. Chapter 7 reports on the glasshouse trial for screening transgenic potato lines for increased resistance to *V. dahliae* infection. Chapter 8 is the concluding discussion of the relevance of the results obtained.

CHAPTER 2

Literature review

2.1 Introduction

This literature review will describe the disease caused by the specific interaction between potato and *V. dahliae*, namely *Verticillium*-wilt. It will continue to briefly introduce the principle of resistance of plants to fungal pathogens. The topics of fungal polygalacturonases as pathogenicity factors, and plants' mechanism to defend against fungal infection, polygalacturonase-inhibiting proteins, are reviewed. Some background information on the pathogen-inducible promoter that will be used in this study, the *gst1* promoter from *Arabidopsis thaliana* (L.) Heynh, is given. Lastly, it will highlight the importance of *A. thaliana* as a model plant for research.

2.2 Potato as an economically important crop

Potato (*Solanum tuberosum* L.) is an important component in intensive agricultural systems in areas of high population density, since it can produce a large mass of high-value food in a short time (Hijmans, 2001). It is an important staple food and animal feed especially in the northern hemisphere. A study of global potato production over the period from 1950 to 1999 has shown a stable production of 30 million tonnes per year. This is due to overall increasing yields, despite the area grown with potato decreasing stably at 92000 ha per year. On a global scale, 51% of potato area is in Europe, 35% in Asia and the rest equally distributed between North America, South America and Africa. South Africa is the second largest potato producer in Africa, with 61000 ha potato area, whereas Egypt has 82561 ha. Ten thousand hectares of the total potato production area in South Africa are for seed potato production. Seed potatoes are certified disease free by a seed certification scheme administered by Potatoes South Africa. These are then supplied to table potato producers that produce potatoes for the consumer market (Berger, 2000). The total value of the potato harvest in South Africa is in excess of two billion rand (Potatoes South Africa, 2001 potato harvest figures).

2.3 *Verticillium*-wilt of potato

Verticillium-wilt is a major disease limiting potato yield under arid growing conditions (Corsini and Pavek, 1996). The main causal organism is *V. dahliae* and to a lesser extent *V. nigrescens* (Millard and Denner, 2001). *V. dahliae* belongs to the Kingdom Mycetae, Division Eumycota, subdivision

Deuteromycotina, class Hyphomycetes, and genus *Verticillium*. *V. dahliae* is a destructive soil-borne fungal pathogen that causes vascular wilt diseases on more than 160 plant species, which includes cotton, tomatoes and potatoes. The fungus penetrates the host through its roots, spreads systemically through the xylem, and subsequently leads to the appearance of wilt symptoms (James and Dubery, 2001).

2.3.1 Source of fungal inoculum

Infected seed potatoes serve as the inoculum source, although it can also occur on roots of natural plant growth. It has a wide host range, so the fungus can survive at low levels on roots of many crop and weed species (Rowe, 1985). The fungus can survive up to 10 years in soil as dormant microsclerotia. As soon as the amount of microsclerotia in the soil exceeds a certain threshold, the disease develops (Millard and Denner, 2001).

2.3.2 Symptoms of *Verticillium*-wilt

Visually there is no difference between *Fusarium*-wilt and *Verticillium*-wilt symptoms. The disease is characterised by chlorosis and wilting of lower leaves, followed by browning and drying-out, after which the symptoms spread to the rest of the stem or even the whole plant. Cross-sections of wilted stems and tubers show the discoloration of the vascular tissue. In potato early dying (PED) disease, where nematodes are also involved in the complex, dying-off of the plant occurs from the flowering stages. The yield of potatoes is then reduced because the growing period is shortened, resulting in a reduction in size of the daughter tubers (Millard and Denner, 2001).

2.3.3 Distribution of the disease in South Africa

Verticillium-wilt is predominantly distributed in the Sandveld-production area, with 53% of incidence in this region. Since 1998, however, it has also become a major problem in other areas of South Africa (Millard and Denner, 2001).

2.3.4 Life cycle of *V. dahliae*

V. dahliae overwinters as microsclerotia in the soil. After planting, root exudates from the developing potato plant stimulate the microsclerotia in the soil to germinate. Hyphae penetrate the roots and move to the inside of the vascular tissue, where they impede effective transport of water and nutrients by the xylem. The mycelium forms a specialised hypha (conidiophore) in which an asexual fungus spore (conidia) is produced. The conidia are produced continually by the mycelium as long as environmental conditions are favourable. The conidia that are formed within the xylem vessels are transported with the transpiration stream. They cause systemic colonisation of the entire plant by germinating at remote locations (Powelson and Rowe, 1994). Symptom development, including chlorosis and wilting, occurs as a result of toxin production and vascular dysfunction (Rowe, 1985).

Dying off of plants can occur from the flowering stages, leading to the name potato early dying disease. When the plant senesces and dies, the fungus colonises the dead tissues saprophytically. Microsclerotia are formed on the dead stems, which are often ploughed into the field during harvesting (Millard and Denner, 2001). Microsclerotia and conidia inoculum in the soil can be disseminated to uninfected fields by wind, water, etc. When the inoculum level in a field reaches a high enough value, it causes the infection of plants by the fungus.

2.3.5 Potato early dying (PED)

Potato early dying is a syndrome caused by a complex of interacting organisms. The same symptoms can be caused by infection by a variety of combinations between nematodes, *Colletotrichum*, *V. dahliae*, *V. albo-atrum*, *Erwinia* etc. (Rowe, 1985). PED is characterised by premature vine death and declining yields. Symptoms of PED are difficult to distinguish from normal senescence and initially the affected plants show only slower growth. Chlorosis and necrosis of the plant start at the bottom leaves, often occurring on only one side of the plant or on individual leaves.

The environment also has an influence on the development of PED. External abiotic factors such as temperature and moisture are implicated (Powelson and Rowe, 1993). Fungal infection of a plant is usually enhanced when the plant is stressed. The optimum growth temperature of potato plants is 18 - 20°C, while the optimal temperature for *V. dahliae* growth is 21 - 27°C. Therefore, the disease severity in potatoes infected with *V. dahliae* increases when the average air temperature rises from 20 to 28°C.

Wheeler *et al.* (1994) noted that when nematodes and *V. dahliae* both infect roots of potato, a disease complex can result that leads to yield losses higher than the sum of those expected for the two pathogens separately. The root lesion nematode *Pratylenchus penetrans* has been shown to interact with *V. dahliae* as a critical component of PED disease (Wheeler *et al.*, 1994). The exact mechanism of the *Pratylenchus-Verticillium* interaction in PED is unknown. Root wounding by nematodes was first believed to provide entry into the root for fungal pathogens. However, evidence from several pathosystems indicates that the interaction is biological or physiological, rather than physical, in nature. Perhaps the nematode feeding on the plant elicits physiological changes in the plant, which then favour infection by *V. dahliae* (Powelson and Rowe, 1993).

The role of nematodes was not determined in a recent survey of *Verticillium*-wilt of South Africa, so the prevalence of PED disease in this country is not known (C. Millard, personal communication).

2.3.6 Management of *Verticillium*-wilt

Management of this disease focuses on integrated control measures, where more than one strategy is followed simultaneously. Seed tubers are tested by enzyme-linked immunosorbent assay (ELISA) to certify them free from *Verticillium* and suitable for planting. Sanitation of equipment, such as farm implements, shoes, crates etc., that comes into contact with contaminated soil and tubers are important to prevent spreading of the fungus from one plot to another. Ploughing the topsoil layer, containing the microsclerotia, deep into the soil may bring cleaner soil to the surface and prevent contact between the pathogen and the roots.

2.3.6.1 Crop rotation

The crop used in crop rotation with potatoes should be chosen with care, since *V. dahliae* has a wide host range. Long rotation periods are necessary to decrease the inoculum levels of microsclerotia. At least three years without potato is required to adequately lower microsclerotia levels (Wheeler *et al.*, 1994). Brassicas, such as cabbage crops, are good choices since they act as biofumigants. They provide sulphur-containing compounds called glucosinolates that form fungal toxins upon degradation. In intact cells, the glucosinolates are separated from the enzyme myrosinase, a thioglucosidase, by subcellular compartmentalisation. In response to tissue damage, the myrosinase hydrolyses the glucosinolates to form unstable aglycone intermediates. These are converted into biologically active molecules, which include the volatile isothiocyanates, nitriles and thiocyanates (Buchanan *et al.*, 2000). Crop rotation with broccoli was shown to be effective against *Verticillium*-wilt of cauliflower (Millard and Denner, 2001).

2.3.6.2 Soil fumigation

Examples of chemical soil fumigants include methyl-bromide and metamsodium. It has been shown that it can control PED effectively in sandy soils, but the potential of this management tool is reduced by limited effectiveness in heavier soils (Wheeler *et al.*, 1994). Soil fumigation is also not a popular control strategy because of cost and safety concerns.

2.3.6.3 *Verticillium*-resistant cultivars

Resistant or tolerant cultivars are the most efficient approach to control of *Verticillium*-wilt of potato and PED disease. Most local cultivars, including BP1, are susceptible. Potato breeding programs of commercial potato resistant to *Verticillium* spp. began in 1949 in Idaho, USA (Wheeler *et al.*, 1994). A few *V. dahliae* resistant cultivars include Reddale, Russette, Gemchip and Ranger Russet (Wheeler *et al.*, 1994; Corsini and Pavek, 1996). Cultivars labelled *V. dahliae* tolerant include Alpha, Kondor, Desiree and Spunta (Tsror and Nachmias, 1995; Nachmias *et al.*, 1990). Désirée is a susceptible cultivar according to Kawchuk *et al.* (2001).

South African cultivars have been tested for *Verticillium*-resistance, but none were found (unpublished results). Currently there is no breeding program in South Africa for resistance against *Verticillium*-wilt (C. Millard (ARC-Roodeplaat), personal communication). ARC-Roodeplaat is involved in breeding strategies against common scab, bacterial wilt and *Fusarium* dry rot of potato.

2.3.7 Problems with breeding for resistance to *Verticillium*-wilt

Disease resistance breeding is made difficult by the complex inheritance of many traits of interest in potato and the problems associated with inbreeding. In particular, resistance to *Verticillium*-wilt appears to be a multigenic trait that makes selecting for it difficult. The breeders developing potato varieties have to select simultaneously for a combination of multigenic traits, including acceptable levels of resistance or susceptibility to diseases. When selecting for a single resistance gene, the population can be screened first for resistance to the disease, and a high proportion of the population will remain as the basis for variety development. However, early screening for resistance to *Verticillium* tends to eliminate clones with acceptable tuber maturity characteristics (Corsini and Pavek, 1996). This is because plants that stay in a non-tuberising juvenile condition do not become systemically infected with *Verticillium* and do not show wilt symptoms. Thus, researchers found it inefficient to select for *Verticillium* resistance at the early stages of variety development. They suggested to rather select for yield and other agronomic criteria in *Verticillium* infested fields.

2.3.8 Resistance to *V. dahliae*

Plant resistance to this disease is defined as the inability of the pathogen to penetrate the roots, to colonise the host tissue, to inactivate toxic elements or to inhibit fungal sporulation (Tsror and Nachmias, 1995). The genetic basis of *Verticillium* resistance has only been characterised in a few crops. Cotton has two dominant genes that control resistance to the disease (Tsror and Nachmias, 1995). Tomato has a single dominant resistance gene (*Ve*) that confers race-specific resistance to infection by *Verticillium* species. Some biotypes are not pathogenic on (*Ve*) tomato. *V. dahliae* is able to overcome the resistance by the formation of new pathotypes or races. A *V. dahliae* biotype called race 2 is pathogenic on tomatoes that contain the *Ve* gene (Nachmias *et al.*, 1987). The genetic basis of resistance has not been characterised in other crops.

Some potato varieties are tolerant to *Verticillium*. They show delayed or reduced colonisation by *V. dahliae* but do not show severe wilt symptoms or yield loss (Nachmias *et al.*, 1990). This phenomenon is referred to as 'Alpha-type tolerance' since it was first observed in the *Verticillium* disease-tolerant cultivar Alpha. This mechanism of tolerance, which does not exert genetic pressure on the pathogen, has not been explained yet (Tsror and Nachmias, 1995).

During reciprocal rootstock grafting experiments between resistant and susceptible cultivars, the root was established as the major site of resistance to *Verticillium*-wilt in tomato and tolerance in potato (Tsror and Nachmias, 1995). However, it appears that potato has additional defence mechanisms that are controlled by other genes. Two races of tomato *V. dahliae* show differential pathogenicity on susceptible and race 1-resistant (*Ve*) tomato cultivars (Nachmias *et al.*, 1987). *V. dahliae* toxin peptides were isolated from the culture fluid of the two races and used in several bioassays. (*Ve*) tomato cultivars are resistant to race 1, but race 2 forms symptoms on all (both resistant and susceptible) cultivars. The two toxins differ in amino acid composition, and it is believed that the toxin from race 2 has evolved to escape recognition by the (*Ve*) tomatoes. Race 1 *V. dahliae* does colonise (*Ve*) plants, but doesn't lead to the development of any disease symptoms. The data therefore suggests that the *Ve* gene doesn't have an effect on *V. dahliae* multiplication but instead limits damage. Experiments with these two races on four potato cultivars (three tolerant and one susceptible) did not show any differential activity between race 1 and race 2. Thus, it appears that a gene similar to *Ve* in tomato does not exist in potato (Tsror and Nachmias, 1995).

The *Ve* locus of tomato was characterised and two closely linked inverted genes were isolated (*Ve*1 and *Ve*2) (Kawchuk *et al.*, 2001). The *Ve* disease resistance genes were found to encode cell surface-like receptors. When the two *Ve* genes conferring resistance in tomato against *V. dahliae* race 1 was transformed into susceptible potato (cultivar Désirée), both genes independently conferred resistance to an aggressive race 1 isolate of *V. albo-atrum*. This resistance to a different pathogen species is a contradiction of the view of highly specific interaction between race-defining *R* genes. Thus, the *Ve* gene of tomato is pleiotropic because it can distinguish between race 1 and 2 of *V. dahliae* and possess the capacity to recognise another *Verticillium* species in a different host, the potato. Resistance of the transgenic potato lines against *V. dahliae* was not tested. *R* genes are thus able to retain their biological activity in other plant genera, and may be valuable in other agricultural crops that are infected by *Verticillium* species.

The deduced primary structure of *Ve*1 and *Ve*2 indicated that they are cell-surface glycoproteins with receptor-mediated endocytosis-like signals (Kawchuk *et al.*, 2001). Both have a hydrophobic N-terminal signal peptide, leucine-rich repeats (LRR) containing 28 or 35 potential glycosylation sites, respectively, a membrane-spanning domain and a C-terminal domain with endocytosis signals. The LRR is often associated with protein-protein interactions and ligand binding (Kobe and Deisenhofer, 1995). Since it is of the extracytoplasmic type, it could facilitate the recognition of an extracellular pathogen ligand. Eukaryotic cells use receptor-mediated endocytosis to communicate or to respond to external stimuli. In *Ve*, receptor-mediated endocytosis may be a mechanism to selectively bind ligands and then to remove the receptor-ligand complex from their surfaces, thereby responding to changing disease pressures.

The *Ve* cell surface-like receptor may transmit a ligand-induced conformational change from the extracytoplasmic to the cytoplasmic domain (Kawchuk *et al.*, 2001). Since it lacks a kinase domain, the cytoplasmic domain will need to interact with a kinase that phosphorylates the various downstream signalling proteins, thereby activating the subsequent signal transduction pathway. Alternatively, the receptor-mediated endocytosis may allow the extracellular domains and the ligands of the *Ve* receptor to directly stimulate signal transduction.

Elemental sulphur (S^0) was implicated as a phytoalexin of fungal vascular pathogens in the xylem of resistant lines of tomato (Williams *et al.*, 2002). It was accumulated much more rapidly in response to *V. dahliae* infection in disease-resistant than in disease-susceptible lines. It is fungitoxic to *V. dahliae*, inhibiting spore germination and mycelium growth. Elemental sulphur was predominantly localised in vascular structures that are in potential contact with the xylem-invading pathogen *V. dahliae* and thereby linked to defence against it.

2.3.9 Phylogeny of *V. dahliae*

The genus *Verticillium* includes various species, which falls into diverse econutritional groups (Bidochka *et al.*, 1999). Some are pathogens of insects, plants, mushrooms, nematodes, spiders and saprobes. The two commercially important plant pathogens, *V. dahliae* and *V. albo-atrum*, have very wide host ranges with little host specificity. Therefore, few physiological races have been found and characterised. Prior to the early 1970's, both species were considered forms of *V. albo-atrum*, but they are now considered to be separate species (Rowe, 1985). The two are separated on the basis of their survival structures, with *V. dahliae* forming true microsclerotia while *V. albo-atrum* only forms melanised hyphae. They also differ in their temperature sensitivity, with *V. albo-atrum* preferring cooler temperatures (up to 24°C) while *V. dahliae* grows well up to 27°C.

The internal transcribed spacer (ITS) and small nuclear (SN) rRNA regions of several *Verticillium* isolates were used to construct a phylogenetic tree (Bidochka *et al.*, 1999). Strains capable of infecting insects (*V. indicum*, *V. lecanii*) are present in divergent groups in the consensus tree, suggesting that this ability has evolved independently a few times. The plant pathogens (*V. dahliae*, *V. albo-atrum* and *V. nigrescens*) form a clade. They all produce pectinase enzymes able to degrade plant cell walls, while the insect and mushroom (*V. fungicola*) pathogens cannot degrade pectin. They, on the other hand, produce high levels of a subtilisin-like protease, capable of degrading insect cuticles. Together with the nematode pathogens, the insect and mushroom pathogens are distinguishable from the plant pathogens by their ability to produce chitinases. Strains of *Verticillium* therefore show enzymic adaptation to the polymers present in the integument of their particular host, being either plant or insect depending on their ecological niche (St Leger *et al.*, 1997).

V. dahliae is not host-specific, since it can infect a wide host range. Its host range includes trees, ground covers, shrubs, vines, fruits, vegetables, field crops, herbaceous ornamentals and many weeds (Powelson and Rowe, 1994). Plants regarded as resistant to *V. dahliae* include ferns, gymnosperms, many monocots and the cactus family. Isolates from a specific host are able to infect other hosts. *V. dahliae* isolates from cotton, potato, tomato and avocado were all pathogenic on tomato and were able to induce foliar symptoms (Visser, 1999). *V. dahliae* races 1 and 2 from tomato were used in potato inoculation experiments, and caused disease symptoms (Tsror and Nachmias, 1995). They did not, however, show differential pathogenicity on tolerant potato cultivars as is observed with (*Ve*) tomatoes.

2.4 Fungal virulence and plant resistance genes

There are many fungal - plant interactions in nature, some causing fungal plant diseases and some that do not (Laugé and De Wit, 1998). Interactions that do not lead to disease mostly occur on plants that are not hosts to the fungus, perhaps because the fungus lacks the pathogenicity factors to cause disease. Plants are hosts when the fungus is known to be a pathogen of the given plant. Not all combinations of fungal strains and host plant cultivars will, however, lead to disease. Therefore, interactions of the "host"-type can be divided into compatible and incompatible interactions. Compatible interactions occur when a susceptible plant is attacked by a virulent pathogen, and become diseased. Incompatible interactions are when a resistant plant is attacked by an avirulent pathogen, and does not become diseased.

The fact that specific interactions occur during compatible and incompatible interactions were demonstrated when Flor (1946) found that virulence appeared to be recessive and avirulence dominant. The gene-for-gene concept was formulated to state that for every dominant gene determining resistance (*R*) in the host, there is a matching dominant avirulence (*Avr*) gene in the pathogen. This accounts for the species-specificity of some fungi that can infect various plant species, but each strain can only infect one or a few host plants.

The elicitor-receptor model has been proposed for the *Avr* and *R* genes to give a biochemical explanation of the gene-for-gene concept. The specific elicitor, a product of an *Avr* gene of the pathogen, is recognised by a receptor, the product of a matching *R* gene in the resistant plant. This interaction activates a signal transduction pathway that leads to disease resistance, often through the hypersensitive response (HR). *Avr* genes and their matching *R* genes can be exploited in molecular resistance breeding against any pathogen that can be inhibited by HR (Laugé and De Wit, 1998).

From the perspective of the pathogen, why would it make sense to have an *Avr* gene if its product leads to a defense response in the plant? A recent idea is that the *Avr* gene products may act as virulence factors under certain conditions, for example on different host plants, or it may have another role that is not related to plant infection. Some *Avr* genes are always maintained within a pathogen population. There often appears to be a fitness penalty when avirulence mutates to virulence. This indicates that the gene products have important roles in pathogenicity (Hammond-Kosack and Jones, 1997).

Plant *R* genes have been used for a long time in breeding for disease control (Rommens and Kishore, 2000). Most *R* genes have only limited durability, which necessitates the continued discovery and introgression of new *R* genes. Because they can be linked to undesirable traits and the phenotype difficult to score, introgression using classical breeding approaches can be time-consuming and laborious. *R* gene-mediated resistance through genetic engineering is suggested to be the solution. *R* genes can be transferred within and across plant species, and have been shown to retain their activity. This approach also eliminates the retention of unwanted genetically linked germplasm. Non-host plants can be a source of extremely durable *R* genes, and they can be transferred to susceptible plants even though they are sexually incompatible.

2.5 Plant defence mechanisms

A plant`s first line of defence against colonisation by microbial pathogens is a preformed physical and chemical barrier. Superimposed on this is an array of inducible responses, and their activation depend upon recognition of the invading pathogen (Hammond-Kosack and Jones, 1997). This recognition event is very specific and is mediated by the interaction between the products of a microbial avirulence *Avr* gene and the corresponding plant disease resistance *R* gene. Following successful pathogen recognition, an oxidative burst and the hypersensitive response (HR) will occur. HR is the programmed death of challenged host cells, producing a visible area of cell death around the site of attempted pathogen invasion.

The oxidative burst is the production of reactive oxygen intermediates (ROIs), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), at the site of attempted invasion. This is one of the most rapid responses following pathogen recognition. The oxidative burst and the transduction of the cognate redox signals play an important role in the diverse array of plant defence responses (Grant and Loake, 2000). ROIs integrate a diverse set of defence mechanisms, resulting in plant disease resistance. The oxidative burst is biphasic, the first phase being associated with wounding or infection by virulent microbial pathogens, while the second is correlated with the establishment of disease resistance. ROIs mediate redox signalling due to their ability to carry unpaired electrons. They oxidise nucleophilic

centres or coordinate interactions with transition metals at the allosteric or active sites of target proteins, thereby modulating their activity.

ROIs are generated by various potential mechanisms of which NADPH-dependent oxidase and peroxidases are the most studied (Grant and Loake, 2000). Nitric oxide (NO) has also been shown to accumulate during HR formation. A plant gene encoding NO synthase (NOS) has not been identified, but NO can be produced as a by-product from NO_2 accumulation by alternative mechanisms such as respiration, denitrification and nitrogen fixation. ROIs and NO may function in combination, possibly after reacting to form peroxynitrite ($ONOO^-$), to drive host cell death during HR (Delledonne *et al.*, 1998).

ROI production needs to be tightly regulated since it is highly cytotoxic (Grant and Loake, 2000). Ca^{2+} release following pathogen recognition is thought to be a trigger. It can either directly activate NADPH oxidase or indirectly by activating NAD kinase by binding to its activating protein, calmodulin. Ca^{2+}- mediated modulation of peroxidase activity is also involved in the regulation.

ROIs have various actions in facilitating defence against pathogens. They can cause strengthening of the cell wall, a physical barrier against pathogen penetration, by oxidatively cross-linking cell wall structural proteins, phenolics and polysaccharides (Bradley *et al.*, 1992). It also has direct antimicrobial activity. ROIs generated by the oxidative burst play a role in host cell death by initiating the development of the HR. ROIs stimulate Ca^{2+} influx into the cytoplasm, which is important for HR cell death, supposedly because of the requirement of cell death effectors for Ca^{2+} for their activity (Grant and Loake, 2000; Grant *et al.*, 2000).

Systemic acquired resistance (SAR) is the establishment of immunity to secondary infections in systemic tissues. It is long lasting, extends to tissues distant from the initial infection site and provides protection against a broad spectrum of microbial pathogens. It requires the accumulation of salicylic acid (SA) for its expression (Gaffney *et al.*, 1993; Delaney *et al.*, 1994). SA is proposed to act as an endogenous signal molecule required for inducing SAR. SA inhibits the activity of some antioxidant enzymes and positively enhances the production of superoxide (O_2^-). It can induce plant defence gene expression and systemic acquired resistance, but not cell death (McDowell and Dangl, 2000). ROIs may also act as systemic signals to establish plant immunity (SAR), by causing the deployment of cellular protectant functions in distal cells (Grant and Loake, 2000; Grant *et al.*, 2000).

Two other plant hormones frequently implicated in plant disease resistance are jasmonic acid and ethylene. Jasmonic acid and its cyclopentanone derivatives are synthesised by the octadecanoic pathway from linolenic acid in undamaged tissues and another pathway in wounded tissues (Xie *et al.*,

1998). It affects a variety of processes in plants, such as root growth, fruit ripening, senescence, pollen development and defence against insects and pathogens. Jasmonic acid alters gene transcription, RNA processing and translation. Ethylene is a simple two-carbon olefin, a plant hormone that is a potent modulator of plant growth and development (Wang *et al.*, 2002). It is involved in many aspects of the plant life cycle, such as seed germination, root hair development, flower senescence, abscission and fruit ripening. Its production is regulated by internal signals and from external stimuli such as pathogen attack and abiotic stresses. Ethylene plays an important role in plant disease resistance pathways. Depending on the type of plant and pathogen, its role may be dramatically different. Plants deficient in ethylene signalling may show increased susceptibility or increased resistance. In general, ethylene seems to inhibit symptom development during necrotrophic pathogen infection, but enhances cell death caused by other types of pathogen infection. The ethylene and jasmonic acid (ET-JA) and salicylic acid responses are mutually inhibitory. ET-JA dependent defence responses are activated by necrotrophic pathogen attack, while the SA-dependent response is triggered by biotrophic pathogens (McDowell and Dangl, 2000).

2.6 Polygalacturonases (PG)

Endopolygalacturonases (endoPGs) are found in a variety of organisms, such as bacteria, fungi and plants, where they are involved in the degradation and remodelling of the plant cell wall. They are poly[1,4-α-D-galacturonide] glycanohydrolases (EC 3.2.1.15), and they hydrolyse α-1-4 glycosidic linkages between galacturonic acids in homogalacturonans. Plants use their PGs in processes such as growth, fruit softening, root formation, organ abscission and pollen development. Phytopathogenic organisms use their PGs to penetrate and colonise their host plant tissues (De Lorenzo *et al.*, 2001). This review will focus on fungal PGs, and endoPGs in particular.

2.6.1 Fungal endoPGs

Endopolygalacturonases are the first detectable enzymes secreted by plant fungal pathogens when cultured *in vitro* on isolated plant cell walls (English *et al.*, 1971). The order, in which polysaccharide-degrading enzymes are secreted by plant fungal pathogens when they are cultured on isolated cell walls, may reflect the order in which they must work to degrade the cell walls. A "wall modifying enzyme" was found to be necessary before other enzymes, such as glycosidases, cellulases, hemicellulases and pectinases (pectin hydrolase, lyase and esterase), could degrade the cell wall polysaccharides (Karr and Albersheim, 1970). EndoPGs facilitate the ability of other fungus-secreted plant cell wall-degrading enzymes to attack their substrates, which are other plant cell wall components.

Fungal endopolygalacturonases play an important role during the early stages of plant pathogenesis (Karr and Albersheim, 1970). This enzyme spreads into the host tissue in advance of the invading fungal mycelium, and hydrolyses the pectic components in primary plant cell walls and middle lamellas (Yao *et al.*, 1996). This causes the cells to separate and the host tissue macerates, facilitating pathogen penetration and colonisation of the plant tissues. Extensive degradation of the plant cell walls lead ultimately to the death of the host cell.

Functional evidence for the role that endoPGs play in the virulence of a fungal pathogen was obtained when a mutation in one member of the PG family in *Botrytis cinerea* (*ΔBcpg1*) resulted in a reduction of virulence on tomato and apple (ten Have *et al.*, 1998). The mutants were still pathogenic and produced the same primary infections as control strains, but secondary infection, i.e. expansion of the lesion, was significantly decreased. The *Bcpg*1 gene is therefore required for full virulence.

While the fungal endoPG is disrupting the plant cell wall, the products of the degradation process are used by the fungus as a nutrient source for growth (Karr and Albersheim, 1970). It is also a potential avirulence factor by releasing cell wall fragments that signal the plant defence responses.

An example of an endoPG eliciting defence responses in plants is its induction of β-1,3-glucanases in *Phaseolus vulgaris* (Lafitte *et al.*, 1993). Purified endoPG from race β of *Colletotrichum lindemuthianum* was absorbed into near-isogenic lines of *Phaseolus vulgaris*, the one resistant and the other susceptible to this fungus. Induction of β-1,3-glucanase activity was earlier and the level higher in the resistant than in the susceptible isoline. This endoPG-mediated defence seems to be dependant on the release of pectic fragments of a critical size from the cell wall, because defence elicitation was abolished by the addition of an exopolygalacturonase to the bioassay. It degraded the oligogalacturonides released by endoPGs to elicitor-inactive monomers.

EndoPGs and mycelium from the fungus *Aspergillus niger* elicited necrosis on pods from cowpea (*Vigna unguiculata*) (Cervone *et al.*, 1987a). Oligogalacturonides with a degree of polymerisation greater than four and PG-released oligosaccharides from *Vigna* cell walls also elicited necrosis. PGs inactivated by heat or by antibodies were unable to elicit this response, indicating that the catalytic activity of the PG is required for its function as an elicitor. The plant may thus sense either the PG or the oligogalacturonides produced by it, or both, as elicitors of plant defence responses. This experiment supports the view that oligogalacturonides of a critical length are necessary for the induction of defence responses.

2.6.2 Fungal exoPGs

In addition to endoPGs, fungi also produce exo-polygalacturonases. They are not subject to inhibition by PGIPs (described in the next section) as are endoPGs (Hoffman and Turner, 1984; Cervone *et al.*, 1990). Two exoPGs from *B. cinerea* are described by Johnston *et al.* (1993). They release reducing sugars from pectin at a much lower rate than endoPGs, and because they are not inhibited by PGIP, they are responsible for part of the background activity in reducing sugar assays with fungal PG preparations. They also reduce the viscosity of a pectin solution at a slower rate than endoPGs. They are implicated in degrading oligogalacturonides released by endoPGs to elicitor-inactive monomers (Lafitte *et al.*, 1993).

2.6.3 The plant cell wall as defence mechanism

The plant cell wall is composed of complex polysaccharides, phenolics and structural proteins. It has important functions in maintaining the cell and tissue integrity. It also plays a complex role in resistance to invading pathogens. It is firstly a physical barrier to infecting pathogens (Karr and Albersheim, 1970). It acts as a source of nutrients for the pathogen and controls the production of degradative enzymes by the pathogen. It is composed of polysaccharides capable of regulating gene expression and defence responses. Enzymes and proteins involved in host defence mechanisms are localised in the plant cell wall.

Pectin is a complex saccharide that is present in the plant cell wall. It contains large amounts of galactosyluronic acid residues (York *et al.*, 1985), and can be broken down by a range of enzymes, such as endo- and exo-polygalacturonases, pectate lyases, pectin lyases and pectin methylesterases.

2.7 Polygalacturonase-inhibiting proteins (PGIPs)

Polygalacturonase inhibiting proteins (PGIPs) are basic proteins present in the cell wall of most dicotyledonous plants. PGIPs are specific, reversible, saturable, high-affinity 'receptors' for fungal, but not plant, endopolygalacturonases (Cervone *et al.*, 1987b, 1989, 1990). PGIPs reduce the activity of endoPGs to different extents and are highly specific. PGIP is structurally related to several resistance gene products, since it belongs to the super-family of leucine-rich repeat (LRR) proteins (Mattei *et al.*, 2001). These LRR proteins are specialised for the recognition of non-self molecules and rejection of pathogens.

2.7.1 Action of PGIP

When fungal endoPGs attack plant cell wall pectic polymers, they produce oligomeric α-1,4-linked oligogalacturonides. Oligomers with especially 10-13 residues are elicitors of plant defence

responses. The endoPGs then rapidly depolymerise them into shorter inactive molecules (Cervone *et al.*, 1989).

Cervone *et al.* (1987b) performed several experiments *in vitro*, which lead to the formulation of a hypothesis for the action of PGIP in plant defence responses. Fungal PGs were incubated with polygalacturonic acid in the absence and presence of bean PGIP. PGIP affected the amount of oligogalacturonides with a degree of polymerisation of four and higher, by retarding the PG-catalysed hydrolysis of polypectate to mono- and digalacturonate.

The elicitor-activity of oligogalacturonides produced from polygalacturonic acid by fungal PGs in the presence and absence of bean PGIP was also assayed (Cervone *et al.*, 1989). The degree of polymerisation of oligogalacturonides was reduced at a much slower rate in the presence of PGIP. The elicitor activity of the digestion products in the presence of PGIP was much higher. The most active group of oligomers was of an intermediate size, with polymerisation between 10 and 13.

The hypothesis based on the *in vitro* evidence states therefore that the complex formed between the endoPG and PGIP leads to more stable oligogalacturonides that have elicitor activity (Cervone *et al.*, 1989; De Lorenzo *et al.*, 1990). The elicitor active molecules accumulate at the site of infection. The PGIP can therefore act against fungal invasion of the plant by causing fungal PGs to increase their elicitation of plant defence responses (Cervone *et al.*, 1987b).

In an experiment with pear PGIP and a complex mix of *B. cinerea* PGs, Sharrock and Labavitch (1994) attempted to verify the hypothesis of Cervone, namely that PGIP modifies cell wall degradation to result in more elicitor active pectic fragments of a specific size class. They attempted to replicate the *in vivo* environment of PGIP by incubating the enzymes with pear cell walls. They found, however, that Cervone's hypothesis is only a model system that either works only *in vitro* or it may only work in some PG:PGIP interactions e.g. bean PGIP but not pear PGIP. Oligomeric breakdown products did not accumulate due to the presence of a component that rapidly degraded intermediates to monomers and dimers. This component was demonstrated to be isozymes of *B. cinerea* polygalacturonase that remained uninhibited by pear PGIP.

2.7.2 History of discovery of PGIP

Proteins were extracted from various tissues that were able to inhibit the activity of fungal polygalacturonases to various extents (Albersheim and Anderson, 1971). The protein from bean hypocotyls formed a complex with a very low dissociation constant with the polygalacturonase from *C. lindemuthianum*. The existence of PGIP was discovered by the apparent lack of extractable fungal PG from infected plant tissues. Lack of pectinase activity in a 1 M NaCl extract of *Cladosporium*

cucumerinum infected cucumber hypocotyls was thought to occur by the co-extraction of a component from the cell wall that specifically inhibited the pectinase (Skare *et al.*, 1975). The apparent absence of PG in various fruit infected with plant pathogenic fungi lead to the discovery of a protein capable of inhibiting the fungal PG (Fielding, 1981). The author found the inhibitory activity of PGIP to separate from the PG during isoelectric focusing. No inhibitors were found in extracts of healthy tissues. It appears as if the infection process stimulated the formation of the inhibitors. Little or no endoPG activity was found in *Capsicum* fruits infected with *Glomerella cingulata* (Brown and Adikaram, 1982) and pear fruit infected with *B. cinerea* and *Dothiorella gregaria* (Abu-Goukh and Labavitch, 1983). It was shown that these fruits contained proteins that inhibited pectinases.

2.7.3 PGIP discovered in various plants

PGIP has since been discovered in the cell wall of all dicotyledonous plants that have been examined so far, as well as a few monocots (onion and leek). The gene encoding PGIP has subsequently been cloned from several plants. Table 2.1 provides a summary of the PGIP proteins and genes studied as well as the literature references.

2.7.4 Protein structure of PGIP

PGIP has not yet been crystallised, so the protein structure presented here is only a prediction. The predictions are based mostly on amino acid sequence predicted from the DNA sequences, and modelling by Mattei *et al.* (2001).

2.7.4.1 N-terminal signal peptide

All mature PGIPs are preceded by a 24 amino acid hydrophobic signal peptide. It is almost identical in pear and apple PGIP (only two substitutions, Yao *et al.*, 1999), and the potential cleavage site (Ala-Leu-Ser) for the signal peptidase is conserved between the apple, pear, bean and raspberry PGIPs (Yao *et al.*, 1999; Ramanathan *et al.*, 1997). The alanine is substituted with a serine in the cleavage site of tomato PGIP (Stotz *et al.*, 1994). The signal peptide targets the PGIP through the endomembrane system (Von Heijne, 1985), for targeting to the apoplast or translocation into the endoplasmic reticulum (De Lorenzo *et al.*, 2001). This is consistent with the proposed cell wall localisation of PGIP (Abu-Goukh *et al.*, 1983b), the observation that PGIP is in the extracellular matrix in bean hypocotyls and that it is secreted into the medium by suspension-cultured bean cells (Salvi *et al.*, 1990).

Table 2.1 **Summary of characterised PGIP proteins and genes.** The plants in which they were identified, the literature references as well as the tissue source from which the protein was isolated are indicated.

Plant	Scientific name	Reference		
		Protein/ Extract	Plant tissue	Gene
Alfalfa	*Medicago sativa*	Degrá *et al.*, 1988	callus	
Apple	*Malus domestica*	Fielding, 1981;	fruit	Yao *et al.*, 1999;
		Brown, 1984;		Arendse *et al.*, 1999
		Yao *et al.*, 1995		
		Müller and Gessler, 1993	leaves	
Arabidopsis	*Arabidopsis thaliana*			De Lorenzo *et al.*, 2001
Bean	*Phaseolus vulgaris*	Albersheim and Anderson, 1971;	hypocotyl	Toubart *et al.*, 1992;
		Anderson and Albersheim, 1972;		Berger *et al.*, 2000
		Cervone *et al.*, 1987b;		
		Berger *et al.*, 2000		
Green peppers	*Capsicum annuum*	Brown and Adikaram, 1982	fruit	
Cotton	*Gossypium hirsutum*	James and Dubery, 2001	hypocotyl	
Cucumber	*Cucumis sativus*	Skare *et al.*, 1975	hypocotyl	
Eucalyptus spp.				Chimwamurombe *et al.*, 2001
Leek	*Allium porrum*	Favaron *et al.*, 1993, 1997;	stalk	
		Favaron, 2001		
Lupin	*Lupinus albus*	Costa *et al.*, 1997	root	
Onion	*Allium cepa*	Favaron *et al.*, 1993	bulb	
Pea	*Pisum sativum*	Hoffman and Turner, 1982, 1984	leaflets	
Pear	*Pyrus communis*	Abu-Goukh *et al.*, 1983a, 1983b;	fruit	Stotz *et al.*, 1993
		Abu-Goukh and Labavitch, 1983;		
		Stotz *et al.*, 1993		
Potato	*Solanum tuberosum*	Machinandiarena *et al.*, 2001	leaves	
Raspberry	*Rubus idaeus*	Johnston *et al.*, 1993;	fruit	Ramanathan *et al.*, 1997
		Williamson *et al.*, 1993		
Soybean	*Glycine max*	Favaron *et al.*, 1994	seedlings	Favaron *et al.*, 1994
Tomato	*Lycopersicon esculentum*	Brown and Adikaram, 1983;	fruit	Stotz *et al.*, 1994
		Stotz *et al.*, 1994		

2.7.4.2 Leucine-rich repeats (LRR)

The mature PGIP protein consists of three domains, namely the central LRR region, and two cysteine-rich flanking regions (Mattei *et al.*, 2001). Figure 2.1 is a schematic drawing of the secondary structure of PGIP indicating these domains. The internal domain consists of tandem repeated units, each a modification of a 24 amino acid peptide. Alignment of 24 different mature PGIP sequences revealed that they consist of 10 repeats of modifications of the 24 aa leucine-rich peptide (De Lorenzo *et al.*, 2001). Leucine residues are regularly spaced in the consensus sequence of GxIPxxLxxLKnLxxLdLSxNxLx, with residues conserved in at least four repeats indicated with capital letters, x's being non-conserved residues and small letters residues identical in five or less repeats. The LRR matches the extracytoplasmic consensus also found in other *R* genes that participate in gene-for-gene resistance.

Leucine-rich repeat (LRR) proteins are found in a variety of organisms and have diverse functions and cellular locations. In most of the cases, they play a role in protein-protein or protein-ligand interactions (Kobe and Deisenhofer, 1995). It is the LRR motif in these proteins that is responsible for the protein-protein interactions (De Lorenzo *et al.*, 2001). They are mostly membrane associated or involved in signal transduction, so proteins containing a domain of tandem Leu-rich repeats may be receptors for other macromolecules (De Lorenzo *et al.*, 1994). The consensus sequence for the repeat motif is very similar between proteins, considering their diverse functions and wide distribution of organisms. This indicates a strong selection pressure for the conservation of this structure.

In plants, LRR proteins play an important role in defence, by facilitating host-pathogen interactions. Most plant *R* genes encode leucine-rich repeat (LRR) proteins (Hammond-Kosack and Jones, 1997), indicating that protein-protein interactions play an important role in plant disease resistance. The functions of plant *R* genes are to recognise pathogen *Avr* gene products and to initiate an induced response. Some of the *R* gene products do this through the LRR that acts as a putative receptor. Sequence variation in the LRRs is thought to influence the recognition specificity of these *R* gene products. The role of the LRR domain in other *R* gene products can, however, also be dimerisation or interaction with upstream or downstream signalling components.

As already mentioned, PGIP of plants also has the LRR structure. PGIP is not directly involved in pathogen recognition, but does bind to fungal endoPGs (Stotz *et al.*, 2000). It is one of the few plant LRR protein for which the ligand is known, so it may prove useful in studying the structural bases of recognition specificity of other plant LRR *R* proteins (Leckie *et al.*, 1999). PGIP is proposed to be the secreted receptor component of the cell-surface signalling system involved in the recognition event between plant and fungi. The LRR of PGIPs may be required for the interaction with and inhibition of fungal PGs (Stotz *et al.*, 1994).

The porcine ribonuclease inhibitor (PRI) was studied, and proved valuable in understanding the interaction between PGIP and PG (Kobe and Deisenhofer, 1995). It consists of a repeated β-strand/β-turn structural unit, arranged into a parallel β-sheet, with the molecule having a horseshoe shape. The motif xxLxLxx is repeated so that the leucines form a hydrophobic core and the sidechains of the flanking amino acids are solvent exposed and able to interact with the ligand. It is proposed that PGIP is also composed of a parallel stacking of β-strand/β-turns, forming an arch-shaped protein with a solvent exposed surface (Leckie *et al.*, 1999). The amino acids that determine the specificity and affinity of PGIP to its target fungal PG may be displayed on the solvent-exposed area of β-strand/β-turn region (De Lorenzo *et al.*, 2001).

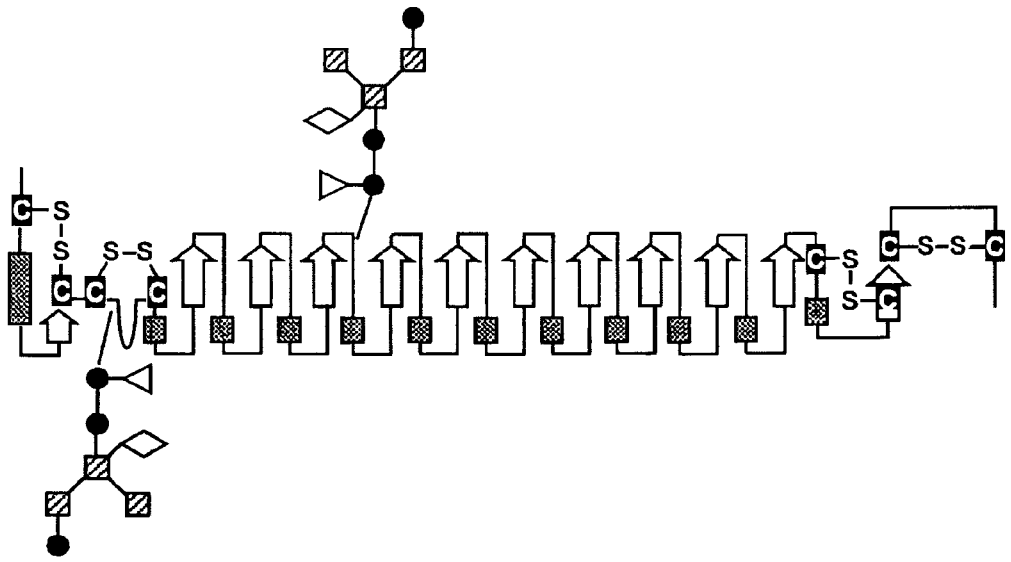


Figure 2.1 **Schematic drawing of PGIP secondary structure elements.** Arrows indicate β-strands and boxes indicate α-helices. Glycan structure has been sketched as follows: (●) GlcNAc (*N*-acetyl glucosamine), (□) Man (mannose), (▽) Fuc (α(1,3)-fucose), (◊) Xyl (β(1,2)-xylose). (Mattei *et al.*, 2001).

2.7.4.3 Cysteine residues

Conserved positions of eight cysteine residues, clustered at the N- and C-terminus of the mature peptide (Figure 2.1), suggest disulphide bonds are involved in stabilising the tertiary structure of PGIP (tomato, pear and bean PGIP: Stotz *et al.*, 1994). This may be responsible, at least partially, for the heat stability and resistance to proteases of PGIPs. They may also be required for the correct folding of the extracellular peptide (Ramanathan *et al.*, 1997).

2.7.4.4 Glycosylation

PGIP is a glycoprotein, containing seven conserved N-glycosylation sites. Differentially glycosylated forms of PGIP account for the heterogeneity in molecular mass (44 - 54 kDa) observed for PGIP that has not been deglycosylated (soybean: Favaron *et al.*, 1994; tomato: Stotz *et al.*, 1994; transgenic pear PGIP in tomato: Powell *et al.*, 2000). Chemically deglycosylated PGIP from pear, tomato, apple and lupin has the same molecular mass of 34 kDa (Stotz *et al.*, 1993, 1994; Yao *et al.*, 1995; Costa *et al.*, 1997). Glycosylation of PGIP may influence inhibitor activity, since differentially glycosylated pear PGIP expressed in transgenic tomato were not equally effective as fungal PG inhibitors (Powell *et al.*, 2000). Figure 2.1 shows the typical structure of plant N-linked oligosaccharides as was determined for bean PGIP-2. The innermost *N*-acetyl glucosamine (GlcNAc) contains an α(1,3)-fucose (Fuc). N-glycans were identified to be attached to the Asn residuces of the predicted Asn-Xxx-Thr/Ser consensus site (Mattei *et al.*, 2001).

2.7.5 Gene structure of PGIP

The gene for PGIP is a single open reading frame of approximately 1000 bp. It codes for a mature polypeptide between 300 and 315 aa in length and with a molecular mass around 44 kDa (De Lorenzo *et al.*, 2001). The nucleotide sequence of the PGIP transcript is colinear with the genome sequence in apple, pear, tomato and bean PGIP (Yao *et al.*, 1999; Stotz *et al.*, 1993, 1994; Toubart *et al.*, 1992) but *A. thaliana* and raspberry PGIP are exceptions (De Lorenzo *et al.*, 2001; Ramanathan *et al.*, 1997). They contain introns of which the positions are conserved in both the *A. thaliana* and raspberry genes (De Lorenzo *et al.*, 2001).

2.7.6 Evolution of PGIP genes

PGIP inhibition specificities and kinetics vary within and between species. This reflects counter-adaptations between fungal PGs and plant PGIPs that lead to specialisation (Stotz *et al.*, 2000). PGIP genes diverged by evolutionary adaptation to different forms of PG encountered, either because of variation in the distribution of different fungal pathogens producing the PGs or because of the evolutionary response of the PG to effective inhibition.

Evolution of PGIP genes occurred by advantageous substitutions, adapting in response to natural selection. Most amino acid substitutions occurred in the solvent-exposed β-strand/β-turn segment of the LRR of PGIPs (Stotz *et al.*, 2000). Amino acid replacements in the hydrophobic core were mostly conservative, indicating the structural role. Differences among PGIPs from different plants mainly consist of substitutions and insertions or deletions of a few amino acids. This indicates that duplication and point mutations are the major driving force in the evolution of the *pgip* families (De Lorenzo *et al.*, 2001).

Compared to other resistance genes containing LRRs, *pgip* appears to be constrained in the number of sites that could change adaptively (Stotz *et al.*, 2000). This can be explained by the fact that PGIP might need to recognise much less divergent forms of PG whereas *R* genes need to evolve rapidly to recognise highly divergent elicitors. Other possible reasons include that PGIP needs to maintain inhibition of multiple fungal PGs and that it must not inhibit endogenous plant PGs. For these reasons, there might only be a few residues that can change inhibition specificity advantageously and without deleterious effects.

2.7.7 Specificity of interaction with PGs

Conclusions on the specificities of PGIP-PG interactions were based on experiments with purified PGIP and PG extracts. Plant PGIPs can interact with endoPG from fungi, but not endoPGs of plant or bacterial origin. Plant PGIP can also not inhibit fungal pectin and pectate lyases or fungal exoPGs (Hoffman and Turner, 1982, 1984; Brown and Adikaram, 1983; Abu-Goukh and Labavitch, 1983; Cervone *et al.*, 1990; Johnston *et al.*, 1993).

Fungi produce many PGs, and each has a different expression pattern *in planta* and *in vitro* (Wubben *et al.*, 1999). PGIPs differ in their PG-target specificity within and between plant species. A particular plant PGIP can also selectively inhibit individual PG isoforms produced by a single fungus. For example, the major pear PGIP that inhibits only certain PG isozymes from *B. cinerea* (Sharrock and Labavitch, 1994).

Positively selected amino acids of fungal PGs are surface exposed and able to interact with other proteins, such as PGIPs. They are distributed into several distinct regions, making it impossible for a single plant PGIP to contact them all at once. Rather, different PGIPs target different domains of PG, none of which are the active site. This explains the competitive and non-competitive inhibition kinetics found for different PGIPs (Abu-Goukh *et al.*, 1983a; Johnston *et al.*, 1993; Stotz *et al.*, 2000), the fact that endogenous fruit PG is not inhibited (pear PG: Abu-Goukh and Labavitch, 1983) and that mutations of the active site abolishes PG activity but does not prevent PGIP binding (Caprari *et al.*, 1996).

Variations in the LLR structure of PGIP influence recognition specificities. The solvent-exposed residues in the β-strand/β-turn motifs of the LRRs determine the specificity and affinity of PGIP to its target fungal PG (De Lorenzo *et al.*, 2001). Even one amino acid substitution is sufficient to alter the ability of PGIP to interact with its ligand (Leckie *et al.*, 1999). This can be illustrated by the example of bean *pgip1* and *pgip2*, where single amino acid differences in the β-strand/β-turn region causes them to have distinct specificities. Each bean *pgip*'s PG-interacting specificities can be conferred onto the other when certain amino acids are substituted with their counterparts'. The ability of bean *pgip2*

to inhibit *Fusarium moniliforme* PG was conferred to bean *pgip1* when one amino acid was changed from a lysine to a glutamine, the corresponding amino acid in bean *pgip 2*. Synonymous nucleotide changes between bean *pgip1* and *pgip2* corresponded mostly to residues located outside the β-strand/β-turn structural motif.

2.7.8 Gene families of PGIP

Most plants studied contain a small family of PGIP genes. Small variations in the structure of different PGIPs from a specific plant might provide resistance to a variety of pathogens (Pressey, 1996). A pure extract of PGIP from bean contains a mixture of PGIPs that is difficult to separate biochemically due to their similar characteristics. Nevertheless, they have different specificities against fungal PGs, and may therefore protect the plant against a variety of fungi (Desiderio *et al.*, 1997; Pressey, 1996).

At least two closely related copies of PGIP are found in pear (Stotz *et al.*, 1993) and tomato (Stotz *et al.*, 1994). The different PGIPs from the same plant tissue may have different target PG specificities. PGIP sequences are particularly divergent in the LRR domains, while the N- and C-terminal portions of the proteins are more conserved (De Lorenzo *et al.*, 2001). It may be these sequences that are responsible for the differences in inhibition kinetics and interaction with different fungal PGs (Stotz *et al.*, 1993, 1994). These differences in specificity might explain the observed host-pathogen compatibility. Heterologous PGIPs may be especially useful to improve disease resistance of crops to certain fungal pathogens.

PGIPs from pear and apple (*Malus domestica)* are very closely related, and may be orthologous genes, that is, direct evolutionary descendants from a common ancestral gene. Because bean and soybean PGIP differ so much, they may be paralogues, descendants of duplication events that occurred prior to speciation. Duplications also occurred after divergence of the species, as evident by the pairs of PGIP genes in *A. thaliana*, bean and soybean (Stotz *et al.*, 2000).

Apple and raspberry have low copy number gene families (Yao *et al.*, 1999; Ramanathan *et al.*, 1997). There are multiple copies of PGIP genes in the bean genome (Frediani *et al.*, 1993). They are clustered and cytologically localised to one chromosomal region, the pericentromeric heterochromatin of chromosome pair X. PGIP genes from five commercially important *Eucalyptus* species were cloned and the sequences compared (Chimwamurombe *et al.*, 2001). They were found to be highly conserved, with 98 to 100% identity. The fact that PGIPs have high homologies within genera strengthens the belief that PGIP plays an important role in plants.

2.7.9 Localisation of PGIP in the plant

Various indications exist that PGIP is localised in the plant cell wall (Albersheim and Anderson, 1971; Skare *et al.*, 1975; Abu-Goukh *et al.*, 1983b; Salvi *et al.*, 1990; Johnston *et al.*, 1993). It is bound ionically to cell walls since it is extractable by salt. PGIPs are basic proteins, and their high pI values may facilitate binding of the positively charged molecules to negatively charged pectin in the plant cell wall. Soluble forms of the PGIP protein have also been discovered (Hoffman and Turner, 1982).

A basal level of PGIP is present in the cell wall of uninfected cells, but levels increase significantly upon fungal attack. PGIP accumulates preferentially in the epidermal cells immediately surrounding the infection site, as in the example of *C. lindemuthianum* infection of *P. vulgaris* leaves and hypocotyls (Bergmann *et al.*, 1994; Devoto *et al.*, 1997). This indicates the role of PGIP as one of the first defence gene products in plant disease resistance, since epidermal cells present the first structural barrier to invading pathogens. Induction of PGIP in the surrounding epidermal cells may occur due to the local signals generated during fungal penetration, namely the oligogalacturonides produced by endoPG:PGIP interaction, or fungal oligoglucosides generated from the fungus itself.

PGIP is localised in the apoplast of *P. vulgaris* stems. PGIP levels are the lowest at the roots and highest at the vegetative apex and the flower (Salvi *et al.*, 1990). It is speculated that PGIP activity is low in roots so as not to prevent invasion by beneficial mycorrhizal fungi, which are expected to produce the same kind of polygalacturonase as phytopathogenic fungi. Because of the occurrence of pectic enzymes in pollens and PGIP in flowers, such as *P. vulgaris* flowers, it is an interesting question whether PGIP might act as a recognition factor for alien pollens.

2.7.10 Timing of PGIP expression

Levels of PGIP activity are higher in immature than in mature fruit: pear (Abu-Goukh *et al.*, 1983b), apple (Yao *et al.*, 1999), raspberry (Johnston *et al.*, 1993) and tomato (Powell *et al.*, 2000). It is suggested that the pre-existing PGIP in young, developing tomato fruit could limit tissue colonisation by inhibiting fungal PGs (Powell *et al.*, 2000). As fruit mature, less PGIP is present and defence against pathogens decline. The decomposition of the cell wall by pathogens may be advantageous to the plant, because it would facilitate the release of mature seeds into the environment.

Expression of the PGIP gene may, however, continue at a consistent level in all the developmental stages, as detected in raspberry using RT-PCR and northern analysis (Ramanathan *et al.*, 1997). If it is assumed that constant levels of PGIP transcript causes constant rates of PGIP expression, the drop in PGIP activity in ripe raspberry fruit may be caused by post-translational modification or a factor present during fruit ripening that downregulates the activity of PGIP.

2.7.11 Inhibition kinetics of PGIP

Kinetic studies have shown that PGIPs inhibit fungal PGs by different types of mechanisms. PGIP showing competitive inhibition is those of pear (Abu-Goukh *et al.*, 1983a) and bean PGIP-2 in its interaction with *F. moniliforme* PG (Federici *et al.*, 2001).

Mutation studies with *F. moniliforme* PG indicated that Histidine-234, located in the active site, is critical for the enzymatic activity (Caprari *et al.*, 1996). Modifying this residue did not alter the capacity of the PG molecules to interact with bean PGIP-2. The authors thus proposed that the site responsible for PGIP recognition must reside in a domain different from the active site. Normally this would represent a situation of non-competitive inhibition.

The crystal structure of a PG from *F. moniliforme* was determined and the amino acids important for interaction with *Phaseolus vulgaris* PGIP-2 elucidated by site-directed mutagenesis (Federici *et al.*, 2001). Three amino acids, located inside and on the edge of the active site cleft, were found to be critical for the formation of the complex. This was consistent with the competitive inhibition effect that PGIP-2 has on the PG. By substituting residues of the fungal PG with residues found in plant PGs, the enzyme was unable to interact with the PGIP. This suggests how plant PGs may escape recognition by PGIPs and maintain their function in the presence of PGIP. The two residues located inside the active site cleft, Arginine-267 and Lysine-269, are likely to play a role in substrate binding. Mutations of these two residues also negatively affected the interaction of the PG with PGIP. Thus, the binding of the PGIP to these two residues prevents the binding of the substrate to the enzyme and thereby inhibits its activity. Other catalytic residues of the active site were shown not to form contacts with PGIP-2. This result corresponds to the result obtained by Caprari *et al.* (1996). The proposed mechanism of PG inhibition by PGIP is therefore the prevention of substrate access while at the same time covering the active site cleft (Federici *et al.*, 2001).

PGIP showing non-competitive inhibition include that of raspberry (Johnston *et al.*, 1993) and tomato (Stotz *et al.*, 2000). In this type of inhibition, the inhibitor binds to a site on the PG molecule that is different from the active site. Mutations of the active site abolish PG activity but do not prevent PGIP binding.

PGIP from apple shows mixed inhibition kinetics with PG from *A. niger* (Müller and Gessler, 1993; Yao *et al.*, 1995). Cotton PGIP also has a mixed or non-competitive effect on *A. niger* PG (James and Dubery, 2001). Cotton PGIP interacts in a positive cooperative manner with an extracellular endoPG from *V. dahliae* (James and Dubery, 2001). A sigmoidal rather than typical Michaelis-Menten curve was obtained. Cooperative allosteric interactions occur when the binding of one ligand at one site is influenced by the binding of another ligand at a different (allosteric) site on the enzyme (Palmer,

1995). The positive cooperative inhibition indicates allosteric interactions between the PG enzyme, inhibitor protein and polygalacturonic acid (PGA) substrate. The reaction rate is reduced at low substrate concentrations, but not at others. The PGIP reduced the reaction rate and decreased the substrate affinity of the PG, thereby contributing to the accumulation of elicitor-active oligogalacturonides.

Since these studies used distinct PGs, the differences in inhibition kinetics may be due to the specific target PG, and not the PGIP properties. The mixed results may be due to impure preparations of PG or PGIP, or both.

2.7.12 Inducers of PGIP expression

PGIP is induced by various stress stimuli, such as:

- Wounding (Soybean: Favaron *et al.*, 1994; Bean hypocotyls: Bergmann *et al.*, 1994; Apple: Yao *et al.*, 1999; Potato: Machinandiarena *et al.*, 2001).
- Elicitors (of plant or fungal origin): elicitor-active oligogalacturonides or fungal glucan. Bergmann *et al.* (1994) showed that the accumulation of bean PGIP in suspension cultures in the response to elicitor treatment was correlated with an increased accumulation of *pgip* mRNA.
- Pathogen infection, especially incompatible interactions with the fungus, manifested by the hypersensitive response (*C. lindemuthianum* and bean hypocotyls: Bergmann *et al.*, 1994; Nuss *et al.*, 1996 and Devoto *et al.*, 1997).
- Salicylic acid treatment causes an increase in PGIP levels (Cotton hypocotyls: James and Dubery, 2001; Bean hypocotyls: Bergmann *et al.*, 1994; Potato leaves: Machinandiarena *et al.*, 2001). Salicylic acid is a molecule implicated in the systemic induction of plant defence responses. During pathogen attack, salicylic acid increases both locally and systemically.
- Developmental factors. PGIP activity and transcripts were found at different levels in different organs of bean seedlings and plants (Salvi *et al.*, 1990; Devoto *et al.*, 1997).

2.7.13 Examples of PGIP

2.7.13.1 Apple PGIP

Much work has been done on the PGIP from apple (*M. domestica*) (Brown 1984; Müller and Gessler, 1993; Yao *et al.*, 1995, 1999; Arendse *et al.*, 1999). Inhibitor activity against endopolygalacturonases from several fungi was first discovered in the cell walls of four apple cultivars, namely Granny Smith, Golden Delicious, Cox's Orange Pippin and Bramley's Seedling (Brown, 1984). It was shown that the cultivar with the highest PGIP levels displayed the least tissue maceration and rot expansion during fungal inoculation experiments.

Proteinaceous inhibitors of fungal PGs were previously only detected in the cell walls of infected apple fruits (Fielding, 1981), but Brown (1984) showed the endoPG inhibitor to be present also in healthy fruits of at least some cultivars. It is suggested that it plays a role in cultivar resistance to some pathogens. It has since been found also in leaves of apples (Müller and Gessler, 1993).

Protection against pectic enzymes by oxidised polyphenols declines as the sucrose levels of fruit increase (Brown, 1984). Therefore, PG inhibitors become more important as protectors against rot as fruit ripens. Unfortunately, the endoPG inhibitor was only present in pre-climacteric, ripening and rotted apple fruit, and seemed to decline when fruits became very ripe (Brown, 1984). This then leads to a conflict, with fruits becoming more susceptible to fungal infection as they near ripening. If PGIP can be expressed during the ripe stages, fruit will be much better protected against fungal infection. According to the hypothesis of the action of PGIP, it will firstly inhibit colonisation and secondly activate host defence responses at the site of infection.

PGIP from Golden Delicious apple fruit was characterised by Yao *et al.* (1995). It was found to be a glycoprotein, with the chemically deglycosylated polypeptide having a molecular mass of 34 kDa. It had a mixed inhibition effect on PG II produced by *B. cinerea* in liquid culture. Other PGs secreted in liquid culture were inhibited differentially, while PG produced in apple fruit inoculated with *B. cinerea* was not inhibited at all. An apple *pgip* gene from *M. domestica* cv. Granny Smith leaves was cloned using degenerate oligo-primed PCR and Inverse PCR (Arendse *et al.*, 1999). Its sequence was found to be identical to that of a PGIP cloned from cv. Golden Delicious by Yao *et al.* (1999). The gene has 990 nucleotides and contains no introns. It encodes a predicted polypeptide of 330 amino acids, of which the first 24 amino acids are the signal peptide. The full-length mRNA transcript is 1.3 kb in size. The predicted mature protein has a calculated molecular mass of 34 kDa and a pI of 7.0. It is predicted to contain 10 imperfect leucine-rich repeat (LRR) motifs that span 80% of the mature peptide. The apple PGIP polypeptide sequence is more homologous to that from pear (Stotz *et al.*, 1993) and other fruit than those from bean and soybean (vegetables). A Southern blot indicated that apple *pgip* might be part of a small gene family of PGIP homologous genes (Yao *et al.*, 1999).

The gene is developmentally regulated since different PGIP transcript levels were present in fruit collected at different maturities (Yao *et al.*, 1999). It is also induced by fungal infections and tissue wounding, with PGIP transcripts increasing locally in the decayed and surrounding areas, but the gene is not activated systemically in tissue distant from the site (Yao *et al.*, 1999). Storage of fruit results in the reduction of the PGIP transcript level, which coincides with increased susceptibility to fungal colonisation and maceration.

2.7.13.2 Pear PGIP

Abu-Goukh and co-workers (Abu-Goukh and Labavitch, 1983; Abu-Goukh *et al.*, 1983a, 1983b), made a detailed study of polygalacturonase inhibitors from "Bartlett" pear fruits. Very young fruit resisted infection by several fungal pathogens, but fruit resistance declined steadily with maturation. PGIP levels also decreased as the fruit matured (Abu-Goukh *et al.*, 1983b). This direct correlation between decreased resistance and decreased PGIP activity suggests a specific role of PGIP in defence.

The PG inhibitor of pear was found to have no effect on pear PG, indicating that the inhibitor has no direct role in fruit ripening (Abu-Goukh and Labavitch, 1983). PGIP might, however, have an effect on a fruit's disease susceptibility, since fungi whose PGs were not successfully inhibited by pear PGIP produced lesions that expanded much faster than those that were inhibited. Pear PGIP was shown to be specific against pathogen PG enzymes, but could distinguish between PG secreted by different pathogenic fungi (*B. cinerea*, *Dothiorella gregaria* and *Penicillium expansum*).

2.7.13.3 Potato PGIP

A PGIP was purified from potato cv. Spunta leaves (Machinandiarena *et al.*, 2001). It had a molecular mass of 41 kDa, and was cell wall bound. It showed broad inhibitory activity against crude PG preparations from several fungi, including *A. niger*, *F. moniliforme* and *F. solani*. Exo-enzymes might have been present in the crude PG extracts from the other fungal cultures that were not inhibited by potato PGIP. Otherwise, they might have contained endoPGs that escaped potato PGIP inhibition. PGIP expression was induced in potato leaves by wounding, treatment with salicylic acid and incompatible interactions with *Phytophthora infestans*.

2.7.14 Role for PGIP in plant defence

Several features point to an important role of PGIP in defence against plant pathogens (De Lorenzo *et al.*, 2001). Just like other known defence genes, expression of PGIP is induced by stress- and pathogen-derived signals. They also share similarities in structure and specificity with *R* gene products. Synthesis and accumulation of PGIP is an active as well as constitutive defence mechanism in dicots (Bergmann *et al.*, 1994). In incompatible interactions between plant and fungi, PGIP mRNA accumulation was much more rapid and intense than in compatible interactions, and is correlated with the expression of the hypersensitive response (Nuss *et al.*, 1996). Circumstantial evidence for their role in defence is that the level of PGIP correlates with increased resistance to fungi, e.g. increased susceptibility of ripening fruit to fungal attack as PGIP levels declines (pear and raspberry: Abu-Goukh *et al.*, 1983b; Johnston *et al.*, 1993). In an experiment where tomato was transformed with pear *pgip*, grey mould symptoms were less on transgenic plants than on control untransformed plants (Powell *et al.*, 2000). PGIP attenuated the disease symptoms by modulating the fungal pathogen

maceration of plant tissues. PGIP therefore does not prevent establishment of the initial plant-pathogen interaction, but influences the expansion of the fungal mass.

The following points summarise the evidence that PGIP plays an important role in defence against fungal pathogens:

- PGs are produced early in a plant-fungus interaction and contribute to the expansion of the infection site (ten Have *et al.*, 1998);
- PGIPs inhibit some (but not all) fungal PGs *in vitro* (Yao *et al.*, 1995; Sharrock and Labavitch, 1994), especially those from pathogens that are least virulent on a plant, suggesting their *in planta* function (Abu-Goukh and Labavitch, 1983; Abu-Goukh *et al.*, 1983b);
- PGs that are inhibited by PGIP *in vitro* produce oligogalacturonides that induce plant defence responses (according to the hypothesis by Cervone *et al.*, 1989);
- PGIP contain the LRR structural motif that is also present in other disease resistance genes (De Lorenzo *et al.*, 1994; Stotz *et al.*, 1994).

2.7.15 Use of PGIP in creating disease resistant crops

Discoverers of PGIP in immature raspberry fruit proposed to use this gene in breeding programs to enhance the disease resistance of ripe fruits against *B. cinerea* (Williamson *et al.*, 1993). Pear PGIP was 20 times more active against PG from *B. cinerea* than tomato PGIP (Stotz *et al.*, 1994). Tomatoes were transformed with pear *pgip*, and it was still an active inhibitor of *B. cinerea* endoPGs (Powell *et al.*, 2000). It slowed the expansion of disease lesions and associated tissue maceration on infected transgenic tomato fruit and leaves, compared with infections of control plants. In contrast, bean *pgip-1* did not enhance the tomato's resistance against fungal infection (Desiderio *et al.*, 1997).

Different PGIPs expressed in the same plant may have different fungal PG-specificities. When individual bean PGIPs were tested *in vitro* against fungal PGs, they exhibited inhibiting abilities different from bulk bean PGIP (Desiderio *et al.*, 1997). The broad activity of bulk bean PGIP appear thus to be the result of the presence of different PGIP molecules with narrow specificities. It must therefore be taken into consideration that by expressing one PGIP in a transgenic plant, inhibiting activity against only a certain number of specific fungi will be obtained. If a general enhanced resistance to fungi needs to be obtained, more than one PGIP gene needs to be transformed.

2.8 *GST1* promoter of *Arabidopsis thaliana*

During this project, a construct was prepared containing the apple *pgip1* gene under control of the *gst1* promoter of *A. thaliana*. The aim is to drive pathogen-inducible expression of the apple *pgip1* gene in transgenic *A. thaliana*, and ultimately in other crops of importance.

The glutathione S-transferases (GSTs) are a family of enzymes that protect cellular macromolecules from various toxic xenobiotics (Yang *et al.*, 1998). GST catalyses the conjugation of thiol groups of the tripeptide glutathione (GSH) (γ-L-glutamyl-L-cysteinylglycine) to a variety of electrophilic substrates, e.g. in plants to detoxify herbicides (Itzhaki *et al.*, 1994). They function as homodimers or heterodimers of subunits. *GST* gene expression in various plants has been shown to respond to many inducers. These include ethylene, herbicide safeners, auxin, pathogen attack, salicylic acid, H_2O_2, dehydration, wounding, low temperature, high salt and DPE (diphenyl ether) herbicide treatment (Dudler *et al.*, 1991; Itzhaki *et al.*, 1994; Yang *et al.*, 1998). Examples of plants of which the *GST* gene was studied include wheat, carnation and *A. thaliana*.

The *GST* gene family of *A. thaliana* consists of six members, and is induced by the various means as listed before. The cDNA and corresponding genomic sequence of *GST1* have been cloned (Yang *et al.*, 1998, accession number Y11727). The gene has an open reading frame coding for 208 amino acids, and is interrupted by two introns. *GST1* cDNA was found to be induced by pathogen infection and dehydration, as well as wounding, high salt, low temperature and DPE herbicide treatment. Its promoter region contains motifs (ethylene responsive elements (ERE) and other motifs such as the TCA motif and the G-box) conserved amongst stress-inducible gene promoters.

Ethylene is a phytohormone that is involved in the regulation of plant growth and development and response to biotic and abiotic stresses. It influences plants by changing gene expression, through ethylene-responsive elements (EREs) conserved in the promoter regions of several genes. The ERE in *A. thaliana GST1* gene promoter (ATTTCAAA) is inversely repeated and found at positions -183 and -737 (Yang *et al.*, 1998). The ethylene responsive *cis*-sequence elements of the *GST* gene of carnation were studied and the same 8 bp sequence was found (Itzhaki *et al.*, 1994). Two putative TCA motifs are found at positions -845 and +32 of the *GST1* gene promoter of *A. thaliana*, indicating that gene expression may be regulated by salicylic acid. A G-box motif, conserved in plant genes associated with response to diverse environmental stresses, was found at position -369 (Yang *et al.*, 1998).

Another example that is evidence for the pathogen-inducibility of *GST* genes, is the putative *GST1* gene of wheat (*Triticum aestivum*). Its transcript was highly induced in leaves infected with the incompatible pathogen *Erysiphe graminis* f. sp. *hordei* (Dudler *et al.*, 1991). Pathogen-induced genes

usually code for products that are involved in host defences against pathogens. Why would *GST* be one of the host genes induced by pathogens, and how can it be involved in defence? In plants it is usually associated with detoxifying herbicides, but in animal systems it protects against oxidative tissue damage by de-toxifying products of membrane lipid peroxidation. Reactive oxygen species (ROS) and membrane lipid peroxidation are also known to occur in plants in response to tissue damage, elicitors and pathogen attack. Thus, GST may play a similar protective role in plants. It may be a member of a class of general stress response genes that is activated by many different stimuli.

The *gst1* gene of *A. thaliana* was used as a molecular marker for ROI accumulation since it is an antioxidant defence gene (Grant *et al.*, 2000). The WS *GST1* sequence (Grant *et al.*, 2000) exhibited 98% DNA sequence identity to the sequence from the Ler accession (Yang *et al.*, 1998), indicating that they may encode the same protein. The promoter of the *gst1* gene was characterised to be 909 bp, and the translated region 1092 bp with two introns of 92 and 110 bp each. The *gst1* promoter contains two EREs, a G-box and a TCA element at the 3' end, the same as was found by Yang *et al.* (1998). The presence of the TCA and ERE motifs is in accordance with previous observations that *GST1* is induced following treatment with ethylene and salicylic acid.

The nucleotide sequence of the *gst1* promoter obtained from G. Loake, University of Edinburgh, contained eight nucleotide differences and had 99% sequence identity to that from the published sequence (Yang *et al.*, 1998, accession number Y11727). The sequence of the *gst1* promoter obtained from G. Loake and the published sequence (Yang *et al.*, 1998) are aligned in Figure 2.2. Identical nucleotides are indicated by an asterisk (*). Both sequences were selected to start at a *Kpn*I restriction site, and included the ATG initiation codon (boxed) as well as a few nucleotides of the *gst1* gene.

The promoter consists of a 909 bp sequence upstream of the translation start site (boxed). The differences between the two sequences may be explained by the fact that the *gst1* promoter obtained from G. Loake was isolated from the ecotype Ws (Grant *et al.*, 2000) while the Y11727 accession was from the Landsberg ecotype (Yang *et al.*, 1998). The nucleotide differences did not affect any of the putative regulatory motifs. G. Loake's *gst1* promoter was shown to be active in directing the expression of a *luciferase* reporter enzyme, a molecular marker for ROI accumulation (Grant *et al.*, 2000).

Seq 1 G. Loake

Seq 2 Yang *et al.*, 1998

KpnI

```
Seq 1  GGTACCATAAGAAGAAGAATTAATCTTATAATCTTGTGATGTACTTTCGGTGATTTCTTTAAGTTTGATG   70
       ******************************************************************* ******
Seq 2  GGTACCATAAGAAGAAGAATTAATCTTATAATCTTGTGATGTACTTTCGGTGATTTCTTTAAGATTGATG   70
                                                                      ERE

Seq 1  TTATGAATAAAAGGCAAAGCTTTTGCAAAAATCACTCTTTTTTTTG-CCATAATGATTTCAAAATTCCAA  139
       ************************************ ******** *************************
Seq 2  TTATGAATAAAAGGCAAAGCTTTTGCAAAAATCACTCATTTTTTTGGCCATAATGATTTCAAAATTCCAA  140

Seq 1  AGAATAATATATACTTCAATATACATGTCACAAGATTAAATGTCAAAGTTGTTTATAATGAGCATTTTTG  209
       ********************************** ***********************************
Seq 2  AGAATAATATATACTTCAATATACATGTCACAAGCTTAAATGTCAAAGTTGTTTATAATGAGCATTTTTG  210

Seq 1  TGGATGTGGAAAATGTGAAAATTACTCTGTTCCTTTGAATGTTTCTATACGAAATTATAGTTAGGATTTA  279
       ********************************** ***********************************
Seq 2  TGGATGTGGAAAATGTGAAAATTACTCTGTTCCTTCGAATGTTTCTATACGAAATTATAGTTAGGATTTA  280

Seq 1  GTAATGTATTTCTCCATAATTATGCTAAATTTAGTTAGTTACTTCAATATGATTAAATACTTTATTGACC  349
       ************************************************************************
Seq 2  GTAATGTATTTCTCCATAATTATGCTAAATTTAGTTAGTTACTTCAATATGATTAAATACTTTATTGACC  350

Seq 1  CCAAATTTGTAATTGTACCAGATTGTCAAAAGTGTGAAACCAAATTTTCTTTTCTTTTGATATTGTTTTG  419
       ********************************************** ********************
Seq 2  CCAAATTTGTAATTGTACCAGATTGTCAAAAGTGTGAAACCAAATTTTCCTTTCTTTTGATATTGTTTTG  420

Seq 1  TTTCTTATTATCTCTCGTTCTATCGAATGATCTAACTCGAGCATCCAACGATCTAACTCGAGCATCTAAC  489
       ************************************************************************
Seq 2  TTTCTTATTATCTCTCGTTCTATCGAATGATCTAACTCGAGCATCCAACGATCTAACTCGAGCATCTAAC  490
          G-BOX
Seq 1  GATCCACGTGGACCCAACAACGTCGGTCAGAGTTTGACTAGTAGATGAAGGACTATTCTTGTGGTCGTTG  559
       ***********************************************************************
Seq 2  GATCCACGTGGACCCAACAACGTCGGTCAGAGTTTGACTAGTAGATGAAGGACTATTCTTGTGGTCGTTG  560

Seq 1  TCACGCGGTGGCTGATATTTTCTCTTATTTTTATTTTATTTTAACTATTTTTTACGTTATATTTAAGTCT  629
       ************************************************************************
Seq 2  TCACGCGGTGGCTGATATTTTCTCTTATTTTTATTTTATTTTAACTATTTTTTACGTTATATTTAAGTCT  630
                                                        ERE
Seq 1  TGAACCAATAGAAACGACGAATCATACATTACTCAGCTTGACTTTGAAATAATCCTATAACAAAAGAGCA  699
       ************************************************************************
Seq 2  TGAACCAATAGAAACGACGAATCATACATTACTCAGCTTGACTTTGAAATAATCCTATAACAAAAGAGCA  700
```

```
Seq 1   TTCCAAGAATTTTATCCAAAAAA-CAAAATAAAAAAGAGTATTCAAGCTTGGTGGCGCCGTTTGTTTTTG   769
        ********************** ******************************** **************
Seq 2   TTCCAAGAATTTTATCCAAAAAAACAAAATAAAAAAGAGTATTCAAGCTTGGTGGAGCCGTTTGTTTTTG   770
                                    TATA-box
Seq 1   GTTTATTCACTAAAGTTACTCTGTTTTAGTTGTATAAATACACACTCCCATTTGTGTATTTCTTTTCATC   838
        ******************************************************************************
Seq 2   GTTTATTCACTAAAGTTACTCTGTTTTAGTTGTATAAATACACACTCCCATTTGTGTATTTCTTTTCATC   840
                                                        TCA
Seq 1   AATCACAAAGATCTCTCTACTTCAATAAATCTCCACCTTACTTTAAGAACAAGAAAAACACAGTATTAAC   908
        ************************************************************************
Seq 2   AATCACAAAGATCTCTCTACTTCAATAAATCTCCACCTTACTTTAAGAACAAGAAAAACACAGTATTAAC   910

Seq 1   AAATGGCAGGAATCAAAGTTTTCGGTCACCCAGCTTCCACAGCCACTAGA                       958
        ***********************************************
Seq 2   AAATGGCAGGAATCAAAGTTTTCGGTCACCCAGCTTCCACAGCCACTAGA                       960
```

Figure 2.2 **Comparison of the nucleotide sequence of the *gst1* promoter from G. Loake with the published sequence (Yang *et al.*, 1998).** The ethylene responsive elements (ERE), G-box, TATA box and TCA element are indicated in red. The restriction site *Kpn*I is indicated in green. The start codon is boxed.

2.9 *Arabidopsis thaliana* as a model experimental plant

A. thaliana is a small diploid angiosperm of the mustard family (Cruciferae or Brassicaceae) that has become a model system for plant research. Its life cycle, from germination of seeds, formation of the rosette plant, bolting of the main stem, flowering and maturation of the first seeds, is completed in six weeks. This rapid growth cycle makes it very useful for research. Its flowers are 2 mm long, self-pollinated and develop into siliques that contain seeds of 0.5 mm. The rosette plant grows to 2 to 10 cm in diameter while mature plants are 15 to 20 cm in height. Several thousand seeds can be collected from a single plant (Meinke *et al.*, 1998).

The *Arabidopsis* Genome Initiative (AGI) was established in 1996 to facilitate coordinated sequencing of the *A. thaliana* genome, which was completed in 2000. The genome sequence of *A. thaliana* was published in *Nature* (chromosomes 2 and 4 in 1999 and chromosomes 1, 3 and 5 in 2000). It is extensively discussed in several articles (Goodman *et al.*, 1995; The Arabidopsis Genome Initiative, 2000). The original idea behind using *A. thaliana* as a model system was to help in the identification of related genes important in crop plants. The *A. thaliana* genome is small, only 120 mega bases, organised into five chromosomes and contains an estimated 26 000 genes. It contains a relatively

small amount of interspersed repetitive DNA, making sequencing of its genome a cost-effective method in identifying every gene in a representative flowering plant (Meinke *et al.*, 1998).

Mutants defective in almost every aspect of plant growth and development have been identified and studied by various research groups over the world. Random large-scale insertional mutagenesis by T-DNA and transposon insertion is used to create gene knockouts so that reverse genetic screens can be applied to deduce the functions of the sequenced genes (Parinov and Sundaresan, 2000). To name a few examples: mutants were used for dissecting the mode of ethylene action in plants (Guzmán and Ecker, 1990); defence-related mutants were used to dissect the plant defence response to pathogens (Ausubel *et al.*, 1995; Glazebrook and Ausubel, 1994; Xie *et al.*, 1998); and to study the effect of plant hormones and signalling molecules on pathogen-induced defence gene expression (Feys *et al.*, 1994; Penninckx *et al.*, 1996).

2.10 *Agrobacterium tumefaciens*-mediated plant transformation

2.10.1 *Agrobacterium tumefaciens* transformation system

Agrobacterium tumefaciens causes crown gall on dicotyledonous plants. It confers this tumorous phenotype by introducing a DNA segment into the plant cell and stably integrating it into the plant chromosome (Hooykaas and Schilperoort, 1992). This was viewed as a useful method of delivering genetic material into plants to create stable transgenics. The transferred DNA is called the T-DNA and is carried on the tumour-inducing plasmid (Ti-plasmid), together with the virulence (*vir*) region that provides the *trans*-acting factors for creating the T-DNA copy, structural elements for the transfer intermediate and components of the transfer apparatus. Only sequences flanked by the T-borders (25 bp direct repeats) are transferred to the plant cell. In wild-type *Agrobacterium*, the genes contained between the T-borders are enzymes for plant growth regulators and opines. In transformation vectors, these sequences are replaced with the gene(s) of interest.

A different method of plant transformation involves a particle gun. Small tungsten or gold particles are coated with DNA and shot directly into plant tissues. The DNA with the particle that reaches the nucleus may integrate into the genome and be expressed. It has the disadvantages of scrambling the DNA copies and integration of multiple DNA copies that may lead to rearrangement, recombination or silencing.

Using the *A. tumefaciens*-mediated transformation method, the copy number of the T-DNA in transformed plant lines is usually low, varying from one to a few copies (Hooykaas and Schilperoort, 1992). Rarely, lines with up to a dozen copies are found. Multiple copies may be located at different loci in the plant genome, or occur at a single locus as direct or inverted repeats. The complex details of the formation of the transfer DNA complex are reviewed by Zupan and Zambryski (1997). The

Agrobacterium system thus doesn't have the disadvantages of the particle gun system, probably due to the structure with which the T-complex is delivered into the plant cells.

2.10.2 Plant transformation vectors

Binary vectors used for plant transformation were created to simplify the cloning of exogenous DNA into the large Ti-plasmid of *A. tumefaciens* (An, 1986). The *vir* region was removed from the Ti-plasmid, to result in a binary vector that contains an artificial T-DNA and carrying only the *cis*-acting elements. The *cis*-elements include the T-DNA borders, selectable marker expressible in plants (e.g. kanamycin resistance), cloning sites and a wide host range replicon. The binary vector needs to be transformed into an *Agrobacterium* strain containing a helper Ti-plasmid with an intact *vir* region but lacking the T-region (Hooykaas and Schilperoort, 1992). The helper Ti-plasmid and the *A. tumefaciens* genome provides *in trans* the other functions required for plant transformation.

2.10.3 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* by the floral-dip method

The floral-dip method for *Agrobacterium*-mediated transformation of *A. thaliana* allows transformation of the plant without the need for tissue culture. The flowering plant is simply dipped into a solution of *Agrobacterium* and allowed to produce seed (Clough and Bent, 1998). The target for *Agrobacterium* transformation is the ovules of young flowers. To achieve efficient transformation, the *Agrobacterium* has to be delivered to the interior of the developing gynoecium before the locules close (Desfeux *et al.*, 2000). Transformants derived from seed from the same seedpod will contain independent T-DNA integration events. The *Agrobacterium*-treated T_0 plant is not treated with selection agents such as herbicide or antibiotics. Rather, the progeny seed is harvested and selection is applied to the resultant T_1 seedlings as they germinate. The T_1 transformants are found to be mostly hemizygous and there is an absence of homozygous self-fertilised offspring. Thus, the transformation event is thought to occur in the germ-line cells after divergence of female and male gametophyte cell lineages, or the T_1 embryo is transformed soon after fertilisation.

2.10.4 Selection of transgenic plants

During transformation, not all cells are transformed. Selecting for a plant selectable marker or a reporter gene can identify cells that have been transformed. Selection markers are based on the sensitivity of plant cells to antibiotics and herbicides (Hooykaas and Schilperoort, 1992). Expressing bacterial genes of detoxifying enzymes can make plants resistant to these compounds. For example, kanamycin resistance is mediated by neomycin phosphotransferase (NPTII), hygromycin resistance via the hygromycin phosphotransferase (HPT) gene and bialaphos (a herbicide) resistance via the *bar* gene from *Streptomyces hygroscopicus*.

Plant reporter genes can also be used to identify transformed cells, by linking the reporter gene to the gene of interest and screening for the expression of the reporter gene. Examples of reporter enzymes include luciferase, β-galactosidase and β-glucuronidase (Hooykaas and Schilperoort, 1992). The firefly luciferase enzyme generates emitted light when it hydrolyses its substrate luciferin. Since many plant tissues have endogenous β-galactosidase activity, β-glucuronidase is preferred as a reporter enzyme. β-glucuronidase enzyme activity can be measured quantitatively by fluorometrically detecting the umbelliferone that is released from umbelliferyl derivatives. It can also be determined histologically by staining transformed tissues. β-glucuronidase enzymatically converts 5-bromo-4-chloro-3-indolyl derivatives to indigo-blue compounds (Hooykaas and Schilperoort, 1992).

This chapter gave a brief overview of polygalacturonase-inhibiting proteins and how they play a role in the defence against phytopathogenic fungi. It also reviewed the specific interaction between a plant and fungus, namely *Verticillium*-wilt of potato. In the following chapters, a brief overview will be given on the topics covered and the methodology used in each.

CHAPTER 3

Cloning of the apple *pgip1* gene under control of the *gst1* promoter of *Arabidopsis thaliana*

3.1 Introduction

In an attempt to test the functionality and pathogen inducibility of the *gst1* promoter from *Arabidopsis thaliana* (L.) Heynh (Yang *et al.*, 1998; Grant *et al.*, 2000), a construct was prepared where it was cloned upstream of the apple *pgip1* gene. This gene codes for a protein product (apple PGIP1) that is able to inhibit the PGs secreted *in vitro* by the fungal pathogen *Verticillium dahliae*. It is reported that the *gst1* promoter is induced by various stress conditions (Yang *et al.*, 1998). The fungal inducibility of the *gst1* promoter would be valuable in the transgenic expression of antifungal resistance genes in crops of importance. The construct will first be tested in *A. thaliana*, a plant that can be rapidly transformed (Chapter 4). It is advantageous to use this model plant since transformants can be obtained without the methods of tissue culture regeneration, and the plant has a relatively short life-cycle (under optimal conditions, six weeks from germination to seed set).

The aim of this chapter was thus to prepare a plant transformation construct in which the apple *pgip1* gene is under control of the pathogen-inducible *gst1* promoter. This will be used in subsequent transformations of *A. thaliana* (Chapter 4).

Construction of plasmids for plant transformation

A. Apple *pgip1* gene under control of a constitutive promoter (CaMV e35S promoter)

Previously, a PGIP gene from apple was cloned at ARC-Roodeplaat using inverse polymerase chain reaction methods (Arendse *et al.*, 1999). The sequence obtained was identical to that published by Yao *et al.* (1999, accession number U77041). A chimeric e35S-*pgip1* construct for the constitutive overexpression of the apple *pgip1* in transgenic plants has been generated (Arendse and Berger, unpublished). The apple *pgip1* gene was amplified from genomic DNA by designing the appropriate primers, including restriction enzyme sites at the ends of the primers. The PCR fragment was directionally cloned into the pRTL2 vector (Cassidy and Nelson, 1995), placing the apple *pgip1* gene under control of the enhanced CaMV 35S promoter (e35S) (a standard dicot constitutive promoter) and the TEV leader (Tobacco Etch Virus leader element, which is a translational enhancer), followed by the CaMV 35S terminator. The cassette, containing the promoter, TEV leader, apple *pgip1* gene and terminator, was subcloned into the binary vector pCAMBIA2300 for plant transformation.

Constructs containing the cassette in either orientation were obtained. The construct pCAMBIA2300-apple*pgip*1B (**Appendix C**) was chosen as the constitutively expressed apple *pgip1* construct for transformation of *A. thaliana* (covered in Chapter 4).

B. Apple *pgip1* gene under control of a pathogen-inducible promoter (*gst1* promoter)

The preparation of a plant transformation vector containing the apple *pgip1* gene under control of the *gst1* promoter from *A. thaliana* required three steps. Firstly, the *gst1* promoter was amplified from a previous construct (p*GST1*-BluescriptSK$^-$) using the polymerase chain reaction, and cloned into the pMOSBlue blunt-end cloning plasmid vector. Secondly, the *gst1* promoter PCR fragment was subcloned into the plant transformation vector pCAMBIA2300. Lastly, the apple *pgip1* gene, as part of a cassette containing the TEV leader and CaMV 35S terminator, was cloned downstream of the *gst1* promoter in pCAMBIA2300. The resultant construct was called *GST1*prom-ap*pgip1*-pCAMBIA2300 (Figure 3.18).

The promoter consists of a 909 bp sequence upstream of the translation start site (Figure 2.2). A primer was designed to amplify the *gst1* promoter fragment from a previous construct (p*GST1*-BluescriptSK$^-$) using the polymerase chain reaction (PCR). The primer (GSTreverse) is complementary to a sequence at the 3' end of the *gst1* promoter, immediately upstream of the ATG start codon of the *gst1* gene (refer to Figure 3.1). The start codon needs to be excluded from the amplified promoter, to ensure correct translation initiation of the apple *pgip1* gene in the transgenic plant. A vector-specific primer was used as the second primer in the primer-pair needed for PCR. PCR exponentially amplifies selected regions of DNA using primers that anneal to denatured dsDNA. A thermostable DNA polymerase such as Taq DNA polymerase, isolated from *Thermus aquaticus*, is used to synthesise the new strands complementary to the denatured template. This polymerase enzyme remains active during the repeated cycles of denaturing of DNA hybrids, annealing of primer to template and extension of the primer complementary to the template.

Restriction enzyme recognition sites were engineered at the 3' end of the *gst1* promoter, to facilitate subsequent subcloning. It was incorporated into the amplification products by adding it to the 5' end of the promoter-specific primer, called GSTreverse. During the first cycle of PCR, a hybrid between template and a primer that is not completely complementary to it will form. After extension the primer will now be incorporated into a daughter molecule, which will serve as a template molecule during the next cycle. In this approach the primer sequence gets incorporated into the PCR amplification products.

This chapter reports on the cloning of the apple *pgip1* gene under control of the pathogen-inducible *gst1* promoter of *A. thaliana* into a plant transformation vector. Chapter 4 will continue with the

transformation of the model plant *A. thaliana* with the *gst1*- and e35S-promoter apple *pgip1* constructs. Molecular analysis of the transformants and PGIP expression studies will also be reported.

3.2 Materials and Methods

All chemicals and reagents used were either analytical or molecular biology grade. Buffers, solutions and media were all prepared using distilled water and were either autoclaved or filter-sterilised through 0.2 µm sterile syringe filters. All buffers, solutions and media used in this study are outlined in **Appendix A**. Ampicillin, calf thymus DNA, the Expand Long template PCR system, Hoechst 33258 DNA binding dye, restriction endonucleases, RNase A, T4 DNA ligase and X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside) were obtained from Roche Diagnostics (Mannheim, Germany). Large scale (Qiagen Midi plasmid purification kit) and mini plasmid DNA preparation kits (Qiaprep and Qiafilter miniprep kits), as well as gel extraction kits (Qiaquick gel extraction kit) were from Qiagen, Germany. Plasmid maps were drawn using Vector NT. Sequence data can be entered and a map generated to scale, indicating restriction enzyme sites and other features at the correct positions.

3.2.1 Plasmid DNA isolation

Isolation of large amounts of plasmid DNA from *Escherichia coli* was performed using the Qiagen Midi plasmid purification kit. Mini preparations of plasmid DNA were performed using the Qiaprep and Qiafilter miniprep kits. Purification using the Qiagen kits relies on the alkaline lysis principle, and the DNA is purified by selective absorption to a silica column in the presence of a chaotropic salt. Contaminants are washed away and the DNA is eluted by a low salt buffer (10 mM Tris, pH 8.5).

For restriction digestion screening of possible recombinants, mini preparations of plasmid DNA was done by the alkaline lysis method as in Sambrook *et al.* (1989) with modifications. A single bacterial colony was grown overnight at 37°C with shaking in 5 ml LB medium containing the appropriate antibiotic (100 µg/ml ampicillin for pMOSBlue constructs and 100 µg/ml kanamycin for pCAMBIA2300 constructs). Cells from 3 ml of overnight culture were collected by centrifugation at 6500×g for 10 min at 4°C. The supernatant was removed and the pellet resuspended in 200 µl Solution I (**Appendix A**) by pipetting or vortexing. Five microlitres of 10 mg/ml RNase A was added and the sample incubated for 5 min at room temperature. The cells were lysed by the addition of 400 µl Solution II (**Appendix A**) and incubated on ice for 5 min. The sample was neutralised by the addition of 300 µl Solution III (**Appendix A**) and incubated on ice for 5 min. The debris was pelleted by centrifugation at 6500×g for 20 min at 4°C. Seven hundred and fifty microlitres of the supernatant was recovered and added to 750 µl isopropanol in a clean tube. The precipitated DNA was pelleted by centrifugation at 6500×g for 15 min at 4°C. The DNA pellet was resuspended in 540 µl dH_2O and 60 µl 5 M $NaClO_4$. Six hundred microlitres isopropanol was added and the mixture was incubated at room temperature for 10 min. The DNA was pelleted by centrifugation at 6500×g for 15 min at 4°C.

washed with 70% ethanol and re-pelleted. After air-drying, the DNA was resuspended in 30 µl 1× TE (pH 8.0) or 10 mM Tris (pH 8.5) by shaking at 37°C for a few minutes. Typically the yield was such that 3 µl was sufficient to be used in restriction digests for screening.

The DNA concentration was determined on a Sequoia-Turner 450 fluorometer (Sequoia-Turner Corporation) according to the manufacturer's instructions. The fluorometer was calibrated with 1× TNE buffer, pH 7.4, containing 0.2 µg/ml Hoechst 33258 DNA binding dye. Calf thymus DNA was used as a DNA standard.

3.2.2 Polymerase Chain Reaction

3.2.2.1 PCR amplification using Taq DNA polymerase (Primer testing)

PCR was conducted in 0.2 ml thin-walled tubes in a MJ Research Minicycler (MJ Research Inc.) with an internal temperature probe. Taq DNA polymerase and 10× reaction buffer [100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton X-100] were from Promega. The reaction mixture, in a total volume of 10 µl, contained 0.5U Taq DNA polymerase, 1× reaction buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer (GSTreverse and SK primer, **Appendix B**) and 0.5 to 10 ng p*GST1*-BluescriptSK$^-$ plasmid DNA as template. The reaction volume was made up to 10 µl with sterile dH_2O and overlaid with mineral oil to prevent evaporation.

The PCR cycling conditions included an initial denaturation step of 94°C for 2 minutes. This was followed by 35 cycles of denaturation at 94°C for 90 s, annealing at 45°C for 90 s and extension at 72°C for 90 s. A final extension step of 7 min at 72°C was included.

3.2.2.2 PCR amplification of the *gst1* promoter using the Expand Long template PCR system

The Expand Long template PCR system was used to amplify the *gst1* promoter for subcloning. The reaction mixture, in a total volume of 50 µl, contained 1× Expand buffer 1 (with a final concentration of 1.75 mM $MgCl_2$), 1.5 µl Expand Long template DNA polymerase mix, 200 µM of each dNTP, 0.5 µM of each primer (GSTreverse and SK primer, **Appendix B**) and 10 ng p*GST1*-BluescriptSK$^-$ plasmid DNA as template. The reaction was duplicated to yield enough PCR products for subsequent cloning. The buffer and polymerase was added to the rest of the components just before the start of the PCR to minimise non-specific amplification. The reaction mixtures were overlaid with mineral oil to prevent evaporation. The same PCR cycling conditions were used as with the Taq DNA polymerase PCR.

3.2.3 Recovery of DNA fragments from agarose gels

DNA fragments from PCR or restriction digestions were separated by electrophoresis through a 1% (w/v) agarose/ 0.5× TAE (pH 8.0) gel containing 0.06 µg/ml ethidium bromide. The DNA was visualised under ultraviolet light and the fragment of interest excised from the gel. The weight of the gel slice was determined and the DNA eluted from the gel using the QIAquick gel extraction kit. The concentration of the eluted fragment was determined by agarose gel electrophoresis alongside a lambda DNA standard series ranging from 5 to 80 ng, and comparing the intensities of the bands to the standards.

3.2.4 pMOSBlue blunt-end ligation

The *gst1* promoter PCR product, that was extracted from the agarose gel, was cloned into the blunt-ended vector pMOSBlue. Ligation reactions were set up as set out in the pMOSBlue blunt-ended cloning kit instruction manual (AEC-Amersham, Little Chalfont, UK). The PCR product was first blunted and phosphorylated in a phosphokinase (pk) reaction containing 1× pk buffer, 5 mM DTT, 1 µl pk enzyme mix, 40 ng PCR product and dH_2O to a final volume of 10 µl. This reaction was incubated at 22°C for 30 min after which the enzymes were heat inactivated at 75°C for 10 min. Complete inactivation of the kinase enzyme is essential to avoid vector phosphorylation, which results in high vector religated background. The reaction was chilled on ice for 2 min to avoid heat inactivation of the ligase enzyme used in the subsequent step. The product of the kinase reaction was then ligated into the blunt dephosphorylated pMOSBlue vector. Ligation was set at a 1:2.5 vector-to-insert ratio. Fifty nanogram pMOSBlue vector (kit) and 1 µl T4 DNA ligase (4 Weiss units) was added to the 10 µl pk product and incubated at 22°C overnight.

3.2.5 Ligation

Vector and insert fragments were recovered from agarose gels and their concentrations determined as described before. Ligation was set at various insert-to-vector ratios and vector DNA quantities. Ligation reactions were prepared containing the appropriate quantities of vector and insert, 1× ligase buffer [66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM ATP, pH 7.5], an additional 5 mM dATP and 5U T4 DNA ligase. The reaction volume was made up to 10 µl with sterile dH_2O. A vector-religated control was included in which insert was omitted to test the ability of vector to re-ligate. The ligation reactions were incubated at 16°C overnight after which they were stored at -20°C until transformation.

3.2.6 Preparation of competent *E. coli* DH5α cells

Competent *E. coli* DH5α cells were prepared using a method involving $CaCl_2$ and $MnCl_2$. The genetic background of these cells is *supE44* Δ(*lac*U169 (Φ80d*lac*ZΔM15)*hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 (Hanahan, 1983).

A single colony of DH5α *E. coli* cells was inoculated into 25 ml of 2× LB medium (containing 0.2% sterile glucose). It was grown overnight at 37°C with shaking. A hundred millilitres of preheated 2× LB medium was inoculated with 1 ml of overnight culture, and grown for 1 hour at 37°C with shaking. One millilitre of sterile 2 M $MgCl_2$ was added and the culture incubated for another 35 minutes at 37°C with shaking. The culture was quick cooled on ice-water, and left on ice for 1 h 30 min. The cells were pelleted in 40 ml sterile centrifuge tubes by centrifuging at 2000×g (Beckman rotor JA-20) for 5 min at 4°C. The supernatant was poured off and the pellets resuspended in 50 ml of ice-cold Ca^{2+} /Mn^{2+} solution (40 mM NaAc, 100 mM $CaCl_2$, 70 mM $MnCl_2.4H_2O$; pH 5.5 and filter-sterilised). The suspension was left on ice for 1 h 30 min, after which the tubes were centrifuged at 700×g (Beckman rotor JA-20) for 5 min at 4°C. The cell pellets were resuspended in 3 ml ice-cold Ca^{2+} /Mn^{2+} solution containing 15% (v/v) sterile glycerol. The cells were kept on ice at all times. The resuspended cells were pooled and aliquotted in 100 µl quantities into pre-chilled sterile 1.5 ml Eppendorf tubes. They were frozen in liquid nitrogen and stored at -70°C. The competency of the prepared cells was tested by transformation with 1 ng pBluescript SK^+ plasmid DNA and plating onto LB-Agar plates containing 100 µg/ml ampicillin.

3.2.7 Transformation of ligation reaction into *E. coli* DH5α

Ligated plasmids were transformed into competent *E. coli* DH5α cells, prepared by the Ca^{2+} /Mn^{2+} method. The 10 µl ligation mixture was added to 100 µl competent DH5α cells and incubated on ice for 30 min. The cells were heat-shocked at 37°C for 1 minute and incubated on ice for 5 minutes. One millilitre of LB medium was added to the cells, after which they were incubated at 37°C for 1 hour with shaking. The cells were pelleted by centrifugation at 700×g for 6 min, and 600 µl of the supernatant was discarded. The cells were resuspended in the remaining supernatant and plated out in two 250 µl aliquots onto LB agar plates containing 100 µg/ml of the appropriate antibiotic. Ampicillin was used for pMOSBlue constructs and kanamycin for pCAMBIA2300 constructs. For blue/white selection, the agar plate was spread with 35 µl of 50 mg/ml X-gal in DMF and left to dry. The agar plates were incubated inverted at 37°C overnight. A positive transformation control in which 100 µl competent cells were transformed with 1 ng of pBluescript SK^+ or 50 ng pCAMBIA2300 plasmid DNA was included to determine the transformation efficiency of the competent cells.

3.2.8 Restriction enzyme digestion

3.2.8.1 Screening of putative transformants by restriction enzyme digestion

Mini preparations of plasmid DNA were performed and analysed by restriction enzyme digestions, as described by Sambrook *et al.* (1989). Plasmid DNA was digested overnight at 37°C in 10 µl reactions containing the appropriate restriction enzyme buffer. Restriction digestion products were analysed by agarose electrophoresis on a 1% (w/v) agarose/ 0.5× TAE (pH 8.0) gel containing 0.06 µg/ml ethidium bromide.

3.2.8.2 Preparation of insert and vector fragments by restriction enzyme digestion

Large-scale restriction enzyme digestions were performed to prepare vector and insert fragments for ligation. Reactions contained the applicable plasmid DNA, the appropriate 1× restriction enzyme buffer and typically 40U of restriction enzyme in a final volume of 50 µl. For the *Kpn*I restriction enzyme 0.01% BSA was included in the reaction. The reaction mixture was incubated at 37°C overnight, after which fragments were separated by electrophoresis on a 1% agarose gel. The applicable fragment was recovered from the gel as described before.

3.2.8.3 Preparation of the apple *pgip1* cassette fragment by partial restriction enzyme digestion

The apple *pgip1* cassette (TEV leader, apple *pgip1* gene and CaMV 35S terminator) was excised from a previous construct, pAppRTL2 (**Appendix C**: Arendse and Berger, unpublished), using *Xho*I and *Pst*I restriction enzyme digestion. The cassette contains an internal *Pst*I site, so to release the full-length cassette (1379 bp), a partial digestion had to be performed after complete digestion by *Xho*I has taken place.

3.2.8.3.1 Complete *Xho*I restriction digestion

Eight microgram pAppRTL2 plasmid DNA was completely digested with *Xho*I. Four replicate reactions were set up to generate enough linearised plasmid for subsequent partial *Pst*I digestions and cloning. After overnight digestion, the reactions were pooled and the linearised vector precipitated. This was done to separate the DNA from the restriction digestion buffer that will interfere with the subsequent partial digests. Precipitation was performed with $1/10^{th}$ volume 3 M NaAc, pH 5.5 and 2.5 volumes 100% ethanol. The sample was incubated at -20°C for 1 hour, after which the DNA was pelleted by centrifugation at 6500×g for 30 minutes at 4°C. The pellet was washed with 70% ethanol, air-dried and dissolved in an appropriate volume of 10 mM Tris (pH 8.5) to give an expected concentration of 450 ng/µl (if 100% recovery after precipitation is assumed).

3.2.8.3.2 Partial *Pst*I restriction digestion

Partial digests were carried out on the basis that limiting the concentration of Mg^{2+} in the reaction buffer can control restriction digestions. The $MgCl_2$ concentration was optimised to determine which concentration gave the highest yield of the 1379 bp cassette. Restriction digestion buffers were prepared which varied only in their $MgCl_2$ concentration [50 mM Tris-HCl, 100 mM NaCl, pH 7.5 with 0, 1 or 10 mM $MgCl_2$]. Four hundred and fifty nanogram samples of *Xho*I-linearised pAppRTL2 plasmid were digested with 0.5U *Pst*I in restriction buffers containing the varying concentrations of $MgCl_2$. The reactions were incubated at 37°C for varying amounts of time (20, 40 and 60 min).

The 450 ng digestion reactions were scaled up to 5.5 µg of linearised pAppRTL2 plasmid for cloning. It was digested with 5.5U *Pst*I in the appropriate buffer (of which the $MgCl_2$ concentration has been experimentally determined to be 1 mM $MgCl_2$) for 20 min at 37°C. The partial digestion products were separated on a 1% agarose gel until the fragments were sufficiently separated. The 1379 bp fragment was recovered from the gel.

3.2.9 Nucleotide sequencing

Plasmid DNA was isolated using the Qiagen Midi plasmid purification kit or Qiafilter miniprep kit. The inserts in the recombinant plasmids were subjected to nucleotide sequencing at the University of Stellenbosch, Department of Genetics. The fragment is amplified linearly in the presence of fluorescently labelled dideoxynucleotides (ddNTPs) (Old and Primrose, 1994). It is based on Sanger's method of sequencing, in which a single-strand primer anneals to a single-strand DNA template, a polymerase synthesises the complementary DNA, and at random positions a ddNTP is incorporated to result in the termination of chain lengthening. A thermostable DNA polymerase isolated from *Thermus aquaticus* is used since it lacks 5`-3` nuclease activity, has a reduced discrimination against fluorescently labelled ddNTPs, and permits a longer length of read. Fragments of every possible length are generated, each ending at the nucleotide incorporated as the ddNTP and labelled by one of the four fluorescent dyes. Each type of ddNTP is labelled with a different coloured dye, and the whole enzymatic reaction is done in the same tube. The labelled extension products are detected in a single lane during electrophoresis on a denaturing polyacrylamide gel, based on the different fluorescence emission properties of the four dyes. The sequence data is captured during the gel electrophoresis run by a focused laser beam. The individual migration fragments that pass a certain point fluoresce at different wavelengths for each of the four dyes. The information is stored electronically, and the DNA sequence output is presented in the form of a dye-specific intensity profile. The nucleotide sequences of the inserts of the recombinant constructs can be inferred from the intensity profiles. Sequence analysis and alignments were done using the computer software *Genepro Version 6.1* (Riverside Scientific Enterprises).

3.3 Results

3.3.1 Subcloning of the *gst1* promoter into pMOSBlue

This section describes the isolation of the appropriate part of the *gst1* promoter and subcloning into pMOSBlue. It is followed by the section in which the *gst1* promoter is subcloned into the plant transformation vector pCAMBIA2300.

3.3.1.1 Primer design and PCR of the *gst1* promoter

The nucleotide sequence information of the *gst1* promoter cloned into pBluescript SK$^-$ (acquired from G. Loake, University of Edinburgh) was available and confirmed by sequencing. The insert cloned into pBluescript SK$^-$ had a *Kpn*I site at the 5' end and an *Eco*RI site at the 3' end. The insert was sequenced from both sides using the T7 and T3 primers (see **Appendix B**). The *gst1* promoter is 909 bp in length. The nucleotide sequence of a section of p*GST1*-BluescriptSK$^-$ is presented in Figure 3.1.

This known sequence enabled the design of a primer complementary to a sequence at the 3' end of the *gst1* promoter, immediately upstream of the ATG start codon of the *gst1* gene. This primer was called GSTreverse, it was designed using the computer program *Primer Designer Version 3.0* (Scientific and Educational Software) and synthesised by Genosys Products. Characteristics of the primer are shown in **Appendix B.** The restriction enzyme sites *Sal*I and *Pst*I was incorporated to the 5' end of the primer to facilitate subcloning of the *gst1* promoter and subsequent cloning of the apple *pgip1* cassette downstream of the *gst1* promoter. The annealing sites of the GSTreverse primer and other primers are indicated in Figure 3.1.

To perform the polymerase chain reaction, a second primer at the 5' end of the *gst1* promoter was needed. Because the *gst1* promoter fragment in pBluescript SK$^-$ is flanked on the 5' end with a *Kpn*I restriction site, it can be used later in the subcloning of the *gst1* promoter PCR product. Therefore, a sequence specific primer was not necessary and a pBluescript SK$^-$ vector specific primer was used as the 5' primer of the primer-pair. Either the T3 or SK primer could have been used in combination with GSTreverse, but the latter was chosen. An amplification product of 1008 bp was expected.

```
                T3  primer                                  SacI                    NotI
   1  GCTCGAAATT AACCCTCACT AAAGGGAACA AAAGCTGGAG CTCCACCGCG GTGGCGGCCG

       SK primer     BamHI  SmaI          EcoRI EcoRV          ClaI    SalI
  61  CTCTAGAACT AGTGGATCCC CCGGGCTGCA GGAATTCGAT ATCAAGCTTA TCGATACCGT

      SalI                  KpnI
 121  CGACCTCGAG GGGGGGCCCG GTACCATAAG AAGAAGAATT AATCTTATAA TCTTGTGATG
 181  TACTTTCGGT GATTTCTTTA AGTTTGATGT TATGAATAAA AGGCAAAGCT TTTGCAAAAA
 241  TCACTCTTTT TTTTGCCATA ATGATTTCAA AATTCCAAAG AATAATATAT ACTTCAATAT
 301  ACATGTCACA AGATTAAATG TCAAAGTTGT TTATAATGAG CATTTTTGTG GATGTGGAAA
 361  ATGTGAAAAT TACTCTGTTC CTTTGAATGT TTCTATACGA AATTATAGTT AGGATTTAGT
 421  AATGTATTTC TCCATAATTA TGCTAAATTT AGTTAGTTAC TTCAATATGA TTAAATACTT
 481  TATTGACCCC AAATTTGTAA TTGTACCAGA TTGTCAAAAG TGTGAAACCA AATTTTCTTT
 541  TCTTTTGATA TTGTTTTGTT TCTTATTATC TCTCGTTCTA TCGAATGATC TAACTCGAGC
 601  ATCCAACGAT CTAACTCGAG CATCTAACGA TCCACGTGGA CCCAACAACG TCGGTCAGAG
 661  TTTGACTAGT AGATGAAGGA CTATTCTTGT GGTCGTTGTC ACGCGGTGGC TGATATTTTC
 721  TCTTATTTTT ATTTTATTTT AACTATTTTT TACGTTATAT TTAAGTCTTG AACCAATAGA
 781  AACGACGAAT CATACATTAC TCAGCTTGAC TTTGAAATAA TCCTATAACA AAAGAGCATT
 841  CCAAGAATTT TATCCAAAAA ACAAAATAAA AAAGAGTATT CAAGCTTGGT GGCGCCGTTT
 901  GTTTTTGGTT TATTCACTAA AGTTACTCTG TTTTAGTTGT ATAAATACAC ACTCCCATTT
 961  GTGTATTTCT TTTCATCAAT CACAAAGATC TCTCTACTTC AATAAATCTC CACCTTACTT
 021  TAAGAACAAG AAAAACACAG TATTAACAAA TGGCAGGAAT CAAAGTTTTC GGTCACCCAG
                GSTreverse  primer
                                                          SalI    EcoRI
1081  CTTCCACAGC CACTAGAAGA GTTCTCATCG CTCTTCACGA GAAGAATGTC GACTTTGAAT

        EcoRV        ClaI      SalI                  KpnI
1141  TCGATATCAA GCTTATCGAT ACCGTCGACC TCGAGGGGGG GCCCGGTACC CAATTCGCCC
1201  TATAGTGAGT CGTATTACAA TTCACTGGCC GTCGTTTTAC AACGTCGTGA CTGGGAAAAC
1261  CCTGGCGTTA CCCAACTTAA TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCGTAAT
        T7 primer
```

Figure 3.1 **Nucleotide sequence of a section of p*GST1*-BluescriptSK⁻ showing restriction enzyme sites and primer annealing sites.** The TATA-box of the *gst1* promoter is indicated in blue, restriction enzyme sites in green and red, the initiator codon of the *gst1* gene is boxed and primer-annealing sequences are underlined.

3.3.1.2 PCR amplification of the *gst1* promoter using Taq DNA polymerase (Primer testing)

The PCR using GSTreverse and SK primers was first optimised with Taq DNA polymerase (Promega) before using a proofreading DNA polymerase enzyme to generate high fidelity products for cloning. The annealing temperature for efficient amplification was determined experimentally to be 45°C (data not shown). PCR was performed in which all conditions were kept constant, except the amount of p*GST1*-BluescriptSK⁻ template was varied. The *gst1* promoter is 909 bp in length (Grant *et al.*, 2000), but with the 19 bp 5'-extension incorporated by the GSTreverse primer, and the 80 bp upstream of the promoter insert amplified by the SK primer, a product of 1008 bp was expected. An amplification

product of approximately 900 bp was obtained for all reactions (Figure 3.2, lanes 1 to 5). The fragment was smaller than expected but nucleotide sequencing later showed it to be correct. A possible reason for this is overloading of the gel (see Discussion). Figure 3.2 shows that a minimum of 0.5 ng template plasmid DNA is sufficient for amplification of the *gst1* promoter using the SK and GSTreverse primers (Figure 3.2, lane 1). As expected, the negative control, containing no template plasmid DNA, did not give any amplification products (Figure 3.2, lane 6).

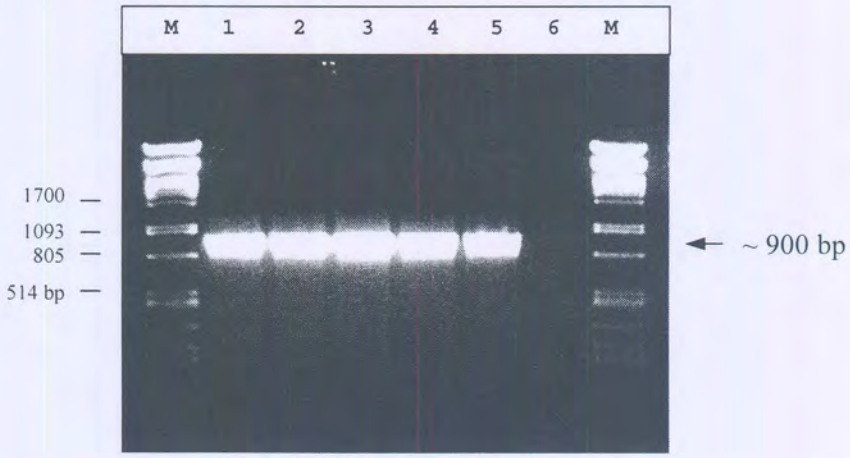


Figure 3.2 **PCR of the *gst1* promoter using Taq DNA polymerase.** PCR was performed with the SK and GSTreverse primers containing varying amounts p*GST1*-BluescriptSK⁻ plasmid as template. M: λDNA/ *Pst*I marker; lanes 1 to 5: 0.5, 1, 2, 5 and 10 ng plasmid as template, respectively; lane 6: negative control containing dH_2O.

3.3.1.3 PCR amplification of the *gst1* promoter using the Expand Long template PCR system

The Expand Long template PCR system was used to accurately amplify the *gst1* promoter for subcloning. It is a mixture of thermostable Taq DNA polymerase and a proofreading polymerase for high fidelity products. The proofreading polymerase is Tgo DNA polymerase, isolated from *Thermococcus gorgonarius*. It is a highly processive 5'-3' DNA polymerase, thermostable and has a better proofreading activity and a higher specificity than Pwo DNA Polymerase. It generates PCR fragments with a mixture of blunt and 3' single A overhangs. PCR using this system also yielded an amplification product of approximately 900 bp (Figure 3.3, lanes 1 and 2).

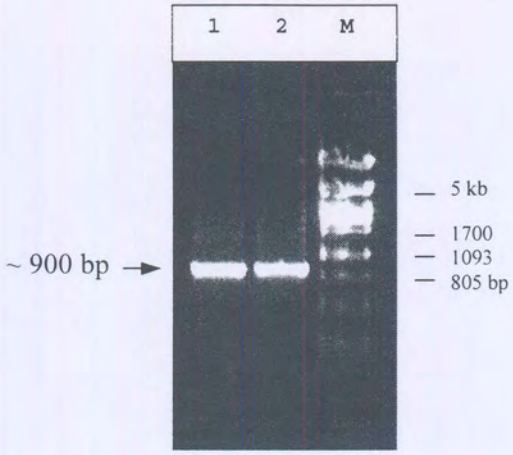


Figure 3.3 **PCR of the *gst1* promoter with the Expand Long template PCR system.** PCR was performed with the SK and GSTreverse primers containing 10 ng p*GST1*-BluescriptSK⁻ plasmid as template. Lanes 1 and 2: 2 µl samples of duplicate 50 µl Expand Long template PCR reactions: M: λDNA/ *Pst*I marker.

3.3.1.4 Recovery of the *gst1* promoter PCR product from the agarose gel

PCR products of the Expand Long template PCR system were pooled and purified from the contaminating PCR components by separating it with agarose gel electrophoresis and eluting the fragment from the gel. The concentration of the eluted PCR fragment was determined to be 40 ng/µl. Figure 3.4 shows the eluted PCR fragment. A faint band of a smaller size fragment (~700 bp) was observed together with the purified PCR fragment of ~900 bp.

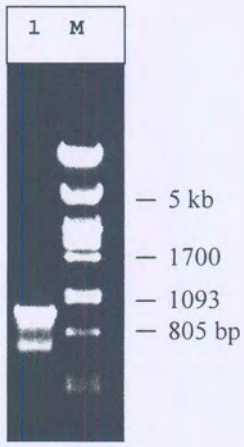


Figure 3.4 ***gst1* promoter PCR fragment eluted from an agarose gel.** Lane 1: 2 µl sample of the 30 µl eluted fragment: M: λDNA/ *Pst*I marker.

3.3.1.5 Ligation of the *gst1* promoter PCR fragment into pMOSBlue

Since ligation of the *gst1* promoter PCR product into the T-A cloning vector pGEM T-Easy (Promega) was not successful (results not shown), it was ligated into the blunt-ended cloning vector pMOSBlue (AEC-Amersham, Little Chalfont, UK).

3.3.1.6 Screening of transformants by miniprep and restriction enzyme digestions

Putative positive clones were identified using blue/white colony selection based on the α-complementation system. Ten white colonies were obtained when the ligation products of the *gst1* promoter PCR fragment and the pMOSBlue vector were transformed into competent DH5α *E. coli*. They were screened for the presence of recombinant constructs containing the *gst1* promoter PCR product insert by extracting plasmid DNA and digesting 3 µl with the restriction enzymes *Kpn*I and *Pst*I.

Of the ten colonies, only three contained putative recombinant plasmids with inserts that could be excised with *Kpn*I and *Pst*I (Figure 3.5, lanes 1, 6 and 7). Since *Kpn*I and *Pst*I sites flank the blunt *Eco*RV cloning site of the pMOSBlue vector, even an insert that is not the *gst1* promoter PCR product will be excised. The expected size of the correct insert excised with *Kpn*I and *Pst*I is 919 bp. While the insert from putative recombinant plasmid clone 1 was too large to be correct (larger than 1200 bp; Figure 3.5, lane 1), the other two clones contained inserts of approximately the correct size. These were clones 6 and 9, with inserts approximately 900 bp in size (Figure 3.5, lanes 6 and 7; and Figure 3.6, lanes 3 and 4).

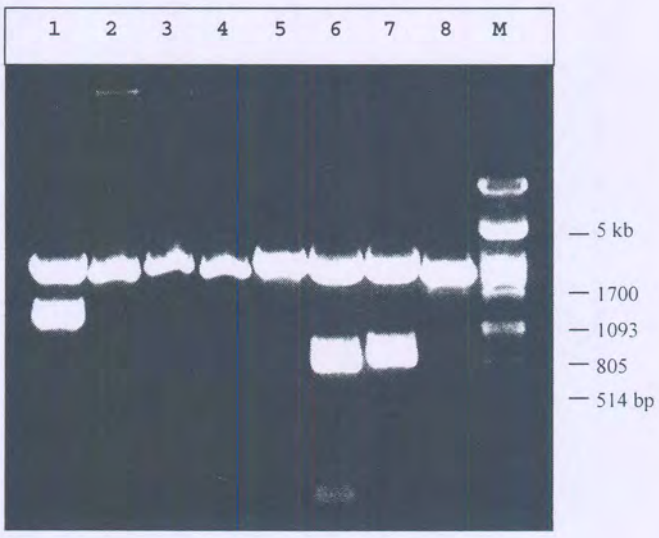


Figure 3.5 **Restriction enzyme screening of eight putative recombinant *GST1*prom-pMOSBlue clones.** Lanes 1 to 8: miniprep plasmid DNA isolated from *E. coli* colonies transformed with putative *GST1*prom-pMOSBlue clones 1, 2, 3, 4, 5, 6, 9 and 11, respectively, and digested with *Kpn*I and *Pst*I: M: λDNA/ *Pst*I marker.

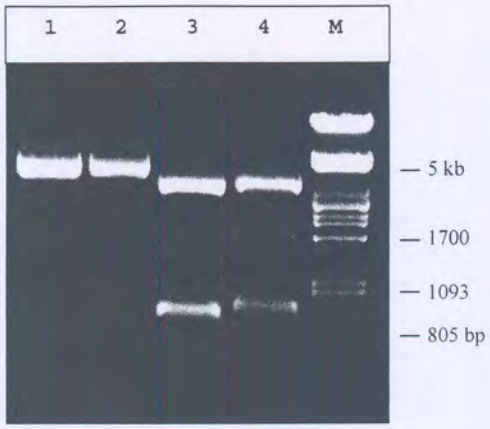


Figure 3.6 **Restriction digestion of putative recombinant *GST1*prom-pMOSBlue clones 6 and 9 plasmid DNA.** Lanes 1 and 2: undigested plasmid DNA from clone 6 and 9, respectively; lane 3 and 4: plasmid DNA of clone 6 and 9, respectively, digested with *Kpn*I and *Pst*I: M: λDNA/ *Pst*I marker.

3.3.1.7 Nucleotide sequencing of *GST1*prom-pMOSBlue clones 6 and 9

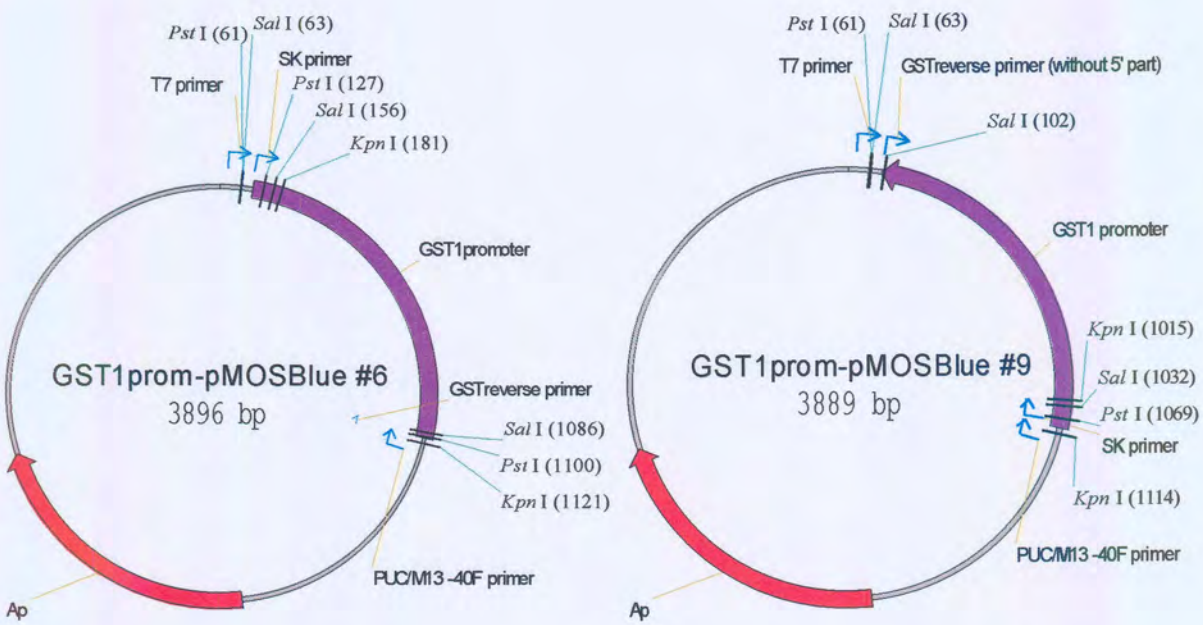
Colonies 6 and 9 were deduced to be likely candidates to contain recombinant *GST1*prom-pMOSBlue constructs. Recombinant plasmid DNA was sent for nucleotide sequencing of the insert using the T7 and PUC / M13-40F primers (**Appendix B**). From the agarose gel results (Figure 3.5 and Figure 3.6), the insert from clone 9 seemed to be slightly larger than the insert from clone 6. The nucleotide sequencing results explained why.

Figure 3.7 is a graphical representation of *GST1*prom-pMOSBlue clones 6 and 9 recombinant plasmids. Nucleotide sequencing of the inserts revealed that in clone 6 the complete GSTreverse primer sequence had been incorporated at the 3` end of the PCR fragment, resulting in a *Pst*I recognition site immediately downstream of the *gst1* promoter. The binding site of the GSTreverse primer is located at the 3` end of the *gst1* promoter, immediately upstream of the ATG start codon of the *A. thaliana gst1* gene. The rest of the *gst1* promoter sequence, up to the binding site of the GSTreverse primer, was identical to the original sequence in p*GST1*-BluescriptSK-. The *gst1* promoter was cloned into pMOSBlue with the same orientation as the ampicillin resistance gene (Ap). The recombinant *GST1*prom-pMOSBlue clone 6 plasmid was chosen for further experiments.

From the nucleotide sequencing results, it was learned that the 5` part (5`-AAACTGCA-3`) of the GSTreverse primer was absent in the sequence of the insert in clone 9. This resulted in the loss of the *Pst*I site incorporated with the GSTreverse primer to the 3` end of the *gst1* promoter. The insert is still excised from clone 9 since *Kpn*I and *Pst*I sites flank the blunt *Eco*RV cloning site in the multiple cloning site of the pMOSBlue vector. *Kpn*I and *Pst*I digestion of clone 9 therefore resulted in a fragment 35 bp larger than expected (954 bp instead of 919 bp). Thirty-five basepairs is the distance

between the *Pst*I site found in the MCS of pMOSBlue and where the *Pst*I site would have been at the 3' end of the *gst1* promoter if it had been incorporated with the GSTreverse primer. The loss of the *Pst*I site of the GSTreverse primer explains the restriction fragment of clone 9 being larger than that of clone 6, as was observed in Figure 3.5 and Figure 3.6.

In clone 9 the *gst1* promoter was cloned into pMOSBlue with the opposite orientation as the ampicillin resistance gene (Ap). Clone 9 also contained an insertion mutation of an Adenine between the 5' end of the *gst1* promoter PCR product and the *Eco*RV blunt cloning site. This may be the result of the Taq DNA polymerase in the Expand enzyme mix, adding single 3' A overhangs to the PCR product during amplification.



***GST1*prom-pMOSBlue #6** ***GST1*prom-pMOSBlue #9**

Figure 3.7 **Graphical representation of *GST1*prom-pMOSBlue clones 6 and 9 recombinant plasmids.** The results obtained for the nucleotide sequencing of the inserts, as well as primer binding sites and selected restriction enzyme recognition sites, are indicated on the maps.

3.3.2 Subcloning of the *gst1* promoter into pCAMBIA2300

*GST1*prom-pMOSBlue #6 was chosen and plasmid DNA isolated using the Qiagen Midi plasmid purification kit for large-scale preparations of plasmid DNA. Plasmid DNA of pCAMBIA2300 was prepared in the same way. Both the insert (*gst1* promoter) and vector (pCAMBIA2300) were prepared with *Kpn*I and *Pst*I restriction enzymes for cloning.

3.3.2.1 Preparation of the *gst1* promoter pMOSBlue insert for subcloning into pCAMBIA2300

Isolation of the *gst1* promoter fragment after *Kpn*I/ *Pst*I digestion of *GST1*prom-pMOSBlue #6 yielded a fragment of ~900 bp (Figure 3.8, lane 1). This correlates well with the expected size of 919 bp. The concentration of the eluted fragment was determined to be ~30 ng/µl.

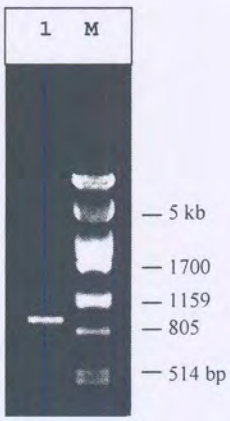


Figure 3.8 ***Kpn*I/ *Pst*I digested *gst1* promoter restriction fragment eluted from an agarose gel.** Lane 1: 2 µl sample of the 30 µl eluted fragment; M: λDNA/ *Pst*I marker.

3.3.2.2 Preparation of pCAMBIA2300

pCAMBIA2300 is a binary plant transformation vector that contains minimal heterologous sequences for plant transformation and selection of transformants (www.cambia.org). A map of pCAMBIA2300 is presented in **Appendix C**. Between the T-borders, it has the CaMV 35S-driven and terminated plant selection neomycin phosphotransferase II (*nptII*) gene that encodes resistance to kanamycin. Indicated also is the lacZα gene, that is interrupted when the insert is cloned into the pUC18 polylinker. This allows for blue/white screening of clones in *E. coli* cells. Outside the T-borders, it has the bacterial kanamycin resistance marker for selection in *E. coli* and *Agrobacterium* strains. It has the wide-host-range origin of replication from the *Pseudomonas* plasmid pVS1, which is very stable in the absence of selection. It also has the pBR322 origin of replication to allow high-yielding DNA preparations in *E. coli*.

Five microgram of pCAMBIA2300 plasmid DNA was digested with *Kpn*I and *Pst*I. The fragment corresponding to the linearised vector (8715 bp) was recovered from the gel. The concentration of the eluted fragment was determined to be ~120 ng/µl.

3.3.2.3 Ligation of the *gst1* promoter into pCAMBIA2300

The 919 bp *gst1* promoter fragment was ligated to the digested pCAMBIA2300 vector in ligation reactions set at various insert-to-vector ratios and vector DNA quantities. The ligation reactions were

transformed into competent *E. coli* DH5α. The cells were plated out onto LB agar plates containing 100 µg/ml kanamycin and that were spread with X-gal.

After ligation and transformation, eight colonies transformed with putative recombinant *GST1*prom-pCAMBIA2300 constructs were identified. Blue/white colony selection, based on the α-complementation system, was used. Two white vector-religated colonies were obtained. No blue colonies were obtained except in the positive transformation control containing pCAMBIA2300 plasmid.

3.3.2.4 Screening of transformants by miniprep and restriction enzyme digestions

Qiafilter miniprep plasmid DNA from eight putative *GST1*prom-pCAMBIA2300 transformed colonies and one vector-religated transformed colony was digested with *Kpn*I and *Pst*I to verify the presence of the *gst1* promoter insert. A fragment of approximately 900 bp was excised with *Kpn*I and *Pst*I from clone 3, indicating it possibly being a recombinant (Figure 3.9, lane 7).

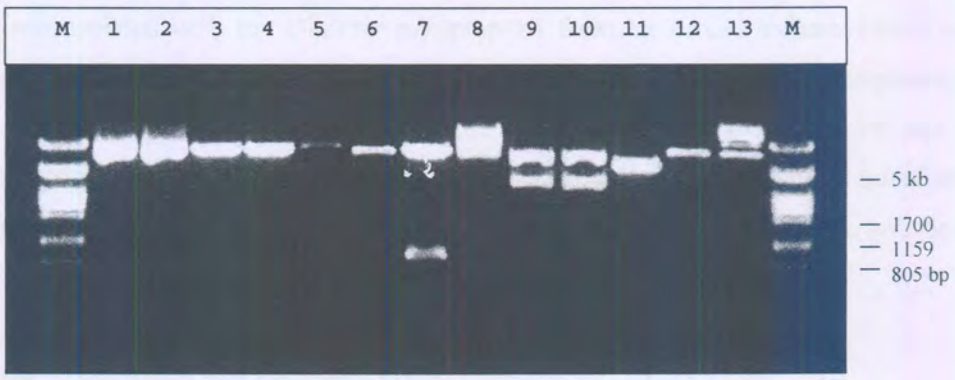


Figure 3.9 **Restriction enzyme screening of eight putative recombinant *GST1*prom-pCAMBIA2300 transformed colonies.** M: λDNA/ *Pst*I marker; lanes 1 and 2: undigested pCAMBIA2300 plasmid DNA; lanes 3 and 4: *Kpn*I/ *Pst*I digested pCAMBIA2300 plasmid DNA; lanes 5 to 12: *Kpn*I and *Pst*I restriction digestion of putative recombinant *GST1*prom-pCAMBIA2300 clones 1 to 8, respectively; lane 13: Religated vector plasmid digested with *Kpn*I and *Pst*I.

*GST1*prom-pCAMBIA2300 #3 plasmid DNA was chosen for sequencing of the insert. When the ligation and transformation was repeated, another eight colonies were obtained. Only one, clone 11, contained an insert that could be excised with *Kpn*I and *Pst*I restriction digestion (Figure 3.10, lane 3). The fragment seemed to be of the correct size, and clone 11 would have served as a backup if the sequence of clone 3 contained errors.

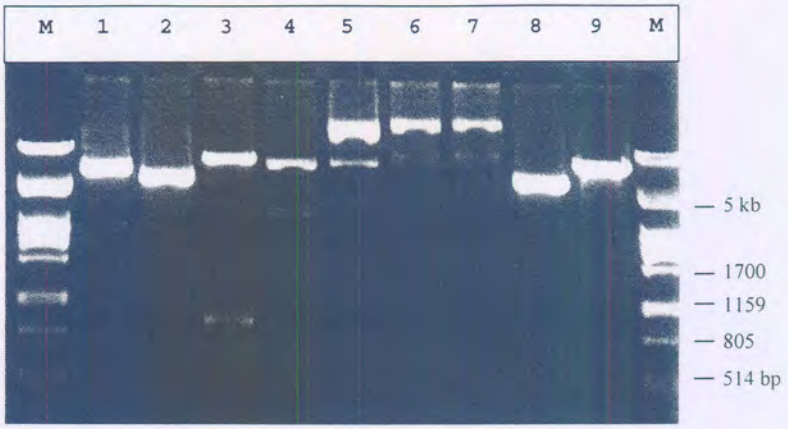


Figure 3.10 **Restriction enzyme screening of putative recombinant *GST1*prom-pCAMBIA2300 clones 9 to 16.** M: λDNA/ *Pst*I marker; lanes 1 to 8: miniprep plasmid DNA isolated from *E. coli* colonies transformed with putative *GST1*prom-pCAMBIA2300 clones 9 to 16, respectively, and digested with *Kpn*I and *Pst*I; lane 9: *Kpn*I/ *Pst*I digested pCAMBIA2300 plasmid DNA.

3.3.2.5 Nucleotide sequencing of *GST1*prom-pCAMBIA2300 #3

Plasmid DNA was isolated with the Qiafilter miniprep kit from an *E. coli* culture containing the *GST1*prom-pCAMBIA2300 #3 construct. The junction points and *gst1* promoter sequences were determined by nucleotide sequencing of the insert. The primers used were PUC / M13 R and PUC / M13-40F. These primer-binding sequences flank the multiple cloning site of the plant transformation vector pCAMBIA2300. Nucleotide sequencing showed a 100% correct sequence of the *gst1* promoter cloned into pCAMBIA2300, with the junction points as expected.

3.3.3 Subcloning of the apple *pgip1* cassette into *GST1*prom-pCAMBIA2300

The previous section described how the *gst1* promoter was cloned into a plant transformation vector to form the construct that is called *GST1*prom-pCAMBIA2300. The aim of this section was to clone the apple *pgip1* gene, in the form of a cassette including also the TEV leader and CaMV 35S terminator, downstream of the *gst1* promoter in pCAMBIA2300. The source of the apple *pgip1* cassette is pAppRTL2 (**Appendix C**), which was previously generated by cloning the apple *pgip1* gene into the pRTL2 vector (Arendse and Berger, unpublished). In this construct, the apple *pgip1* gene is under control of the enhanced CaMV 35S promoter. This vector also provides the TEV leader and CaMV 35S terminator.

3.3.3.1 Preparation of apple *pgip1* cassette with complete *Xho*I and partial *Pst*I restriction digestion

To clone the apple *pgip1* cassette into the engineered *Sal*I and *Pst*I sites of *GST1*prom-pCAMBIA2300 #3, the cassette fragment had to be excised from pAppRTL2 using *Xho*I (5` end) and *Pst*I (3` end)

digestion. *Sal*I and *Xho*I digestion o icky overhangs, thereby facilitating the annealing of the 5' *Xho*I- digested cassette fragment to the *Sal*I-digested vector fragment.

*Pst*I digestion of pAppRTL2, however, posed a problem, since this construct contains three *Pst*I sites (**Appendix C**). Two are located at each end of the cassette and a third site is present within the apple *pgip1* cassette to be subcloned. It lies between the 3' end of the apple *pgip1* gene and the CaMV 35S terminator sequence. Digestion of pAppRTL2 with *Xho*I and *Pst*I would therefore lead to the digestion of the apple *pgip1* cassette into two fragments, 1148 and 231 bp in size. In order to release the full-length cassette (1379 bp), a partial *Pst*I digestion of the plasmid was required, after complete digestion by *Xho*I has taken place. Complete digestion by *Xho*I is necessary to limit the number of fragments that would be generated if *Xho*I digestion, in addition to *Pst*I digestion, was also only partial. The sizes of the expected products are 1379 bp for the full-length cassette, 1148 bp for the truncated cassette and 231 bp for the short 3' piece containing the CaMV 35S terminator.

3.3.3.2 Partial *Pst*I restriction digestion

Partial restriction digests were performed by decreasing the $MgCl_2$ concentration of the restriction buffer. The optimum $MgCl_2$ concentration was determined by preparing buffers that differ only in their $MgCl_2$ concentration, and looking for the greatest yield of the full-length (1379 bp) cassette fragment following restriction enzyme digestion. The digestion times were also varied from 20 to 60 min. Digestion in the presence of buffer H (containing 10 mM $MgCl_2$) and buffer with $1/10^{th}$ the $MgCl_2$ concentration (1 mM) yielded almost similar products for the reactions incubated for 60 and 20 minutes (Figure 3.11, lanes 3, 4, 11 and 12), with 1 mM $MgCl_2$ yielding slightly more of the 1379 bp cassette (Figure 3.11, lanes 4 and 12). Digestion for 40 minutes with 1 mM $MgCl_2$ (Figure 3.11, lane 8) was more complete than at t = 60 min (Figure 3.11, lane 4) and in the presence of 10 mM $MgCl_2$ (Figure 3.11, lanes 3, 7 and 11). This was unexpected, because lanes 3 and 7 were expected to have the most complete digestion. This figure illustrates that all restriction enzymes require Mg^{2+} to function, since without it virtually no digestion took place (Figure 3.11, lanes 5, 6, 9, 10, 13 and 14).

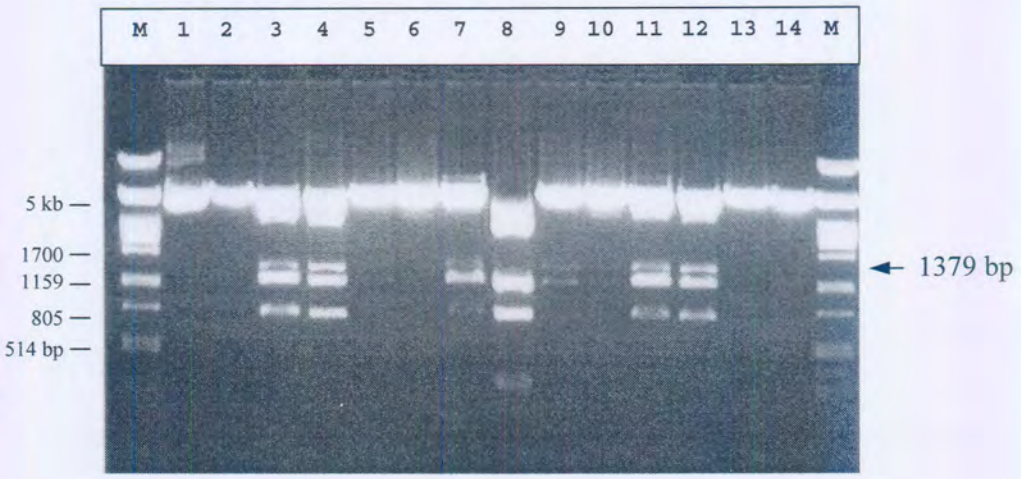


Figure 3.11 **Partial *Pst*I digestion of *Xho*I-linearised pAppRTL2.** *Pst*I digestion of *Xho*I-linearised pAppRTL2 in the presence of different $MgCl_2$ concentrations was performed to determine the optimum $MgCl_2$ concentration where the yield of the full-length cassette fragment is optimal. M: λDNA/ *Pst*I marker; lane 1: undigested pAppRTL2; lane 2: pAppRTL2 linearised with *Xho*I; lanes 3 to 6: 60 minutes digestion with *Pst*I of *Xho*I-linearised pAppRTL2 in the presence of buffer containing 10 mM $MgCl_2$, 1 mM $MgCl_2$, 0 mM $MgCl_2$ and dH_2O, respectively. Lanes 7 to 10 and lanes 11 to 14: 40 min digestion and 20 min digestion in the presence of the same buffers as at 60 min, respectively.

The buffer containing 1 mM $MgCl_2$ was chosen for the subsequent partial digestions. The 20 minute partial digest was scaled up and loaded into a single long well to separate the cassette fragment from the contaminating fragments by agarose gel electrophoresis (Figure 3.12, lane 1).

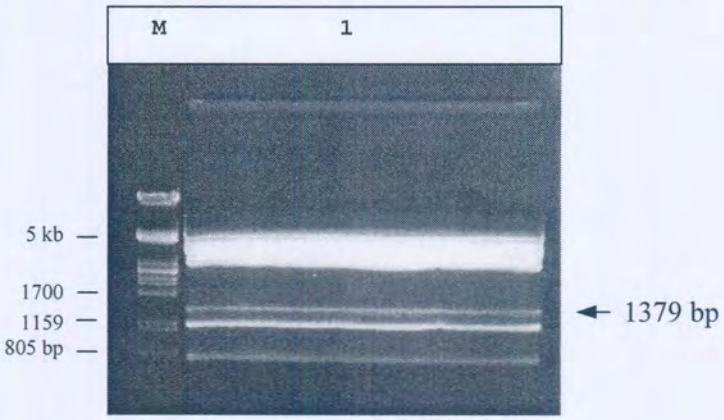


Figure 3.12 **Preparation of the 1379 bp apple *pgip1* cassette insert fragment for cloning.** M: λDNA/ *Pst*I marker, lane 1: Complete *Xho*I-digested, partial *Pst*I-digested pAppRTL2.

The excised 1379 bp apple *pgip1* cassette fragment was contaminated with the shorter 1148 bp cassette fragment (Figure 3.13, lane 5, white arrows). It was co-excised from the gel by accident, but it is unclear why since the two fragments were clearly separated (Figure 3.12, lane 1). Ligation of this

mix of insert fragments would therefore lead to colonies containing the full-length cassette and a shortened cassette, the latter lacking the 3' terminal *Pst*I fragment. Isolation of the 1379 bp fragment was repeated, and again found to be contaminated by the second fragment. The concentrations of the insert fragments recovered from the agarose gel were estimated to be between 15 ng/µl and 30 ng/µl.

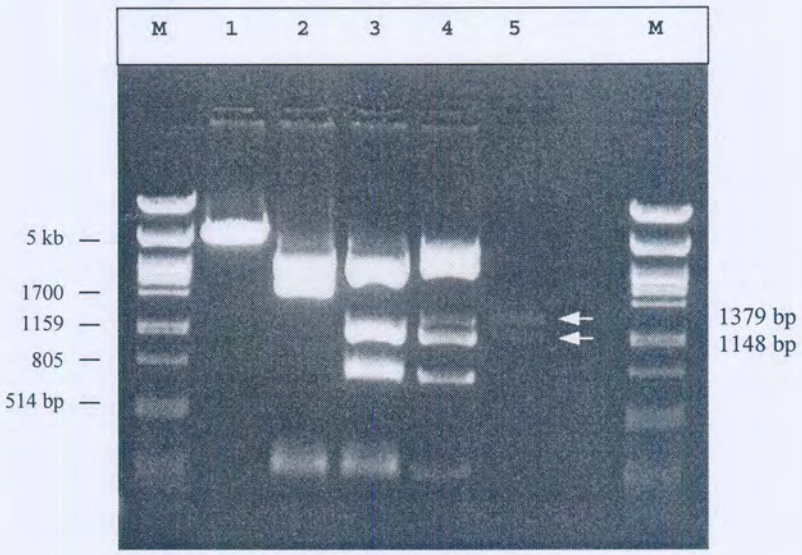


Figure 3.13 **Full and partial digested pAppRTL2 and gel extracted insert fragment containing the contaminating fragment.** M: λDNA/ *Pst*I marker; lanes 1 to 4: pAppRTL2 digested with *Xho*I, *Pst*I, *Xho*I and *Pst*I and *Xho*I & partial *Pst*I, respectively; lane 5: cassette fragment eluted from the gel.

3.3.3.3 Preparation of the *GST1*prom-pCAMBIA2300 #3 vector

The plant transformation vector containing the *gst1* promoter (*GST1*prom-pCAMBIA2300 #3) was prepared with *Sal*I and *Pst*I for cloning of the apple *pgip1* cassette downstream of the *gst1* promoter. Eight microgram *GST1*prom-pCAMBIA2300 #3 plasmid DNA was digested with *Sal*I and *Pst*I restriction enzymes. The fragment corresponding to the linearised vector (9635 bp) was excised and eluted from the gel. The concentration of the eluted fragment was determined to be ~400 ng/µl.

3.3.3.4 Ligation of the apple *pgip1* cassette into *GST1*prom-pCAMBIA2300 #3

The 1379 bp apple *pgip1* cassette fragment (*Xho*I/ *Pst*I digested) was ligated to the *Sal*I/ *Pst*I digested *GST1*prom-pCAMBIA2300 #3 vector in ligation reactions set at various insert-to-vector ratios and vector DNA quantities. Competent *E. coli* DH5α cells were transformed with the ligation reactions as described before. The cells were plated out onto LB agar plates containing 100 µg/ml kanamycin. Several colonies were obtained from two repetitions of ligation and transformation. 24 and 90 colonies, respectively.

3.3.3.5 Screening of putative *GST1*prom-ap*pgip1*-pCAMBIA2300 transformants

Forty-four of the colonies were screened for the presence of recombinant constructs by plasmid DNA isolation and restriction digestion. *Kpn*I and *Pst*I were chosen as restriction enzymes to screen for putative *GST1*prom-ap*pgip1*-pCAMBIA2300 clones, since they can discriminate between positive and negative clones and between full-length and truncated cassette constructs. Three fragments with sizes of 8715, 2053 and 231 bp are expected from positive clones containing the full-length cassette. Fragments corresponding to these sizes were obtained from seven clones (Figure 3.14, lanes 5, 6, 20, 25, 26, 30 and 38). The lanes are numbered the same as the clones. Only two fragments of 8715 and 2053 bp are expected for transformants containing the truncated cassette, lacking the 231 bp fragment. Ten clones that contained the truncated cassette were among the 44 clones screened (Figure 3.14, lanes 4, 24, 28, 29, 31, 32, 39, 40, 41 and 44).

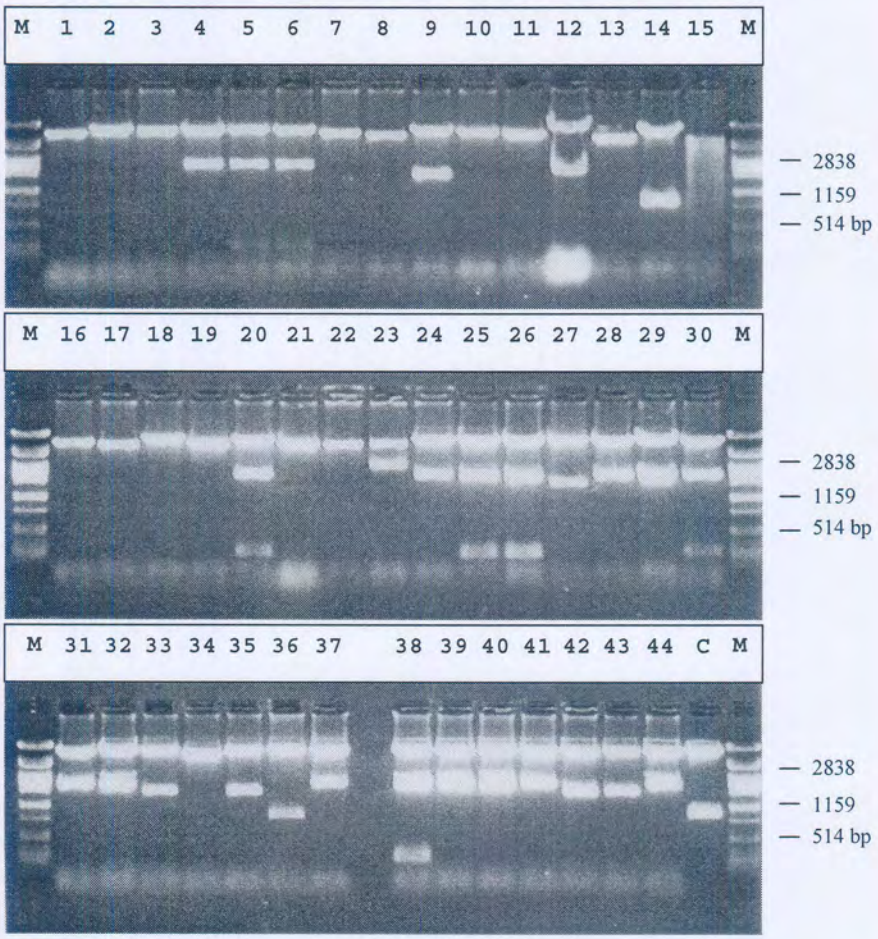


Figure 3.14 ***Kpn*I and *Pst*I digestion of plasmid DNA from 44 putative *GST1*prom-ap*pgip1*-pCAMBIA2300 clones.** M: λDNA/ *Pst*I marker; C: *Kpn*I/ *Pst*I digested *GST1*prom-pCAMBIA2300 #3 (negative control); lanes 1 to 44: lane numbers correspond to the putative recombinant *GST1*prom-ap*pgip1*-pCAMBIA2300 clones digested with *Kpn*I/ *Pst*I.

3.3.3.6 Restriction analysis of seven positive *GST1*prom-ap*pgip1*-pCAMBIA2300 clones

Restriction analysis was performed on plasmid DNA from the seven putative positive clones using the restriction enzymes *Hind*III, and a double digest with *Kpn*I and *Xba*I. The sizes of the expected fragments following *Hind*III digestion are 8789, 1094, 656 and 460 bp for a clone containing the full-length cassette and 8789, 1094, 656 and 229 bp for a truncated cassette. *Kpn*I & *Xba*I digestion liberates fragments with sizes 8936, 1565 and 498 bp from full-length cassette clones and 9203 and 1565 bp from truncated cassette clones. The seven clones showed the expected restriction pattern of a full-length apple *pgip1* cassette cloned downstream of the *gst1* promoter (Figure 3.15 A (*Hind*III digestions) and B (*Kpn*I and *Xba*I digestions), lanes 1 to 7). *Kpn*I and *Xba*I digestion showed conclusively that the 3' terminal *Pst*I fragment of the apple *pgip1* cassette is present in all seven clones, due to the presence of the 498 bp fragment which is not expected from truncated clones.

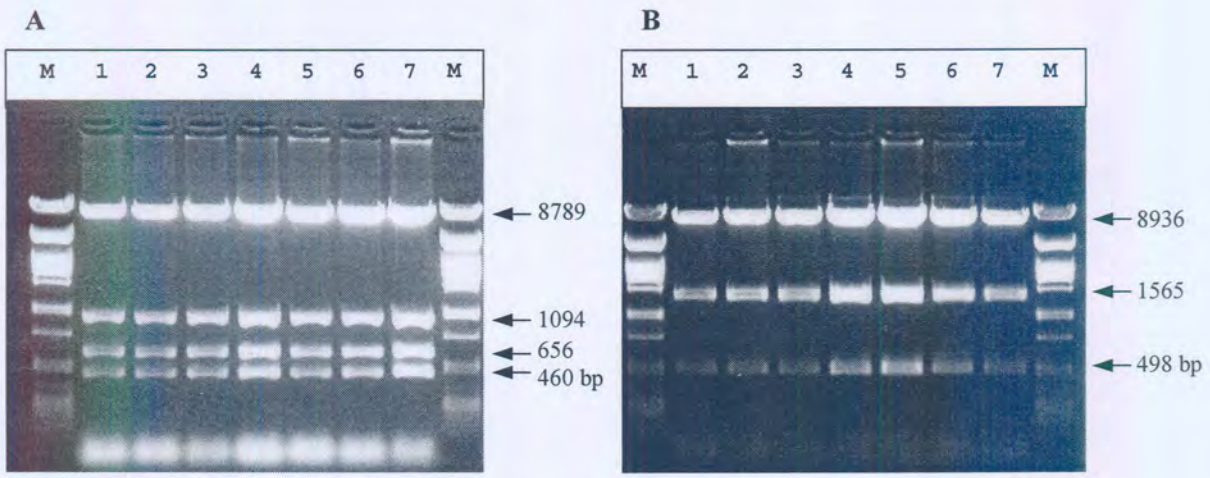


Figure 3.15 **Restriction analysis of seven positive *GST1*prom-ap*pgip1*-pCAMBIA2300 clones.** M: λDNA/ *Pst*I marker; A: lanes 1 to 7: *Hind*III restriction digestion of plasmid DNA from putative *GST1*prom-ap*pgip1*-pCAMBIA2300 clones 5, 6, 20, 25, 26, 30 and 38, respectively. B: lanes 1 to 7: *Kpn*I & *Xba*I digested plasmid DNA from the same clones used in the *Hind*III digests.

3.3.3.7 Nucleotide sequencing of *GST1*prom-ap*pgip1*-pCAMBIA2300

*GST1*prom-ap*pgip1*-pCAMBIA2300 #25 and #30 were selected and sent for nucleotide sequencing of the insert. The primers used for sequencing the junction sites were AP-PGIP-INVR and PUC / M13-40F. AP-PGIP-INVR anneals to a sequence in the middle of the apple *pgip1* gene (Figure 3.16). Using it in the sequencing reaction yielded the sequence upstream of it to the end of the *gst1* promoter. PUC / M13-40F anneals downstream of the CaMV 35S terminator, and gave the sequence up to the middle of the apple *pgip1* gene. Figure 3.16 shows the relative positions and lengths of the sequences obtained for the respective primers used. Sequence analysis showed 100% correct junction points for both clones. The details of the nucleotide sequence between the TATA-box of the *gst1* promoter and

the initiation codon of the apple *pgip1* gene is shown in Figure 3.17. The predicted amino acid sequences are also shown (see Discussion).

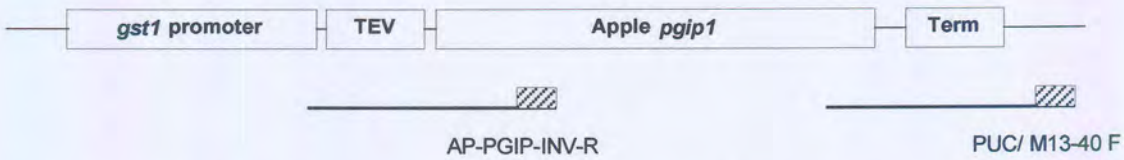


Figure 3.16 **Nucleotide sequencing of the *gst1* promoter - apple *pgip1* cassette inserted into pCAMBIA2300.** Schematic representation of the primer annealing positions and the regions sequenced during nucleotide sequencing of the *gst1* promoter and apple *pgip1* cassette cloned into the plant transformation vector pCAMBIA2300. The hatched boxes represent the primers used for sequencing (AP-PGIP-INVR and PUC / M13-40F) and the lines extending upstream from them represent the relative length of sequencing data obtained. TEV: Tobacco etch virus leader sequence; Term: CaMV 35S terminator.

```
TATAAATACACACTCCCATTTGTGTATTTCTTTTCATCAATCACAAAGATCTCTCTACTT      60
Y  K  Y  T  L  P  F  V  Y  F  F  S  S  I  T  K  I  S  L  L
 I  N  T  H  S  H  L  C  I  S  F  H  Q  S  Q  R  S  L  Y  F
  *  I  H  T  P  I  C  V  F  L  F  I  N  H  K  D  L  S  T  S
↓
CAATAAATCTCCACCTTACTTTAAGAACAAGAAAAACACAGTATTAACAGTCGAGAATTC     120
Q  *  I  S  T  L  L  *  E  Q  E  K  H  S  I  N  S  R  E  F
 N  K  S  P  P  Y  F  K  N  K  K  N  T  V  L  T  V  E  N  S
  I  N  L  H  L  T  L  R  T  R  K  T  Q  Y  *  Q  S  R  I  L

TCAACACAACATATACAAAACAAACGAATCTCAAGCAATCAAGCATTCTACTTCTATTGC     180
S  T  Q  H  I  Q  N  K  R  I  S  S  N  Q  A  F  Y  F  Y  C
 Q  H  N  I  Y  K  T  N  E  S  Q  A  I  K  H  S  T  S  I  A
  N  T  T  Y  T  K  Q  T  N  L  K  Q  S  S  I  L  L  L  L  Q

AGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTCTGAAAATTTTCACCATTTAC     240
S  N  L  N  H  F  F  *  S  K  S  N  F  L  K  I  F  T  I  Y
 A  I  *  I  I  S  F  K  A  K  A  I  F  *  K  F  S  P  F  T
  Q  F  K  S  F  L  L  K  Q  K  Q  F  S  E  N  F  H  H  L  R

GAACGATAGCCATGGAACTCAAG                                          263
E  R  *  P  W  N  S
 N  D  S  H  G  T  Q
  T  I  A  M  E  L  K
```

Figure 3.17 **Nucleotide sequence and predicted amino acid sequence between the TATA box of the *gst1* promoter and the initiator codon of the apple *pgip1* gene in the *GST1*prom-a*ppgip1*-pCAMBIA2300 construct.** The TATA-box of the *gst1* promoter is indicated in blue, the TEV leader sequence is indicated in red, the initiator codon of the apple *pgip1* gene is boxed and the only methionine residue is indicated in green. Amino acid residues are indicated by their one-letter code and stop codons by asterisks (*).

Clone 30 was chosen for further experiments and was named *GST1*prom-ap*pgip1*-pCAMBIA2300#30. The plasmid map of *GST1*prom-ap*pgip1*-pCAMBIA2300 is included in Figure 3.18.

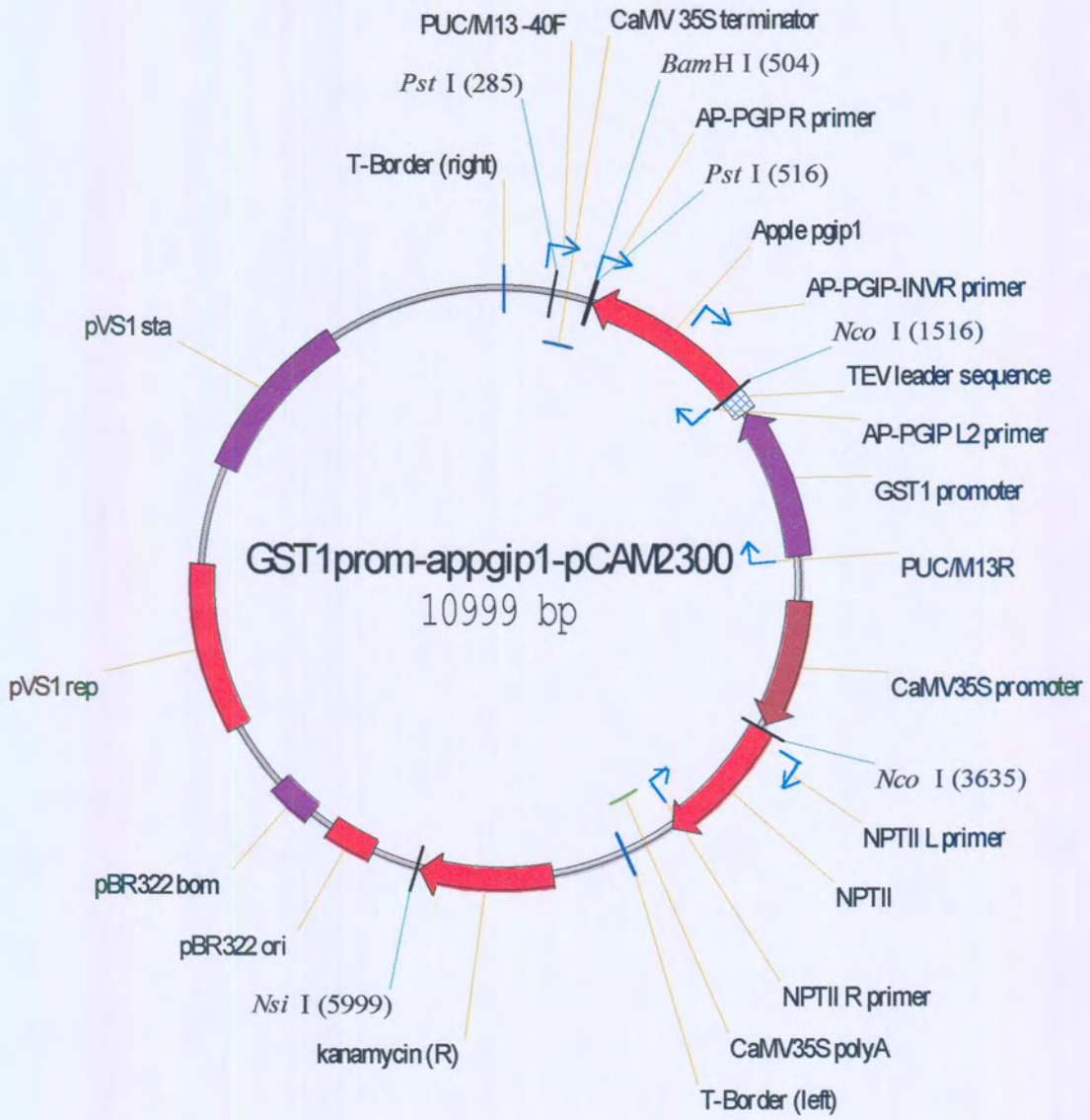


Figure 3.18 **Plasmid map of *GST1*prom-ap*pgip1*-pCAMBIA2300.**

3.4 Discussion

3.4.1 PCR primer design

Before subcloning the *gst1* promoter upstream of the apple *pgip1* gene, it was essential to isolate the appropriate part of the *gst1* promoter from a previous construct (p*GST1*-BluescriptSK$^-$). The polymerase chain reaction (PCR) was employed, since it can selectively amplify a specific region of DNA while at the same time incorporate desired sequences at the ends of the fragment. The GSTreverse primer was designed to amplify the promoter just upstream of the ATG start codon of the *gst1* gene. Translation by plant ribosomes is affected negatively if they encounter extra start codons before the correct one.

In prokaryotes, the initiator AUG codon is preceded by a purine rich "Shine/Dalgarno" sequence centred about ten nucleotides upstream. In contrast, eukaryotic ribosomes bind to the 5' end of the transcript and migrate along it, to start translation at the first AUG triplet it encounters, with a few exceptions. Studies with 79 higher plants confirmed the role of the first AUG codon on the processed mRNA as the translation initiation site. The consensus sequence for the plant initiator site was TAAACAATGGCT (on the plus strand of DNA) (Joshi, 1987), which conforms to the general eukaryotic model of a purine at position -3 and +4. The preferred consensus sequence for eukaryotic (mostly animal) translation initiation sequences is [(A/G)XXAUGG] (Kozak, 1981). Thus, purines are favoured in position -3, suggesting that purines in positions -3 and +4 might facilitate recognition of the AUG codon during formation of the initiation complex. The X's were later established to favour pyrimidines. The sequence around the initiator codon of the apple *pgip1* gene is **G**CCATG**G**. It conforms thus to both the plant and animal eukaryotic translation initiation consensus sequences, having purines at position -3 and +4 (indicated in bold) and pyrimidines at positions -1 and -2.

The nucleotide sequence between the TATA box of the *gst1* promoter and the initiator codon of the apple *pgip1* gene in the *GST1*prom-ap*pgip1*-pCAMBIA2300 construct was presented in Figure 3.17. The transcription initiation site of the *gst1* promoter was mapped by primer extension (Yang *et al.*, 1998) to be 50 bp upstream of the translation initiation codon (indicated by an arrow ↓). The translation into all three reading frames is indicated by the one letter amino acid code. The only methionine residue (M) in all three reading frames is the one corresponding to the initiator codon of the apple *pgip1* gene (indicated in green). This illustrates that there are no other initiator codons in the mRNA transcript than the correct one. Since it will be the first AUG that the eukaryotic ribosome will encounter while it migrates along the mRNA transcript, translation of the apple *pgip1* gene will proceed correctly.

3.4.2 PCR amplification and gel purification of the *gst1* promoter

During PCR amplification of the appropriate part of the *gst1* promoter using the GSTreverse and SK primers, a fragment of 1008 bp was expected. An amplification product of approximately 900 bp was obtained for all reactions using either Taq DNA Polymerase or the Expand Long template PCR system (Figure 3.2, lanes 1 to 5, and Figure 3.3, lanes 1 and 2). This was unexpected, but may have been an artefact of the agarose gel, perhaps being overloaded with the high yield of PCR amplification product. After gel extraction of the PCR product, a faint band of a smaller size (~700 bp) was observed together with the purified PCR fragment of ~900 bp (Figure 3.4, lane 1). The identity of this fragment was unknown, and no colonies transformed with recombinant pMOSBlue containing this smaller insert were obtained. Nucleotide sequencing of the recombinant constructs, *GST1*prom-pMOSBlue #6 and #9, showed that the correct sequence was amplified and cloned. It is speculated that the smaller size fragment is single-stranded DNA that is eluted from the silica column during purification of the fragment using the Qiaquick gel extraction kit. It migrates faster than the double stranded fragment, so that it seems smaller in size. Because it is single-stranded, it cannot ligate to the pMOSBlue vector to produce recombinant constructs.

3.4.3 Subcloning of the *gst1* promoter PCR product into pMOSBlue

The PCR product was cloned into the pMOSBlue vector for two reasons. Firstly, it was to enable nucleotide sequencing of the appropriate part of the *gst1* promoter to confirm the correct sequence before proceeding with the construct preparation. Secondly, it was to efficiently excise the promoter insert from the pMOSBlue vector for subcloning into pCAMBIA2300, since restriction digestion of PCR products is not very efficient. Two colonies transformed with recombinant *GST1*prom-pMOSBlue constructs were obtained, of which only clone 6 had a 100% correct sequence. The 5' part of the GSTreverse primer, containing the *Pst*I site, was missing from the insert cloned in clone 9. The rest of the *gst1* promoter sequence was, however, as expected. Clone 6 was therefore chosen for further experiments, so that the apple *pgip1* cassette could be later inserted into the *Sal*I and *Pst*I sites that was incorporated by the GSTreverse primer.

3.4.4 Subcloning of the *gst1* promoter into pCAMBIA2300

The plasmid pCAMBIA2300 is an *A. tumefaciens* binary vector (www.cambia.com). It is a building block for plant transformation because it has a multiple cloning site between the left and right border sequences in which a transgene can be inserted. It also contains the neomycin phosphotransferase II (*nptII*) gene, encoding kanamycin resistance under control of plant expression signals, to simplify selection of transformed plants. Two colonies transformed with recombinant *GST1*prom-

pCAMBIA2300 constructs were obtained, and nucleotide sequencing of clone 3 showed the correct junction sequences of the *gst1* promoter fragment ligated into pCAMBIA2300.

3.4.5 Subcloning of the apple *pgip1* cassette downstream of the *gst1* promoter in pCAMBIA2300

pAppRTL2 contains the apple *pgip1* gene in an expression cassette with the TEV leader and CaMV 35S terminator (**Appendix C**). Preparation of the cassette for subcloning it downstream of the *gst1* promoter in pCAMBIA2300 required digestion with *Xho*I and *Pst*I. *Xho*I cuts 5` to the TEV leader, and *Pst*I at the 3` end of the cassette. However, an additional *Pst*I recognition site lies between the apple *pgip1* gene and the CaMV 35S terminator. Partial restriction digestion thus had to be performed to isolate the full-length (1379 bp) cassette.

The $MgCl_2$ concentration, restriction enzyme (*Pst*I) concentration and incubation time are important factors to consider during partial digestion. Too much enzyme and too long digestion times will result in complete digestion. 1U Enzyme per 1 µg linearised plasmid, digested for 20 min in 1 mM ($1/10^{th}$ of the optimal) Mg^{2+} concentration, yielded the highest yield of excised full-length cassette (Figure 3.11, lane 12). The only reaction which gave complete *Pst*I digestion, was digestion for 40 minutes with 1 mM $MgCl_2$ (Figure 3.11, lane 8). It is unclear why this reaction was more complete than at t = 60 min or with 100% Mg^{2+} concentration (compare Figure 3.11 lane 8 with lanes 3, 7 and 11). The most logical explanation is that a pipetting error of too much enzyme mastermix was made to this tube, resulting in more enzyme digestion activity being present.

Because the 1379 bp apple *pgip1* cassette fragment, excised from the agarose gel, was contaminated with the 1148 bp shortened cassette fragment (Figure 3.13, lane 5), several colonies containing the truncated cassette (lacking the 3` terminal part) were obtained (Figure 3.14, lanes 4, 24, 28, 29, 31, 32, 39, 40, 41 and 44). Both the full-length and the truncated cassette are flanked by sticky ends of *Xho*I on the 5` end and *Pst*I on the 3` end. This enables them to anneal and ligate to the vector prepared with *Sal*I and *Pst*I. Fortunately, seven of the 44 colonies screened by restriction digestion contained the 3` terminal *Pst*I fragment. Further restriction analysis (Figure 3.15) confirmed the full-length identity of the subcloned apple *pgip1* cassette in all seven clones. Two clones, numbers 25 and 30, were sent for nucleotide sequencing of the junction points, and both contained the correct sequences.

This chapter reported on the cloning of the apple *pgip1* gene under control of the pathogen-inducible *gst1* promoter of *A. thaliana* into a plant transformation vector. The transformation of *A. thaliana* plants with the *gst1* promoter- and e35S- apple *pgip1* constructs, as well as molecular analysis of the transformants and PGIP expression studies, will be reported in Chapter 4.

CHAPTER 4

Transformation, molecular analysis and expression studies of *Arabidopsis thaliana* transformed with apple *pgip1* gene constructs

4.1 Introduction

In Chapter 3, a plant transformation construct was prepared in which the expression of the apple *pgip1* gene is controlled by the *gst1* promoter from *Arabidopsis thaliana* (L.) Heynh. This chapter describes how the construct was transformed into *A. thaliana* in an attempt to test the functionality and pathogen inducibility of the *gst1* promoter (Yang *et al.*, 1998; Grant *et al.*, 2000). The advantages of using this model plant were discussed in Chapter 2. The aim of this chapter was to test whether the hypothesis that the *gst1* promoter is pathogen inducible is true. The fungal inducibility of the *gst1* promoter would be valuable in the transgenic expression of antifungal resistance genes in crops of importance. If the level of expression of the induced *gst1* promoter were higher than the constitutive enhanced CaMV 35S (e35S) promoter, it would also be a significant result. The *gst1* promoter would then be useful as a tool when high expression levels of a gene of interest is required in plants.

This chapter will describe the transformation of *A. thaliana* with the *gst1* promoter-*pgip1* construct as well as a construct containing the apple *pgip1* gene under control of the constitutive enhanced CaMV e35S promoter. The floral-dip method, as described in Chapter 2, was used. The molecular analysis of the transformants and PGIP expression studies will also be reported.

Agarose diffusion assay (ADA)

In this chapter, the agarose diffusion assay was employed to test the PG-inhibiting activity of extracts prepared from apple *pgip1* transgenic *A. thaliana*. The agarose diffusion assay is used to quantify pectolytic enzyme activity. The assay medium, modified from Taylor and Secor (1988), consists of ammonium oxalate and polygalacturonic acid solidified with agarose, in a citric acid-sodium phosphate buffer (pH 4.6). Wells are punched into the solidified medium and filled with the sample, such as fungal culture supernatant, of which the enzyme activity needs to be determined. The agarose diffusion assay is specific for polygalacturonases (PGs), which are enzymes that break glycosidic bonds by hydrolysis. These enzymes have pH optima around pH 5.0, and are inhibited by Ca^{2+}. The ammonium oxalate is included to bind and remove the Ca^{2+} present in the assay solution. The enzymes diffuse into the medium and hydrolyse the substrate. PG activity is represented by the formation of zones around the wells where the substrate has been hydrolysed. Plates are developed

with ruthenium red, which reacts with unhydrolysed polygalacturonic acid. It provides a sharper ring development, and therefore a more sensitive means of detecting pectolytic activity, than another developing method that employs 5 N HCl (not used in this chapter but in Chapter 6).

4.2 Materials and Methods

All chemicals and reagents used were either analytical or molecular biology grade. Buffers, solutions and media were all prepared using distilled water and either autoclaved or filter-sterilised through 0.2 µm sterile syringe filters. All buffers, solutions and media used in this study are described in **Appendix A**.

4.2.1 Transformation of *Agrobacterium tumefaciens*

Competent *A. tumefaciens* GV3101(pMP90RK) was transformed with 5 µg plasmid DNA. The GV3101 strain contains a disarmed Ti-plasmid pTiC58 derivative, pMP90RK, which has proved to be successful in use with *A. thaliana* (Clough and Bent, 1998; Koncz and Schell, 1986). Five microlitres of sterile dH_2O was used as a negative control. Competent *A. tumefaciens* cells were prepared by resuspending the cell pellet of a 100 ml overnight culture in 2.5 ml ice-cold 20 mM $CaCl_2$ and dispensing it in 0.3 ml aliquots. After quick-freezing the cell-plasmid mixture in liquid nitrogen, the cells were thawed by incubating the tubes in a 37°C incubator for 10 min. One millilitre of LB medium was added and the tubes incubated at 30°C for 3 h with shaking. The tubes were centrifuged at 700×g for 10 min in a microcentrifuge, the supernatant discarded and the pellet resuspended in 0.3 ml LB. Hundred microlitre aliquots were plated onto LB-agar plates containing 50 µg/ml rifampicin, 50 µg/ml gentamycin and 50 µg/ml kanamycin. Rifampicin selects for the *A. tumefaciens* genome, gentamycin for the disarmed Ti-plasmid pMP90RK and kanamycin selects for the introduced binary plasmid. The plates were incubated inverted at 30°C for three days for colonies to appear.

4.2.2 Screening of *A. tumefaciens* transformants by PCR

Transformants were screened by the direct colony PCR method as set out in the pMOSBlue blunt-ended cloning kit instruction manual (AEC-Amersham, Little Chalfont, UK). Colonies of *A. tumefaciens* strain GV3101(pMP90RK) transformed with each of the different plasmids were picked with sterile toothpicks and transferred to 1.5 ml microcentrifuge tubes containing 50 µl sterile water. The tubes were vortexed to disperse the cell pellets and boiled for 5 min to lyse the cells and denature DNases. The samples were centrifuged for 5 min at 4°C at 6500×g in a microcentrifuge to pellet the cell debris. PCR was performed on the supernatants of the putative transformants using the AP-PGIP-L2 and AP-PGIP-R primers (**Appendix B**) to screen for the presence of the apple *pgip1* gene (expect an amplification product of 1024 bp). The *nptII* gene (conferring kanamycin resistance) was amplified with NPTII-L and NPTII-R. A product of 699 bp was expected.

PCR was conducted in 0.2 ml thin-walled tubes in a MJ Research PTC-200 Programmable Thermal Controller (MJ Research Inc.). The reaction mixture contained 1× Taq reaction buffer, 200 µM of each dNTP, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase (Promega) and 1 µl of the lysed colony supernatant as template. The reaction volume was made up to 10 µl using sterile dH_2O. The reaction mixture was overlaid with one drop of mineral oil to prevent evaporation. Negative controls, containing all the PCR reagents and untransformed *A. tumefaciens* colony supernatant or dH_2O, were included. A positive control containing 5 ng *GST1*prom-ap*pgip1*-pCAMBIA#30 plasmid DNA was also included.

35 PCR cycles were carried out with the cycle conditions of 94°C for 30 s, 58°C for 30 s and 72°C for 45 s, ended by 1 cycle of 3 min at 72°C. PCR products were analysed by electrophoresis through a 1% (w/v) agarose gel in 0.5× TAE buffer (pH 8.0) containing 0.06 µg/ml ethidium bromide and visualised under UV light.

4.2.3 Transformation of *Arabidopsis thaliana* using the floral dip method

4.2.3.1 Growth of *A. thaliana*

A. thaliana seeds of ecotype Columbia (Col-0) were used. Seeds were placed on potting medium consisting of peat moss, vermiculite and sand (4:1:1), and allowed to vernalise for 48 hours at 4°C after which they were transferred to 20°C. Seedlings were transplanted four to a pot, watered by sub-irrigation and fertilised once a week with Phosphogen^R. Plants were placed in the transgenic greenhouse under long day-length conditions (16 hours light, 8 hours dark). Two weeks after transplantation, emerging bolts were removed to stimulate more bolt formation. One week after cutting of the bolts, the first *Agrobacterium* dip was applied. Transformation is the most efficient when numerous immature, unopened floral buds and a few siliques are present (Clough and Bent, 1998).

4.2.3.2 Floral dip of *A. thaliana*

The floral-dip method for *Agrobacterium*-mediated transformation of *A. thaliana* was used (Clough and Bent, 1998). Colonies of *A. tumefaciens* GV3101(pMP90RK) transformed with pCAMBIA2300, pCAM2300-ap*pgip1*B and *GST1*prom-ap*pgip1*-pCAMBIA#30 were inoculated into 5 ml LB starter cultures containing 50 µg/ml of each gentamycin, rifampicin and kanamycin. After overnight incubation at 30°C with shaking, the starter cultures were used to inoculate 500 ml LB (containing the same antibiotics) in a 2 l conical flask. The cultures were incubated overnight at 30°C with shaking. The optical densities of the cultures were determined at 600 nm. The cells were collected by centrifugation at 1600×g for 20 min in a JA-14 rotor, the supernatant decanted and the pellet

resuspended in 5% sucrose to an OD_{600} of 0.8. Three weeks after transplantation and growth in a glasshouse, the *A. thaliana* flowers were dipped in the *Agrobacterium* solution. Each *Agrobacterium* solution containing a different construct was used to dip 14 pots containing four plants each. Just before dipping the flowers, Silwet L77 (Ambersil Ltd.) was added to a final concentration of 0.05%. After dipping, the pots were placed on their sides inside a plastic container and the plants covered with plastic wrap. The plants were kept in the shade for one day, after which the plastic was removed and the pots placed upright and returned to their shelves in the glasshouse. The second *Agrobacterium* dipping was applied six days after the first. For the second dipping, the Silwet L77 concentration was halved to 0.025%.

Siliques were collected and seed harvested three weeks after the second *Agrobacterium* dipping. The plants were completely senesced and dried out by then. Seeds (termed T1) were collected individually for each construct that was transformed, and from each pot containing 4 plants each. Thus, 14 envelopes of seed were produced for each of the three constructs that were transformed into the plants. From the time of transplanting the seedlings to the collection of putative transgenic seed, the transformation protocol took seven weeks.

4.2.4 *In vitro* kanamycin selection for transgenic *A. thaliana* seedlings

The required amount of seeds was washed with 70% ethanol and sterilised for 30 min in 1.5% sodium hypochlorite while shaking. Seeds were rinsed three times with 1 ml sterile distilled water, and resuspended in 500 µl sterile 0.1% (w/v) agarose. The resuspended seeds were plated out onto MS selection plates [1× MS salts (Sigma M5519 (St Louis, MO, USA) or Highveld Biologicals), 3% (w/v) sucrose, pH 5.9, 0.8% (w/v) agar, 50 µg/ml kanamycin and 250 µg/ml cefotaxime]. After vernalisation for 2 days at 4°C, plates were placed in the growth room at 25°C, covered with aluminium foil. After two days the foil was removed and the seedlings left at 16 h light, 8 h darkness for two weeks. Seedlings with green leaves and healthy roots were transferred to soil (4:1:1 peat moss, vermiculite and sand), covered with plastic for the first day and grown for two months at 22°C in a growth chamber (12 h light, 12 h dark). Seedlings were watered the first few times with a solution of 2.5 g/l Multifeed^{R} (Plaaskem Ltd.) to stimulate root growth. Bolts were removed from plants to stimulate more bolt formation. Mature siliques were collected when the plants started to senesce.

4.2.5 Isolation of genomic DNA from *A. thaliana*

Approximately four small leaves of putative transgenic *A. thaliana* were ground in a 1.5 ml Eppendorf tube with liquid nitrogen and an Ultra Turrox. One millilitre of preheated 2% CTAB isolation buffer containing PVP [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100

mM Tris-HCl (pH 8), 1% PVP] was added and the tube incubated at 65°C for 30 minutes, shaking gently every 10 min. Plant debris was removed by centrifugation at 6500×g for 2 minutes at 16°C, and the supernatant transferred to a 2.2 ml tube. The samples were extracted with an equal volume (1 ml) of chloroform: isoamyl alcohol (24:1) and incubated at room temperature for 5 min. The tubes were centrifuged at 6500×g for 10 minutes at 4°C, and 50 µl of 10% CTAB buffer (10% CTAB, 0.7 M NaCl) added to the supernatant in a fresh 2.2 ml tube. After incubation at 65°C for 10 min, it was extracted with chloroform: isoamyl alcohol as before. The tubes were centrifuged at 6500×g for 10 minutes at 4°C, and 1 ml of ice-cold isopropanol added to the top layer. Nucleic acids were precipitated by incubation at -20°C for 10 min. The tubes were centrifuged at 6500×g for 20 minutes at 4°C, the supernatant decanted and the pellet washed with 500 µl ice-cold 70% ethanol. The pellets were air-dried and resuspended in 400 µl 1× TE (pH 8.0). 2.5 µl RNase A (10 mg/ml) was added and the tubes incubated at 37°C overnight. Four hundred microlitres 1 M NaCl was added and the tubes incubated at room temperature for 30 min with occasional inversion of the tube. Four hundred microlitres isopropanol was added and the DNA precipitated by incubation at -20°C for 10 minutes. The DNA was collected by centrifugation at 6500×g for 20 min at 4°C. The supernatant was decanted, the pellet washed with 500 µl ice-cold 70% ethanol and the tubes centrifuged again for 20 minutes. All liquid was removed and the pellets allowed to air-dry. The pellets were dissolved in 50 µl 1× TE (pH 8.0) and the concentration determined fluorometrically.

4.2.6 PCR screening of putative transgenic *A. thaliana*

PCR was performed on the putative transgenic *A. thaliana* using the AP-PGIP and NPTII primer sets (**Appendix B**) to screen for the presence of the apple *pgip1* gene and the *nptII* gene, respectively. The same amplification products were expected as with the *A. tumefaciens* colony PCR.

PCR was conducted in 0.2 ml thin-walled tubes in a MJ Research PTC-200 Programmable Thermal Controller (MJ Research Inc.). The reaction mixture contained 1× Taq reaction buffer, 200 µM of each dNTP, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase (Promega) and 3 µl of the isolated gDNA (39 - 66 ng) as template. The reaction volume was made up to 10 µl using sterile dH_2O. The reaction mixture was overlaid with one drop of mineral oil to prevent evaporation. Negative controls, containing all the PCR reagents and untransformed *A. thaliana* gDNA or dH_2O, were included. A positive control containing 15 ng *GST1*prom-ap*pgip1*-pCAMBIA#30 plasmid DNA was also included.

The same PCR cycle conditions were used as with the *A. tumefaciens* colony PCR, but the *nptII* PCR had an annealing temperature of 62°C. PCR products were analysed by agarose electrophoresis as described before.

4.2.7 PGIP extraction from *A. thaliana* plants

One hundred to 150 mg *A. thaliana* leaf material was used for PGIP extractions. The samples were ground in a 1.5 ml Eppendorf tube with carborundum and an Ultra Turrox. Two volumes of 1 M NaCl, 20 mM NaAc buffer (pH 4.7) were added and the extracts shaken for two hours at 7°C. The cell debris was sedimented by centrifugation at 6500×g for 20 minutes at 4°C, and the supernatant transferred to a clean tube.

4.2.8 Methyl-salicylate treatment of *A. thaliana* leaves

Ten or more leaves of each transgenic *A. thaliana* line were pressure-infiltrated from the bottom with 10 µl of 1 mM methyl-salicylate (Me-Sa) (MW 152.15 g/mole) in potassium phosphate buffer (pH 5.8) using a syringe (Sambrook *et al.*, 1989). Leaves were infiltrated while still attached to the plants. Leaves were harvested 24 h later and stored at -70°C until PGIP extraction. PGIP extraction was performed on 170 - 275 mg leaf material as described before.

4.2.9 Agarose diffusion assay of PGIP extracts

Sixty-five millimetre diameter Petri dishes containing 10 ml of the assay medium [1% type II agarose, 0.01% PGA and 0.5% ammonium oxalate in citrate-phosphate buffer, pH 4.6] were prepared according to Taylor and Secor (1988) with a few modifications (See **Appendix A**). Holes were punched in the solidified medium using a no. 1 cork borer.

Fifteen microlitres of *V. dahliae* PG (isolation discussed in **Chapter 6**) was incubated with 15 µl of either 20 mM NaAc buffer (pH 4.7) or various PGIP extracts. The reactions were incubated at 25°C for 20 minutes, after which 25 µl was loaded into a well of an ADA plate and left to diffuse into the gel.

The plates were incubated at 27°C overnight. The fungal endopolygalacturonase activity was visualised by staining each plate with 10 ml of 0.05% ruthenium red (Sigma) for 1 h at 37°C. After staining, the plates were rinsed with dH_2O to remove excess dye and left overnight at 4°C before the zone diameters were measured.

4.3 Results

The aim of this section was to transform *A. thaliana* with apple *pgip1* constructs using the *Agrobacterium*-floral dip method. Seed were collected and subjected to kanamycin selection to select for transgenics. The seedlings were transplanted to soil and the process repeated until plants homozygous for the transgene were obtained. Transformants were screened using PCR and PGIP assays were performed to analyse transgene expression. An experiment to induce the *gst1* promoter activity by Me-Sa is also reported.

4.3.1 Creating apple *pgip1* transgenic *Arabidopsis thaliana* plants

4.3.1.1 Transformation of *Agrobacterium tumefaciens* GV3101(pMP90RK) with the apple *pgip1* constructs

The constructs used for transformation of *A. thaliana* were the following: pCAMBIA2300, pCAM2300-ap*pgip1*B and *GST1*prom-ap*pgip1*-pCAMBIA#30 (refer to **Appendix C** and Figure 3.18). The preparation of the latter construct was discussed in Chapter 3. The identities of the latter two constructs were verified by restriction enzyme digestion (Sambrook *et al.*, 1989). *Kpn*I and *Pst*I double-digestion of *GST1*prom-ap*pgip1*-pCAMBIA#30 is expected to excise two fragments with sizes of 2053 and 231 bp (Figure 4.1, lane 2). *Bam*HI, *Bgl*II and *Nco*I digestions of pCAM2300-ap*pgip1*B release fragments of sizes 265, 1522 and 2356 bp (Figure 4.1, lanes 4, 5 and 6), respectively. Fragments were obtained as expected (Figure 4.1). The expected vector fragments were also obtained for each digestion reaction.

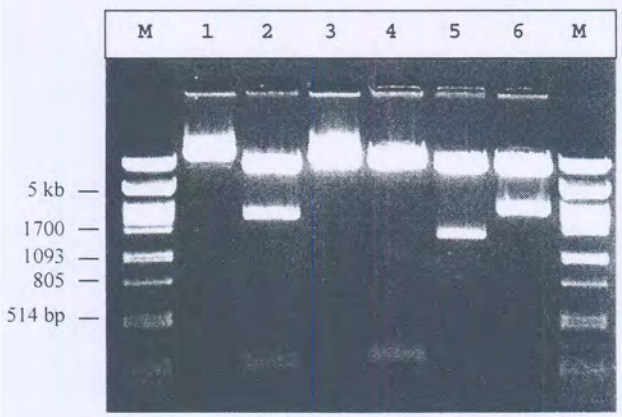


Figure 4.1 **Restriction analysis of constructs used for *A. thaliana* transformation.** M: λDNA/ *Pst*I marker: lanes 1 and 3: undigested *GST1*prom-ap*pgip1*-pCAMBIA#30 and pCAM2300-ap*pgip1*B plasmid. respectively: lane 2: *Kpn*I and *Pst*I double-digested *GST1*prom-ap*pgip1*-pCAMBIA#30 plasmid: lanes 4 to 6: pCAM2300-ap*pgip1*B plasmid digested with *Bam*HI, *Bgl*II and *Nco*I, respectively.

A. tumefaciens GV3101(pMP90RK) was transformed with 5 µg of each construct using a freeze-thaw method. Several colonies were obtained and a number of them screened with PCR

4.3.1.2 Screening of *A. tumefaciens* transformants by PCR

One *A. tumefaciens* colony, transformed with each construct, was selected and used for floral dip transformation of *A. thaliana*. Figure 4.2 shows an agarose gel of the PCR products obtained during screening of this colony using the apple *pgip1* and *nptII* primers.

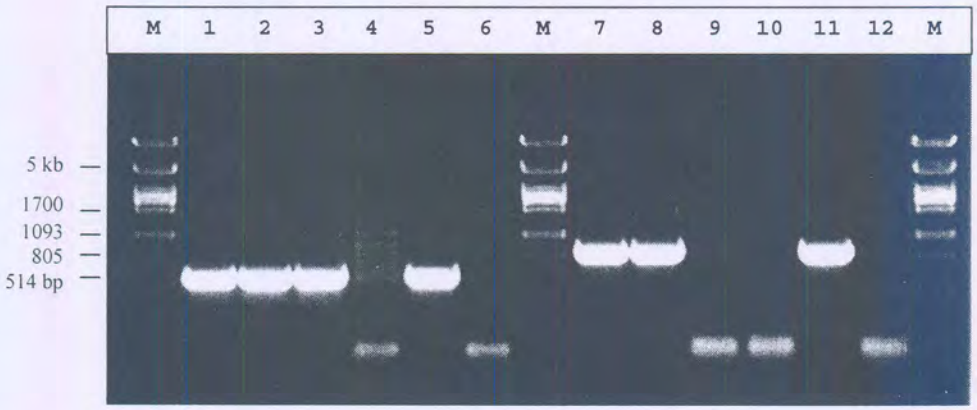


Figure 4.2 **Colony PCR of *A. tumefaciens* GV3101 using AP-PGIP and NPTII primers.** M: λDNA/ *Pst*I marker; lanes 1 to 3: NPTII PCR of colonies transformed with *GST1*prom-ap*pgip1*-pCAMBIA#30, pCAM2300-ap*pgip1*B and pCAMBIA2300, respectively; lanes 4 to 6: NPTII PCR of untransformed *A. tumefaciens* GV3101, positive control and negative water control, respectively; lanes 7 to 12: AP-PGIP PCR with the same templates as the NPTII PCR.

The *nptII* primers yielded a product of approximately 600 bp for all colonies except the untransformed *A. tumefaciens* and dH_2O negative controls (Figure 4.2, lanes 4 and 6). The apple *pgip1* primers yielded only 1 kb products for colonies transformed with *GST1*prom-ap*pgip1*-pCAMBIA#30 and pCAM2300-ap*pgip1*B and the positive control (Figure 4.2, lanes 7, 8 and 11). As expected, the apple *pgip*1 gene was not present in the pCAMBIA2300 vector transformed colonies, the untransformed *A. tumefaciens* or the dH_2O control (Figure 4.2, lanes 9, 10 and 12, respectively). The results are thus as expected, with the *nptII* gene present in all transformed colonies, and the apple *pgip1* gene only in colonies transformed with constructs that have the gene.

4.3.1.3 Transformation and selection of *A. thaliana* accession Columbia

The binary plant transformation vectors pCAMBIA2300, pCAM2300-ap*pgip1*B and *GST1*prom-ap*pgip1*-pCAMBIA#30 were transformed into *A. thaliana* using the floral dip method. The binary vectors were transferred to the plant cells by the vir functions encoded by the disarmed pMP90RK Ti-plasmid. This helper Ti-plasmid was disarmed by deleting the T-DNA phytohormone genes, and

therefore can no longer cause crown gall disease (Koncz and Schell, 1986). Putative transgenic *A. thaliana* seed (T1) were collected and tested *in vitro* for kanamycin resistance by the addition of this antibiotic to the tissue culture medium. The seeds were sterilised and plated onto MS media plates supplemented with kanamycin and cefotaxime. Cefotaxime selects against the growth of *A. tumefaciens*. Kanamycin susceptible seedlings remained yellow and failed to root. Kanamycin-resistant seedlings were transplanted to soil and allowed to set seed. T2 seeds were harvested from them and subjected to a second round of kanamycin selection. Homozygotes and heterozygotes containing the inserted gene (seedlings were able to grow on kanamycin) were selected and again transplanted to soil. T3 seeds were harvested and subjected to kanamycin selection to determine whether the T2 plant was a heterozygote or a homozygote. T3 seedlings from homozygous T2 plants (100% of its seed germinated on kanamycin plates) and T2 kanamycin resistant seedlings were transplanted to soil and leaf material collected for genomic DNA isolations and PGIP extractions.

Three lines transformed with each construct were selected for further studies. Lines labelled with a "g" indicate that the plants were transformed with the *GST1*prom-ap*pgip1*-pCAMBIA#30 construct. Similarly, the "e" is for pCAM2300-ap*pgip1*B transformed plants and "p" for pCAMBIA2300 transformed plants. Two T2 lines transformed with each construct were selected. They were g7-9, g9-13, e10-15, e10-17, p10-2 and p10-9. Because they were only second-generation transformants, it was not known whether they were homo- or heterozygous for the transgene. The homozygous T3 lines chosen were g14.2, e18.2 and p11.3. Each line is expected to be an independent transformation event, since *Agrobacterium*-mediated transformation transforms each seed separately (Clough and Bent, 1998).

4.3.2 PCR analysis of transgene insertion

4.3.2.1 Isolation of genomic DNA from putative apple *pgip1* transgenic *A. thaliana*

Genomic DNA was isolated from the putative transgenic *A. thaliana* lines g14.2, g7-9, g9-13, e18.2, e10-15, e10-17, p11.3, p10-2 and p10-9. A 2% CTAB method containing PVP in the isolation buffer was used, and very low yields of DNA were obtained. The average concentration from ten *A. thaliana* samples was 16 ng/µl, with a yield of 800 ng gDNA per 4 small leaves. The DNA pellets were, however, very clean and appeared glassy on the sides of the tubes.

4.3.2.2 PCR screening of putative apple *pgip1* transgenic *A. thaliana*

PCR was successful on the isolated gDNA, despite the low yield. All putative transgenic plants and the controls showed the expected PCR products. Plants transformed with the *GST1*prom-ap*pgip1*-pCAMBIA#30 construct (lines g14.2, g7-9, g9-13, Figure 4.3 lanes 1 to 3, respectively) and pCAM2300-ap*pgip1*B (lines e18.2, e10-15, e10-17, Figure 4.3 lanes 4 to 6, respectively) contained

amplification products for both the apple *pgip1* gene and the *nptII* gene. Plants transformed with the plant transformation vector pCAMBIA2300 as a negative transformation control (lines p11.3, p10-2 and p10-9) contained only the *nptII* gene (Figure 4.3, lanes 7 to 9, respectively). The dH_2O control and untransformed *A. tumefaciens* gDNA reactions yielded no amplification products, as expected (Figure 4.3, lanes 10 and 11, respectively). The amplification products of the transgenic plants had the same sizes as the positive control containing *GST1*prom-ap*pgip1*-pCAMBIA#30 plasmid as template (Figure 4.3, lane 12).

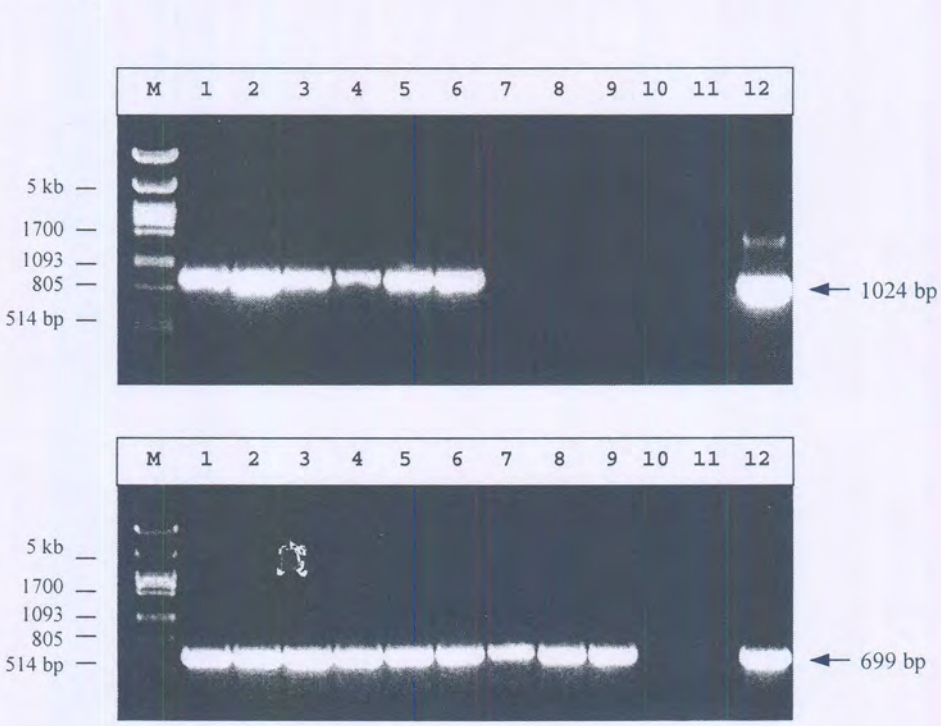


Figure 4.3 **PCR analysis of *A. thaliana* Col-0 transformed with apple *pgip1* constructs, using AP-PGIP and NPTII primers.** A: AP-PGIP PCR; B: NPTII PCR; For both A and B: M: λDNA/ *Pst*I marker; lanes 1 to 12: Amplification products of reactions containing the following as template: lanes 1 to 3: gDNA from lines g14.2, g7-9 and g9-13, respectively; lanes 4 to 6: gDNA from lines e18.2, e10-15 and e10-17, respectively; lanes 7 to 9: gDNA from lines p11.3, p10-2 and p10-9, respectively; lane 10: untransformed *A. thaliana* gDNA; lane 11: negative water control; lane 12: positive *GST1*prom-ap*pgip1*-pCAMBIA#30 plasmid control.

4.3.3 Analysis of transgene expression by PGIP inhibition assays

The *gst1* promoter of *A. thaliana* contains elements that indicate it is inducible by salicylic acid (Yang *et al.*, 1998). Transformed *A. thaliana* were treated with methyl-salicylate in an experiment to induce the expression of the apple *pgip1* gene that is controlled by the *gst1* promoter. Plants transformed with constructs not containing the *gst1* promoter (the e- and p-lines) were also treated to serve as negative controls. Crude PGIP extracts were prepared from untreated and Me-Sa treated putative apple *pgip1*

transgenic *A. thaliana* lines (g14.2, g7-9, g9-13, e18.2, e10-15, e10-17, p11.3, p10-2 and p10-9) and untransformed *A. thaliana* Col-0. The PGIP extracts were used in an agarose diffusion assay with *V. dahliae* PGs. The larger the cleared zone in the solidified pectin medium, the more PG activity is present. Unhydrolysed substrate is stained by ruthenium red.

Figure 4.4 shows the zone diameters obtained during the agarose diffusion assay of *V. dahliae* PG in the presence of various PGIP extracts. A decreased zone diameter indicates inhibiting activity. The blue bars represent PGIP extracts from plants that were not treated with Me-Sa, while the red bars represent PGIP extracts from Me-Sa treated plants. Green bars indicate the activity of *V. dahliae* PG in the presence of NaAc buffer during the two ADA experiments. Purified apple PGIP was used as a positive control (yellow bars). No error bars are indicated since the experiments were not done with replicates. It should be taken into consideration that the diameter of the well in the agarose diffusion plate is 6 mm. Figure 4.4 was sketched so that the graphs start at 6 mm.

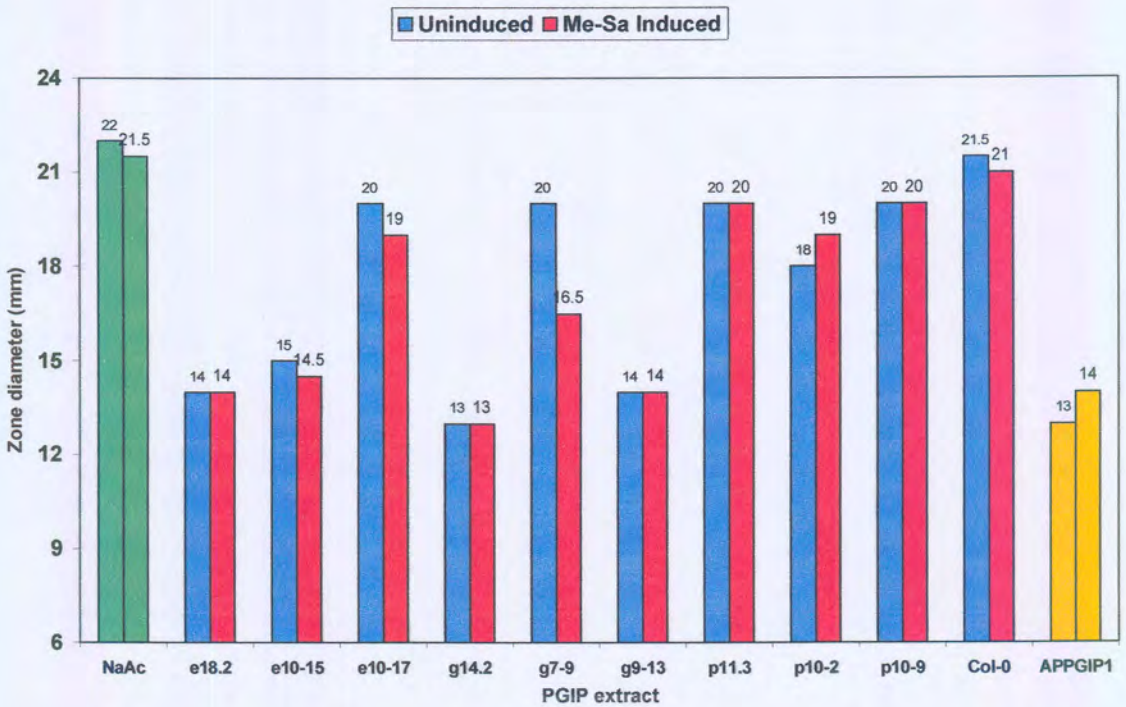


Figure 4.4 **Inhibition of *V. dahliae* PG activity by PGIP extracts from putative apple *pgip1* transgenic *A. thaliana* leaf material.** Zone diameters obtained during an agarose diffusion assay are presented. The activity of PG in the presence of PGIP extracts from each line is measured. Blue bars represent the PGIP extracts from uninduced leaves, and the red bars from Me-Sa treated leaves. PGIP extract from non-transgenic *A. thaliana* Col-0 was included as a negative control (column labelled with Col-0), and purified apple PGIP1 (APPGIP1) as a positive control (yellow bar). The column labelled *NaAc* (green bar) represents the activity of fungal PG in the presence of 20 mM NaAc buffer (pH 4.7) alone.

Two each of the e- and g-lines contained inhibiting activity of the *V. dahliae* PGs. Extracts from these plants (e18.2, e10-15, g14.2 and g9-13) caused a zone diameter reduction from 22 mm in the presence of 20 mM NaAc buffer to 13-15 mm. The two homozygous T3 plants containing the *pgip1* gene (e18.2 and g14.2) showed inhibition. In each case, it was one of the T2 generation plants that didn't show much inhibition (e10-17 and g7-9). Extracts from these lines only decreased the zone diameter to 20 mm. Inhibition by lines e18.2, e10-15, g14.2 and g9-13 compared well with the inhibition obtained by pure apple PGIP1, which formed a zone of 13 mm in diameter (Figure 4.4, yellow bar). None of the lines transformed with pCAMBIA2300 showed a substantial reduction in zone size, i.e. no inhibition. Line p10-2 decreased the zone diameter from 22 mm to 18 mm, while extracts from both p11.3 and p10-9 resulted in a decrease of the zone diameter from 22 mm to 20 mm. Untransformed *A. thaliana* PGIP showed no reduction in zone size, indicating no PG inhibition. This shows that *A. thaliana* PGIP is not effective against *V. dahliae* PGs.

PGIP extracts prepared from plants treated with Me-Sa showed virtually the same results as the uninduced plants. Only an extract from line g7-9 seemed to cause a smaller zone of 16.5 mm in diameter (following MeSA induction) compared to 20 mm obtained with the extract prepared from the uninduced plant.

4.4 Discussion

Transformations of *A. tumefaciens* GV3101(pMP90RK) with three different plasmid constructs were successful. This is a specific strain used for *A. thaliana* transformations (Koncz and Schell, 1986). PCR screening of one selected colony yielded the expected amplification products (Figure 4.2). The floral-dip method of *Agrobacterium*-mediated transformation of *A. thaliana* was followed (Clough and Bent, 1998). The number of transformants obtained on a plant can be increased by a second floral-dip application of *Agrobacterium*, roughly one week after the first application (Clough and Bent, 1998). The transformation efficiency was not determined, but transformation was efficient enough to generate a sufficient number of transgenic seedlings transformed with each construct for this analysis.

Kanamycin selection was applied to the harvested seed to select for homozygotes. Segregation of the transgene was estimated from the proportion of seeds able to germinate on kanamycin. The progeny of a plant containing a single copy of the transgene should show a typical Mendelian segregation (ratio 3:1), while transgenic plants containing more than one copy of the transgene will have nearly all their seeds germinate on selective medium. This method was also used by Desiderio *et al.* (1997) to determine the segregation of transgenic tomatoes. During this study, one line that was determined to be homozygous for the transgene, and two heterozygous lines, for each transformed construct, were chosen for PCR analysis and PGIP extractions. gDNA isolations had very low yields, but the purity of the gDNA was very high to result in successful PCR amplification. PCR of these lines using the apple *pgip1* and *nptII* primer sets yielded the expected amplification products (Figure 4.3).

In this study, PCR and kanamycin resistance provided evidence that the chosen *A. thaliana* lines were transformed and contained the apple *pgip1* gene. The fact that the gene was inherited by the progeny also provided evidence that the transgene had been stably integrated into the genomes of the transgenic *A. thaliana* plants. During PCR, it is possible that contamination by transformed *Agrobacterium* can lead to a false positive result. The probability of this was, however, very low since multiple rounds of cefotaxime selection were applied to the progeny seeds. A complete study of the transgenic *A. thaliana* lines would include Southern, northern and western blot to confirm transformation. These techniques were not applied in this study due to time constraints. The PGIP activity assays are preferred above the northern blot analysis in any case, since the activity of the expressed PGIP was of greater interest than the level of mRNA expression.

Sequences presumed to code for PGIP have been found in the genome of *A. thaliana* (Stotz *et al.*, 2000; De Lorenzo *et al.*, 2001). Two *pgip* genes are located on chromosome five (*Atpgip1* and *Atpgip2*), while two more divergent genes, *FLR1* and *FLR2*, are present on chromosome three. During analysis of transgene expression by PGIP inhibition assays, the PGIP extract prepared from

untransformed *A. thaliana* Col-0 didn't inhibit zone formation at all. This signifies that the endogenous *A. thaliana* PGIP is not active against *V. dahliae* PG (Figure 4.4, column labelled *Col-0*). The plants transformed with the pCAMBIA2300 vector, as a negative transformation control, also didn't show significant inhibiting activity.

Four of the selected six plants, transformed with apple *pgip1* constructs, showed inhibiting activity against *V. dahliae* PG. They were lines e18.2, e10-15, g14.2 and g9-13. The degree of inhibition, as assayed with the agarose diffusion assay, was comparable to purified apple PGIP1. It can thus be deduced that the apple *pgip1* transgene is being functionally expressed in these lines. The two lines that didn't show much inhibition (e10-17 and g7-9) were from the T2 generation plants, which could have been homozygous or heterozygous for the transgene.

Expression of PGIP1 in lines transformed with *GST1*prom-ap*pgip1*-pCAMBIA#30 (the g-lines) indicated that the *gst1* promoter fragment was active and able to direct transcription of the gene. The construct was thus successfully prepared in Chapter 3. Leaves from the g-lines that were not induced with Me-Sa showed expression of the PGIP1 protein. This fact indicated that the *gst1* promoter was either constitutively active (Grant *et al.*, 2000), or that the plants were stressed, which caused the induction of the *gst1* promoter. The possibility of stress was high, since the plants were inadvertently infected with powdery mildew and they were salt-stressed from watering with tap water.

In spite of the activity of the *gst1* promoter to direct transcription of the apple *pgip1* gene in plants that were not induced with Me-Sa, one line seemed to have enhanced PGIP activity after Me-Sa treatment. PGIP activity was higher in line g7-9 after induction with Me-Sa (Figure 4.4, column labelled *g7-9*). This indicates a possible induction of the *gst1* promoter, which activates the transcription of the apple *pgip1* transgene. It should be noted that the inhibition experiments were not done with replicates, so results should be evaluated only as a qualitative indication of inhibiting activity. It is not possible to draw conclusions without replicates, so it is impossible to accept or reject the hypothesis that the *gst1* promoter is pathogen inducible.

Other workers have compared a few different constitutive and inducible promoters in *A. thaliana* (Holtorf *et al.*, 1995). They found the highest expression level with the CaMV 35S promoter, which was enhanced two- to threefold by the addition of a translational enhancer. In their case, the TMV (tobacco mosaic virus) omega element, the 5'-untranslated leader of TMV, was used. Strong expression of the reporter gene (GUS) was found in the roots, cotyledons, leaves and all parts of the inflorescence. An inducible promoter (soybean heat-shock promoter *Gmhsp17.3*) showed the same expression pattern. It is anticipated that the TEV (tobacco etch virus) translation enhancer used in this study will be effective in enhancing translation of the apple *pgip1* transgene in *A. thaliana* (see Figure

3.18 and **Appendix C** for plasmid maps of *GST1*prom-ap*pgip1*-pCAMBIA#30 and pCAM2300-ap*pgip1*B).

In this study, the activity of the *gst1* promoter was tested by the PG-inhibiting activity of the expressed apple PGIP1 during an agarose diffusion assay. The best way of testing the inducibility of the *gst1* promoter in plants would be to fuse it to a reporter gene and then to transform plants with it. The detection of the reporter gene is simplified since its expression can generally be detected using a simple protocol. A reporter gene that has been used previously for the *gst1* promoter from *A. thaliana* is the luciferase reporter gene. The *gst1* promoter was fused to the luciferase gene and used to monitor ROI accumulation (Grant *et al.*, 2000). It was found that the engagement of the oxidative burst and cognate redox signalling functioned independently of salicylic acid, methyl jasmonate and ethylene but required a 48 kDa mitogen-activated protein kinase (MAPK).

Transformation of plants by *Agrobacterium* results in predominantly single-copy integrations (Hooykaas and Schilperoort, 1992). The observed variation in PGIP expression between the lines independently transformed with the same construct is thus mainly due to the position effect which influences the expression of foreign genes in transgenic plants. Using *A. tumefaciens* transformation, vast differences in promoter activity of transferred genes were observed in independently derived cell lines (An, 1986). In his study, the nopaline synthase (*nos*) promoter was used. This position effect may even lead to complete silencing of the genes. This is because foreign gene expression is often dependent on the location of the insertion on the chromosome and the chromatin structure at the insertion site. It may be inserted into a region of low transcriptional activity, such as heterochromatin, which results in a lower expression level of the transgene product. DNA methylation may also affect the expression of genes introduced in the T-DNA (Hooykaas and Schilperoort, 1992). The endogenous *gst1* promoter in *A. thaliana* might also have an effect on the apple PGIP1 expression levels of lines transformed with the *gst1* promoter-*pgip1* construct. It may cause gene silencing due to competition for the same transcription factors.

Yang *et al.* (1998) reported that wounding, low temperature, high salt and DPE herbicide treatment induced the *GST1* of *A. thaliana*. Previously it has been reported to be induced by pathogen attack and dehydration (Yang *et al.*, 1998). In the promoter region of the gene, sequences corresponding to ethylene-responsive elements and other motifs conserved among stress-inducible gene promoters are found (reviewed in Chapter 2; refer to Figure 2.2). The findings of this study may be correlated with the results of Yang *et al.* if it is assumed that the plants were stressed. The induction of PGIP expression in one line of *gst1* promoter-*pgip1* transformed *A. thaliana*, line g7-9, may be due to methyl-salicylate treatment. Due to time constrains, more assays to further investigate the *gst1* promoter-induced expression were not performed. The main aim of the project was to molecularly

characterise apple *pgip1* transgenic potato lines, and to investigate their PGIP expression. Inducers that can be used in the future include ethylene, herbicide safeners, auxin, pathogen infections, salicylic acid, H_2O_2, dehydration, wounding, low temperature, high salt and DPE (diphenyl ether) herbicide treatment (Dudler *et al.*, 1991; Itzhaki *et al.*, 1994; Yang *et al.*, 1998).

Results indicated that there was no induction of the *gst1* promoter under the experimental conditions used. There were no major differences in *pgip* expression levels between the Me-Sa induced and uninduced *A. thaliana* lines transformed with the *gst1* promoter-*pgip1* construct (the g-lines). Because no replicate experiments were performed, there was not enough evidence in favour of the induction of the *gst1* promoter. Both the *gst1* promoter- and e35S promoter- containing constructs were active in expressing functional PGIP1 in two of each of the three lines. So, even though the *gst1* promoter seemed not to be inducible, the fact that it was active in directing expression of the apple *pgip1* gene was still an important result. The ADA only gives a qualitative indication of PG-inhibiting activity in extracts prepared from transgenic *A. thaliana* lines. No conclusions can be made on the exact levels of PGIP1 expression in the two different promoter-driven constructs, since a western blot quantifying the PGIP was not performed. Since transgenic plants that were not treated with methyl-salicylate also showed PGIP expression, it is hypothesised that the plants were already stressed, or that the *gst1* promoter contains a level of constitutive expression. This hypothesis can be tested by growing the *A. thaliana* under more favourable conditions, so that the plants are not physiologically stressed, before repeating the induction experiments. A positive control for determining if Me-Sa is inducing defence gene expression, would be a northern blot for the endogenous *GST1* transcipts or other salicylic acid pathway genes, such as those encoding PR-proteins. For example, PR-1a and the PR-2 genes of tobacco are salicylic acid-inducible (Durner *et al.*, 1997)

This chapter reported on the production and characterisation of *A. thaliana* plants containing the apple *pgip1* gene. The next two chapters will deal with the more important section of this study: the apple *pgip1* transgenic potato cv. BP1 lines. Chapter 5 will report on the molecular analysis of the transgenic lines, while Chapter 6 will give inhibition results of their PGIP extracts against *V. dahliae* PG.

CHAPTER 5

Molecular analysis of the apple *pgip1* gene in transgenic potato

5.1 Introduction

The apple *pgip1* gene has been isolated previously at ARC-Roodeplaat (Arendse *et al.*, 1999). It was inserted into the pRTL2 vector, which provided the enhanced CaMV 35S (e35S) promoter, TEV leader and CaMV 35S terminator to form an expression cassette. The cassette was transferred to the binary plant transformation vector pCAMBIA2300 (**Appendix C**). Constructs containing the cassette in either orientation were obtained. These constructs (called pCAMBIA2300-ap*pgip1*A and pCAMBIA2300-ap*pgip1*B) were transferred to *Agrobacterium tumefaciens* LBA4404 by direct transformation. Potato cv. BP1 was transformed with both constructs using *Agrobacterium*-mediated transformation and 29 independent transgenic lines were generated (A. Veale, ARC-Roodeplaat). They were selected for kanamycin resistance, which is conferred to the plant by the pCAMBIA construct. The *nptII* gene, conferring kanamycin resistance, is located between the T-DNA borders and is transferred to the plant genome together with the apple *pgip1* cassette. This chapter reports on the molecular analysis of these transgenic potato lines. The aim was to determine the presence of the apple *pgip1* transgene in the lines, before analysing the transgene expression by preparing protein extracts and testing them in inhibition assays with fungal PGs.

Two methods were used to characterise the transgenic potato lines at the molecular level. PCR was performed to verify the presence of the transgene in the plant genomic DNA. Southern blot was applied to a few selected lines to confirm the insertion of the transgene into the genomic DNA and to determine the number of copies of the transgene and insertion events that took place during transformation.

Southern blotting

E. M. Southern developed a technique for transferring size-separated DNA fragments from an agarose gel to a membrane where it is then analysed by hybridisation with a DNA probe (Southern, 1975). Several methods exist whereby DNA can be transferred, including vacuum, electro- and capillary transfer. Capillary transfer is the simplest as well as the most efficient method, and it requires no special equipment. It is usually performed overnight to result in the most complete transfer.

The DIG-system from Roche Diagnostics (Mannheim, Germany) is a nonradioactive nucleic acid labelling and detection method. Digoxigenin (DIG) is a steroid hapten that is coupled to dUTP, UTP or ddUTP. It is incorporated into a nucleic acid probe by performing various enzymatic reactions in the presence of these DIG-linked uracil nucleotides. The hybridisation with DIG-labelled probes is done according to standard protocols, except that a special blocking reagent is needed to reduce background. After hybridisation of the labelled probe to the target nucleic acid on a blot, the signals are detected by methods used for western blots. An antibody against digoxigenin is conjugated to alkaline phosphatase, which is detected by colorimetric or chemiluminescent alkaline phosphatase substrates. The colorimetric signal (e.g. NBT (nitroblue tetrazolium) and BCIP (5-bromo-3-chloro-3-indolyl phosphate)) develops directly on the membrane. The chemiluminescent signal is caused by the enzymatic dephosphorylation of CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2`-(5`-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate) by alkaline phosphatase, which leads to light emission at a maximum wavelength of 477 nm at the site of the hybridised probe. This is then recorded on an X-ray film. The advantages of using DIG-labelled probes are that they are much safer than radioactive probes, and are stable for a long period at -20°C.

An alkali-stable form of DIG-dUTP is used for labelling fragments that will be transferred from a gel to a membrane using alkaline blotting techniques. This is useful for labelling a DNA molecular weight marker that will be electrophoresed on the same agarose gel as the genomic DNA samples. The alkali-labile form of DIG-dUTP, on the other hand, is used to prepare a labelled probe using PCR. This enables subsequent rehybridisation of the blots by stripping the DIG molecule from the blot under alkaline conditions.

The hypothesis is that the apple *pgip1* transgene will be present in most, if not all, of the kanamycin-resistant *in vitro* putative transgenic potato lines. It is expected that the gene will still be present in genomic DNA isolated from plants that were grown in the glasshouse, because they are stable transformants. Southern blotting of transgenic potato genomic DNA is expected to show single or few insertions of the transgene into the genome, as is usually observed when plants are transformed by the T-DNA of *A. tumefaciens* (Hooykaas and Schilperoort, 1992).

5.2 Materials and Methods

All chemicals and reagents used were either analytical or molecular biology grade. Buffers, solutions and media were all prepared using distilled water and were autoclaved. They are described in **Appendix A**. Restriction endonucleases, RNase A and DIG probe synthesis and detection kits were obtained from Roche Diagnostics (Mannheim, Germany).

5.2.1 Isolation of genomic DNA from plant leaf material

5.2.1.1 Small scale isolation of plant genomic DNA (2% CTAB method)

Small-scale isolation of genomic DNA was performed using an adapted method from Murray and Thompson (1980). Two leaf disks were collected in 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -70°C. The leaves were thawed before use, a small volume of carborundum (400 grit) was added, and ground in the 1.5 ml tube using an Ultra Turrox. The ground leaf material was resuspended in 400 µl of CTAB DNA extraction buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris (pH 8.0), 0.2% (v/v) β-mercaptoethanol], preheated to 60°C. The samples were then incubated at 60°C for 30 minutes, with mixing every 10 minutes. The samples were allowed to cool to room temperature before being extracted with an equal volume of chloroform: isoamyl alcohol (CIAA, 24:1). After mixing for 5 minutes, the phases were separated by centrifugation at 4500×g for 10 minutes at 22°C. The DNA-containing aqueous phase (400-450 µl) was recovered, transferred to a clean Eppendorf tube and the DNA was precipitated by the addition of an equal volume of ice-cold isopropanol. The tubes were mixed and incubated at -20°C for 30 minutes to overnight.

The precipitated DNA was pelleted by centrifugation at 6500×g for 15 minutes. The DNA pellet was washed with 500 µl 70% ethanol, air-dried and resuspended in 20 to 40 µl 1× TE (pH 8.0). The DNA concentration of each sample was determined fluorometrically using a Sequoia-Turner 450 fluorometer (Sequoia-Turner Corporation) as described before.

To clean up RNA contamination from gDNA that was isolated using the 2% CTAB method, it was treated with RNase A and reprecipitated. The sample's volume was adjusted to 400 µl with 1× TE (pH 8.0). Two and a half microlitres RNase A (10 mg/ml) was added and incubated at 37°C for 3 hours. Four hundred microlitres 1 M NaCl was added and the tubes incubated at room temperature for 30 min with occasional inversion of the tube. The DNA was precipitated with 400 µl isopropanol and 10 min incubation at -20°C. The tubes were centrifuged at 6500×g for 10 min at 4°C, and the pellet washed with 500 µl 70% ethanol. After centrifugation, all the liquid was removed and the pellet allowed to air-dry. The pellet was dissolved in 1× TE (pH 8.0) to a final concentration of 100 ng/µl.

5.2.1.2 Large-scale plant genomic DNA isolation (Dellaporta method)

Large-scale isolation of genomic DNA was performed on 10 g of leaf material, collected from glasshouse plants and stored at -70°C. The method of Dellaporta *et al.* (1983) was used. The material was ground to a fine powder using liquid nitrogen and a mortar and pestle. The ground leaf material was resuspended in 60 ml of DNA extraction buffer [100 mM Tris (pH 8.0), 0.5 M NaCl, 50 mM EDTA, 0.07% (v/v) β-mercaptoethanol] in a 250 ml centrifuge tube. Eight millilitres of 20% SDS was added and the sample mixed thoroughly. The samples were incubated at 65°C for 30 minutes while shaking. Twenty millilitres of 5 M KOAc was added and the samples incubated at 0°C for 20 min. The cell debris was pelleted by centrifugation at 6500×g for 20 minutes at 4°C. The supernatant was filtered through muslin cloth wetted with 40 ml cold isopropanol, and the sample incubated at -20°C for 30 min. The DNA was pelleted by centrifugation at 6500×g for 15 minutes at 4°C. The supernatant was decanted and the pellet dried by inversion of the tube for 10 min. The pellet was resuspended in 3 ml 1× TE (pH 8.0), 150 µl RNase A (10 mg/ml) was added, and the pellets allowed to dissolve overnight at 10°C. The dissolved DNA was split into three 2.2 ml Eppendorf tubes, and each tube extracted twice with 1000 µl phenol: chloroform (1:1). The phenol had been equilibrated with Tris buffer to a pH of 7.9. The phases were separated by centrifugation at 6500×g for 10 minutes at 4°C. Each tube was extracted once with 1 volume (1000 µl) chloroform, and the phases separated by centrifugation. One tenth of the volume (100 µl) 3 M NaOAc and an equal volume (1000 µl) cold 100% ethanol was added to each tube, mixed and incubated at -70°C for 10 minutes. The precipitated DNA was pelleted by centrifugation at 4500×g for 10 minutes at 4°C. The DNA pellet was washed with 1 ml 70% ethanol, pelleted by centrifugation and air-dried. The DNA pellets from the three tubes corresponding to the same plant were resuspended in 150 µl 1× TE (pH 8.0) each and pooled. The tubes were centrifuged twice at 4500×g for 5 minutes and the clear supernatant transferred to a new tube to remove the milky suspension still present in the DNA solution. The DNA concentration of each sample was determined fluorometrically using a Hoefer TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco) as described before.

For PCR it was required to clean up the genomic DNA. The sample's volume was adjusted to 500 µl with 1× TE (pH 8.0). It was extracted once with 1 volume phenol: chloroform (1:1), centrifuged at 6500×g for 10 min at 15°C, and the top layer removed to a new tube. The top layer was extracted once with 1 volume chloroform: isoamyl alcohol (CIAA; 24:1) and centrifuged again. The gDNA was precipitated from the top layer with NaCl and isopropanol using the same method as when cleaning up the small-scale isolated gDNA. The pellet was dissolved in 50 µl 1× TE (pH 8.0) overnight at 4°C and the DNA concentration determined fluorometrically.

5.2.2 PCR with plant genomic DNA for screening of putative apple *pgip1* transformants

PCR was conducted in 0.2 ml thin-walled tubes in a MJ Research PTC-100 or PTC-200 Programmable Thermal Controller (MJ Research Inc.). The reaction mixture, in a total volume of 10 µl, contained 1× Taq reaction buffer, 200 µM of each dNTP, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase (Promega) and 50 - 120 ng gDNA as template. The reaction volume was made up to 10 µl using sterile dH_2O. The reaction mixture was overlaid with one drop of mineral oil to prevent evaporation. Positive controls contained 15 - 30 ng plasmid DNA or apple *pgip1* transgenic tobacco (LA Burley: *pgip1* #8) gDNA. Negative controls containing dH_2O and untransformed BP1 potato gDNA were also included.

The transgene specific primer combinations used for amplification were as follow (see **Appendix B** for sequences):

For amplification of the apple *pgip1* gene: AP-PGIP-L2 and AP-PGIP-R.

For amplification of the *nptII* gene (conferring kanamycin resistance): NPTII-L and NPTII-R.

The PCR cycling conditions included an initial denaturation step of 94°C for 2 min. This was followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 45 s or 1 min. The annealing temperature was adjusted to 62°C for the NPTII primers. A final extension step at 72°C for 2 min was included.

PCR products were separated on a 1% (w/v) agarose gel containing 0.06 µg/ml ethidium bromide in 0.5× TAE buffer (pH 8.0). The DNA was visualised under ultraviolet light.

5.2.3 Southern blot hybridisation of selected apple *pgip1* transgenic potato lines

5.2.3.1 Apple *pgip1* fragment preparation for spiking untransformed genomic DNA during Southern blot hybridisation

Fifteen microgram pAppRTL2 plasmid was digested overnight at 37°C with 100U *Pst*I enzyme in the appropriate restriction enzyme buffer. The amount of pAppRTL2 plasmid DNA, required to represent different numbers of copies of the gene in 10 µg potato gDNA, was calculated. The constant amount of nuclear DNA present in a tetraploid potato (*Solanum tuberosum* L. 2n = 4X) cell is 8.4 pg (Arumuganathan and Earle, 1991). Ten microgram of potato gDNA used for a Southern blot represents 1.2×10^6 genome copies. One picogram of DNA represents 0.965×10^6 kb, thus 1 kb of DNA equals 1.03×10^{-6} pg. The size of pAppRTL2 is 4784 bp, which equals 4.956×10^{-6} pg. The

mass of 1.2×10^6 copies of pAppRTL2 is therefore 5.95 pg. Thus, 5.95 pg pAppRTL2 plasmid DNA represents 1 copy of the apple *pgip1* gene in 10 µg potato gDNA.

5.2.3.2 Preparation of DIG-labelled apple *pgip1* probe

The apple *pgip1* gene was labelled non-radioactively with DIG to use as a DNA probe in Southern blot analysis of apple *pgip1* transgenic potato lines. The probe was prepared in a PCR reaction containing DIG-dUTP (alkali-labile) using the PCR DIG Probe synthesis kit (Roche Diagnostics). The reaction consisted of 30 ng pAppRTL2 plasmid containing the gene as template, 0.5 µM of each PCR primer (AP-PGIP-L2 and AP-PGIP-R), PCR buffer with $MgCl_2$, PCR DIG mix and Expand High fidelity enzyme mix. The reaction was made up to a total volume of 50 µl with dH_2O. It was overlaid with mineral oil and the same PCR cycling conditions was used as for the screening of transformant plants. The labelled PCR product was analysed on a 1% agarose gel alongside an unlabelled PCR product to confirm labelling.

5.2.3.3 End-labelling of λDNA/ *Hind*III with DIG-dUTP to use as DNA molecular weight marker

λDNA digested with *Hind*III (Molecular weight marker (MWM) II from Roche Diagnostics) was labelled with DIG by filling in the ends with Klenow enzyme in the presence of dATP, dCTP, dGTP and DIG-11-dUTP (alkali-stable, Roche Diagnostics). The reaction contained 1 µg λDNA (digested with *Hind*III), 1× buffer B (restriction enzyme buffer from Roche Diagnostics), 200 µM each of dATP, dCTP and dGTP, 40 µM DIG-11-dUTP (alkali-stable) and 3U Klenow enzyme (Roche Diagnostics) in a total volume of 50 µl. The reaction was incubated at 37°C for 3 h, after which the enzyme was heat inactivated at 65°C for 15 min. 60 ng of labelled λDNA/ *Hind*III was electrophoresed together with the samples for Southern blot in an agarose gel, which was subsequently blotted to a membrane.

5.2.3.4 Restriction digestion of potato genomic DNA

Samples of potato gDNA were restriction digested with 3U of enzyme per µg gDNA. Restriction enzymes used included *Nsi*I, *Nco*I, *Bam*HI, *Pvu*I and *Pst*I. The reaction contained the appropriate 1× restriction enzyme buffer, gDNA and restriction enzyme in a total volume of 30 µl and was incubated at 37°C overnight. The digestions were checked by agarose gel electrophoresis.

For Southern analysis, 10 µg genomic DNA was digested with different restriction enzymes (*Nsi*I, *Nco*I and *Bam*HI). The reaction contained the appropriate restriction enzyme buffer in a total volume of 300 µl and was incubated overnight at 37°C. Small samples were analysed by agarose gel electrophoresis to check if digestion was complete. More enzyme was added and the reactions incubated longer if needed. The digested samples were precipitated with 1/20th volume 5 M NaCl and

2.5 volumes cold ethanol and incubation at -20°C for 1 h. After centrifugation at 6500×g for 20 min, the pellet was air-dried and resuspended in 30 µl 1× TE (pH 8.0). The fragments were separated by overnight electrophoresis at 7°C on a 1% agarose gel.

5.2.3.5 Southern blotting of DNA onto nylon membrane

After electrophoresis, the agarose gel containing DNA fragments separated by electrophoresis was stained in 0.5 µg/ml ethidium bromide for 10 minutes while gently shaking. The gel was visualised using a UV illuminator and photographed. Southern blot of the fragments to a nylon membrane was performed using standard protocols (Southern, 1975).

The agarose gel containing DNA fragments separated by electrophoresis was depurinated in 0.25 M HCl for 10 min, denatured for 2 × 15 min in denaturation solution [0.4 N NaOH, 0.6 M NaCl] and neutralised in neutralisation solution [0.5 M Tris [pH 7.5], 1.5 M NaCl] for 2 × 15 min. The Southern blot was set up as follows: a wick, made from Whatman 3MM filter paper (Whatman International), was placed onto a glass plate on top of a plastic support in a shallow tray containing 20× SSC [3 M NaCl, 0.3 M Sodium citrate, pH 7.4]. The gel was placed upside-down onto the wick, and the blotting membrane (Osmonics Magnacharge nylon transfer membrane, Amersham-Pharmacia Biotec, Little Chalfont, UK) cut to the same size and placed on top of the gel. Care was taken so that no air bubbles were trapped between the layers. Two layers of filter paper were cut to the same size as the membrane and placed on top, followed by a stack of dry paper towels. A glass plate with a weight was placed on top of the stack, and held in position by a retort stand. The transfer was allowed to take place overnight. After overnight transfer, the membrane was rinsed in 2× SSC to remove the excess 20× SSC, placed between clean sheets of filter paper and baked for 2 h at 80°C to fix the blotted DNA to the membrane. The baked membrane was stored in aluminium foil at room temperature until hybridisation.

5.2.3.6 Hybridisation and detection of DIG-labelled probe

The DIG-labelled apple *pgip1* probe was hybridised to the blot and the signal detected using the protocols as set out in the DIG System Users Guide for Filter Hybridization (*Boehringer Mannheim*). The membrane was rolled, with the DNA side on the inside, and placed in a hybridisation bottle. It was prehybridised for 5 h at 42°C in 20 ml of DIG Easy HYB solution (Roche Diagnostics) containing denatured salmon testes DNA at a final concentration of 125 µg/ml. Salmon testes DNA was denatured by boiling for 10 min and quick-chilling on ice. For the hybridisation step, 20 ml fresh prehybridisation solution was prepared. Two microlitres of PCR DIG-labelled probe per ml hybridisation solution and salmon testes DNA to a final concentration of 125 µg/ml were denatured the same way and added to the preheated DIG Easy HYB solution. The prehybridisation solution in

the roller bottle was replaced with the hybridisation solution without allowing the membrane to dry out. Hybridisation was performed overnight at 42°C.

Post-hybridisation washes were performed at high stringency, as outlined in the DIG detection kit manual. In short, the protocol consisted of washing the membrane 2 × 5 min in stringency washing buffer I [2× SSC, 0.1% SDS] at room temperature. These washes were followed with 2 × 15 min washes in stringency washing buffer II [0.5× SSC, 0.1% SDS] at 65°C. The membrane was blocked and DIG detected using the DIG Wash and Block Buffer set and the DIG Luminescent detection kit for nucleic acids (Roche Diagnostics). First, the membrane was rinsed in washing buffer, and then blocked in 1% blocking solution for 30 min at room temperature. The membrane was incubated in 75 mU/ml Anti-DIG AP (the Fab fragment of polyclonal sheep anti digoxigenin, conjugated to alkaline phosphatase, diluted 1:10 000 in blocking buffer) for 30 min at room temperature. This was followed by 2 × 15 min washes in washing buffer, after which the membrane was equilibrated in detection buffer for 5 min. The chemiluminescent substrate CSPD (25 mM) was diluted 1:100 in detection buffer, and the membrane incubated with it in a sealed plastic bag for 5 min. Excess liquid was removed, the bag re-sealed and incubated at 37°C for 15 min. The membrane was exposed to X-ray film (Hyperfilm ECL High performance chemiluminescence film, Amersham-Pharmacia Biotec) for 1 h and the film developed.

5.3 Results

5.3.1 PCR analysis of putative apple *pgip1* transformant plants

PCR analysis was performed to verify the insertion of the transgene into the genome of putative transgenic plants. gDNA was isolated from untransformed and putative apple *pgip1* transgenic tobacco and potato plants to use as template in a PCR reaction.

5.3.1.1 PCR of putative apple *pgip1* transgenic potato *in vitro* plants

Small-scale isolation of genomic DNA was performed on *in vitro* leaf material from 29 putative transgenic BP1 potato lines, a positive control LA Burley: *pgip1* #8 tobacco and the negative controls untransformed LA Burley tobacco and BP1 potato. Two leaf disks from separate leaves of each *in vitro* plant were collected into a 1.5 ml Eppendorf tube and genomic DNA extracted from it using a small-scale 2% CTAB method. PCR was performed with 120 ng of this gDNA as template, using the apple *pgip1* and *nptII* primer sets. A 1024 bp fragment was expected for the apple *pgip1* primers and a 699 bp fragment for the *nptII* primers. Figure 5.1 indicates that the apple *pgip1* gene was present in 22 of the 29 putative transgenic *in vitro* potato lines and the transgenic LA Burley: *pgip1* #8 tobacco (indicated by arrows). The PCR-positive potato lines were the following: A3, A5, A6, A7, A8, A9, A10, A11, A12, A14, 7A, B3, B4, B5, B7, B9, B10, B11, B12, B13, B16 and B18. The seven apple *pgip1* PCR-negative lines were A2, A4, A13, B1, B2, B6 and B17. The *nptII* gene was present in all lines except line A4 (Figure 5.2; lane 5).

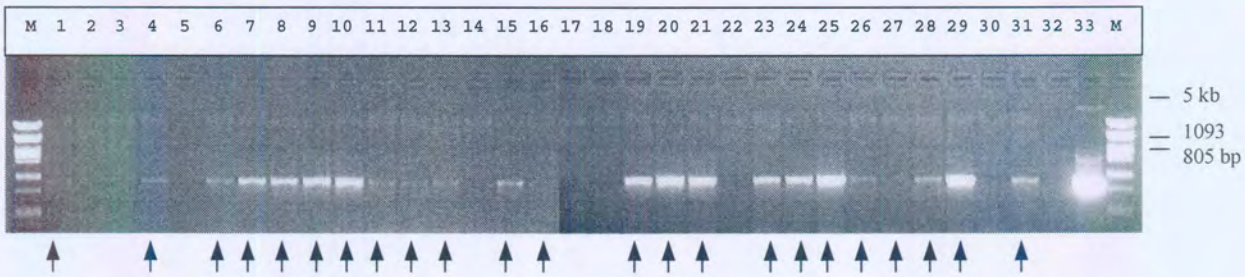


Figure 5.1 **Apple *pgip1* PCR with gDNA from *in vitro* transgenic potato leaf material.** M: λDNA/*Pst*I marker; lane 1: LA Burley: *pgip1* #8 positive control; lane 2: untransformed LA Burley negative control; lanes 3 to 31: putative transgenic potato lines A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, 7A, B1, B2, B3, B4, B5, B6, B7, B9, B10, B11, B12, B13, B16, B17 and B18, respectively; lane 32: negative water control; lane 33: positive control with plasmid as template.

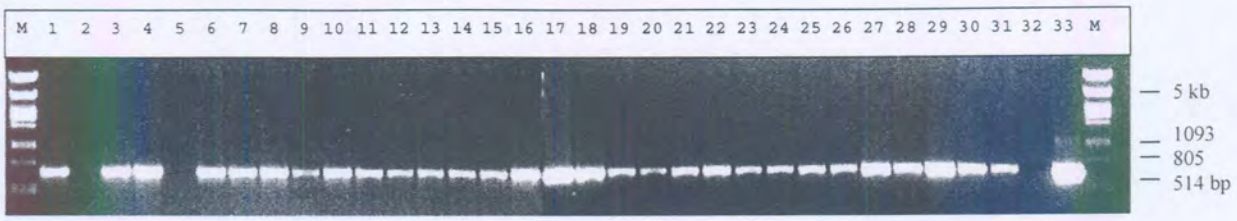


Figure 5.2 ***nptII* PCR with gDNA from *in vitro* transgenic potato leaf material.** M: λDNA/ *Pst*I marker; lane 1: LA Burley: *pgip1* #8 positive control; lane 2: untransformed LA Burley negative control; lanes 3 to 31: putative transgenic potato lines A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, 7A, B1, B2, B3, B4, B5, B6, B7, B9, B10, B11, B12, B13, B16, B17 and B18, respectively; lane 32: negative water control; lane 33: positive control with plasmid as template.

5.3.1.2 PCR to verify the presence of the apple *pgip1* gene in the glasshouse transgenic material

Genomic DNA was isolated from glasshouse leaf material of 20 putative transgenic BP1 potato lines, untransformed BP1 and a positive control transgenic LA Burley: *pgip1* #8 tobacco. gDNA was isolated on a small scale from 14 potato lines, while the Dellaporta *et al.* (1983) large-scale method was used for the other six lines (A5, A6, A9, B10, B11 and B13) and untransformed BP1.

When PCR was performed using this gDNA as template and the apple *pgip1* primers, only one potato line gave a good amplification product (7A, Figure 5.3, lane 12) with another line giving a faint product (B9, lane 15). There were smears visible around the primer-dimers in the lines of which gDNA was isolated using the small-scale CTAB method (Figure 5.3, lanes 2, 5, 6, 8 to 15, 18, 20 and 21).

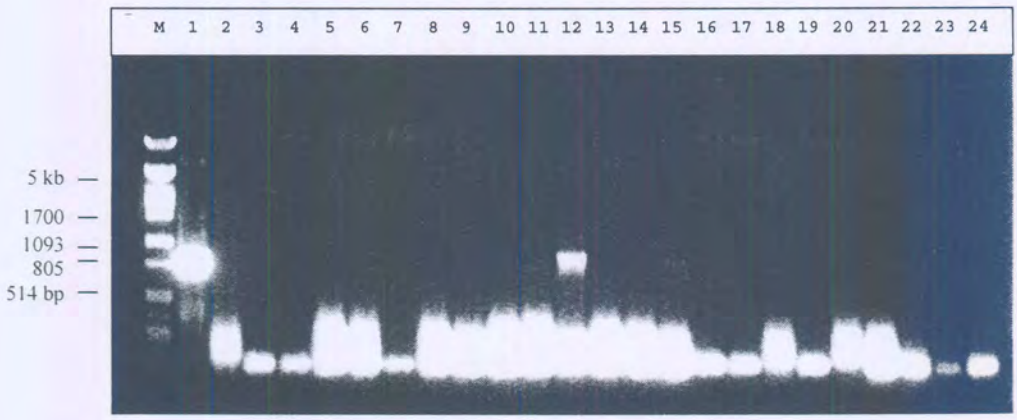


Figure 5.3 **Unsuccessful PCR with gDNA from glasshouse transgenic potato leaf material.** M: λDNA/ *Pst*I marker; lane 1: positive control with plasmid as template; lanes 2 to 21: putative transgenic potato lines A3, A5, A6, A7, A8, A9, A10, A11, A12, A14, 7A, B3, B5, B9, B10, B11, B12, B13, B16, and B18, respectively; lane 22: LA Burley: *pgip1* #8 positive control; lane 23: untransformed BP1 negative control; lane 24: negative water control.

Upon investigation, a high amount of contaminating RNA was observed in these samples (Figure 5.4, lanes 1 to 5). No RNA was present in gDNA isolated from the seven lines using the Dellaporta *et al.* (1983) method (samples A5 and B13 as examples, Figure 5.4, lane 6 and 7, respectively). It seemed as if the RNA inhibited the PCR, so the samples were treated with RNase A and the gDNA reprecipitated. Figure 5.5 shows two examples of the cleaned-up gDNA (lines B3 and B9, lanes 2 and 3, respectively). A small amount of smearing of gDNA is visible (Figure 5.5, lanes 2 and 3), but the RNase A treatment was successful in removing the RNA contamination.

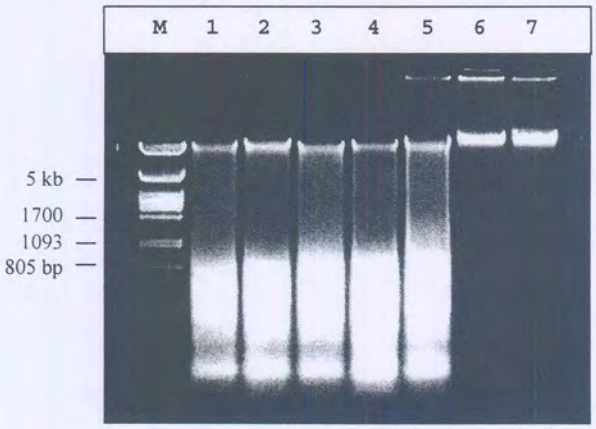


Figure 5.4 **RNA contamination of gDNA** (prepared from glasshouse transgenic potato leaf material). RNA contamination of gDNA extracted from transgenic glasshouse leaf material using the small-scale CTAB isolation method. M: λDNA/ *Pst*I marker; lane 1 to 5: ~300 ng gDNA samples from lines A3, A11, 7A, B9 and LA Burley: *pgip1* #8, respectively, isolated using the small-scale CTAB method; lanes 6 and 7: ~300 ng gDNA samples from lines A5 and B13, respectively, isolated using the large-scale Dellaporta *et al.* (1983) method.

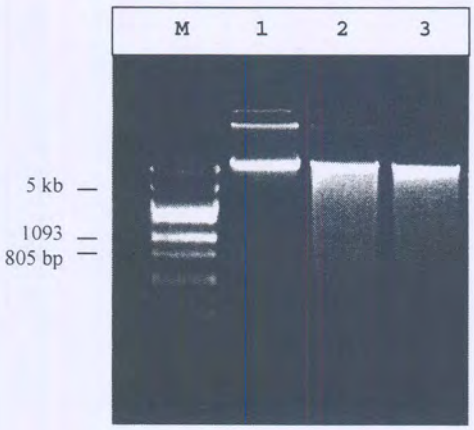


Figure 5.5 **gDNA cleaned up from RNA contamination.** M: λDNA/ *Pst*I marker; lane 1: gDNA from LA Burley: *pgip1* #8 isolated using the large-scale Dellaporta *et al.* (1983) method; lanes 2 and 3: gDNA from lines B3 and B9, respectively, isolated using the small-scale CTAB method and treated with RNase A.

Repeated attempts of PCR using large-scale isolated gDNA as template were unsuccessful (results not shown). Since difficulties were experienced during PCR, it was thought that the preparation contained inhibitors of PCR. An 11 µg gDNA sample of each plant line was re-extracted with phenol: chloroform (1:1) and reprecipitated to clean it up from possible inhibitors. PCR was repeated on the cleaned-up gDNA from both the small-scale and large-scale isolated gDNA samples. This time PCR amplification of the apple *pgip1* gene (Figures 5.6 A and B) and the kanamycin resistance gene (Figure 5.7) from all 20 lines was successful. Line A11 failed to yield an amplification product with the apple *pgip1* primers in this experiment (Figure 5.6 A, lane 8), but it did work during a previous PCR (Figure 5.6 B, lane 1 (arrow)). PCR with transgenic LA Burley: *pgip1* #8 gDNA as template yielded a very faint band with AP-PGIP primers (Figure 5.6 A, lane 22), but other PCRs were successful in amplifying the transgene from this plant (results not shown). The untransformed BP1 and water negative controls yielded no amplification products, as expected.

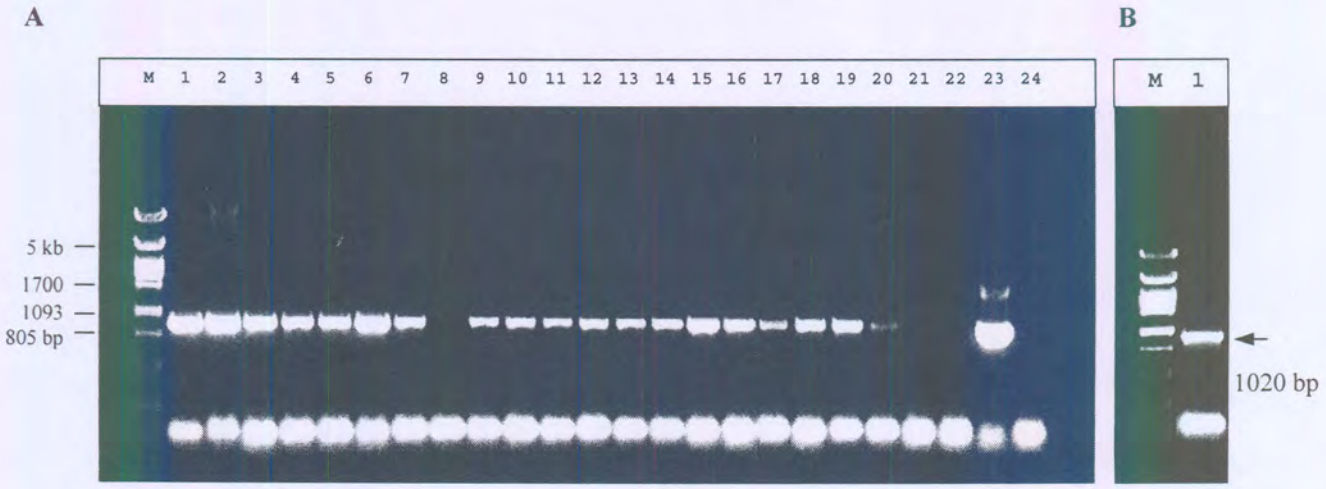


Figure 5.6 **Apple *pgip1* PCR with gDNA from glasshouse transgenic potato leaf material.**

A. All glasshouse potato lines gDNA as template. M: λDNA/ *Pst*I marker; lanes 1 to 20: apple *pgip1* PCR with gDNA from putative transgenic potato lines A3, A5, A6, A7, A8, A9, A10, A11, A12, A14, 7A, B3, B5, B9, B10, B11, B12, B13, B16, and B18, respectively; lane 21: untransformed BP1 negative control; lane 22: LA Burley: *pgip1* #8 positive control; lane 23: positive control with plasmid as template; lane 24: negative water control.

B. Putative transgenic potato line A11 gDNA as template. M: λDNA/ *Pst*I marker; lane 1: apple *pgip1* PCR with genomic DNA isolated from glasshouse leaf material of putative transgenic potato line A11.

The kanamycin resistance gene was amplified from all 20 putative transgenic potato lines as well as LA Burley: *pgip1* #8 using the *nptII* primer set (Figure 5.7). The untransformed BP1 and water negative controls yielded no amplification products, as expected.

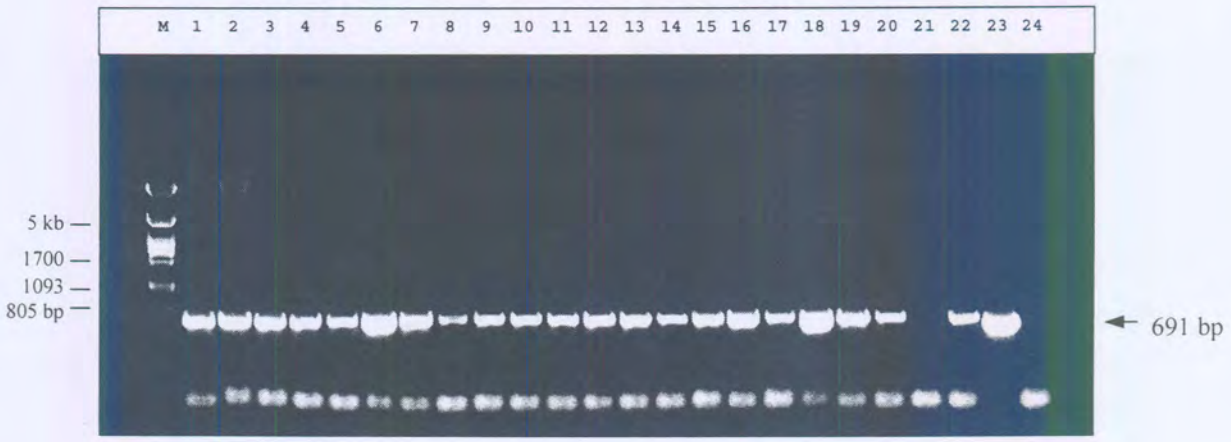


Figure 5.7 ***nptII* PCR with gDNA from glasshouse transgenic potato leaf material.** M: λDNA/*Pst*I marker; lanes 1 to 20: *nptII* PCR with gDNA from putative transgenic potato lines A3, A5, A6, A7, A8, A9, A10, A11, A12, A14, 7A, B3, B5, B9, B10, B11, B12, B13, B16, and B18, respectively; lane 21: untransformed BP1 negative control; lane 22: LA Burley: *pgip1* #8 positive control; lane 23: positive control with plasmid as template; lane 24: negative water control.

5.3.2 Southern blot hybridisation of selected apple *pgip1* transgenic potato lines

Six transgenic potato lines were randomly selected to analyse transgene insertion into the plant genome by Southern blot hybridisation. Untransformed BP1 potato gDNA was included in the Southern blot to serve as a negative control.

5.3.2.1 Apple *pgip1* fragment preparation for spiking untransformed genomic DNA during Southern blot hybridisation

The plasmid pAppRTL2 (**Appendix C**) was used as a source of the apple *pgip1* gene to spike untransformed potato gDNA during Southern blot analysis of selected transgenic potato lines. pAppRTL2 was digested completely with *Pst*I to release the apple *pgip1* gene. In pAppRTL2 the apple *pgip1* gene is part of a cassette containing also the CaMV e35S promoter, TEV leader and CaMV terminator. Digestion with *Pst*I releases a fragment 1893 bp in length containing the CaMV e35S promoter, TEV leader and apple *pgip1* gene. Figure 5.8 shows *Pst*I digested (Figure 5.8, lane 2) and undigested (Figure 5.8, lane 3) pAppRTL2 plasmid DNA. No bands corresponding to undigested DNA are visible in lane 2, indicating that the *Pst*I digestion was complete. This *Pst*I digested pAppRTL2 plasmid DNA was used to spike untransformed gDNA during Southern blot of 10 µg

potato gDNA. The 1893 bp fragment containing the apple *pgip1* gene was not purified from the restriction digestion reaction. One copy of the apple *pgip1* gene in 10 µg potato gDNA was calculated to be represented by 5.95 pg pAppRTL2 plasmid DNA.

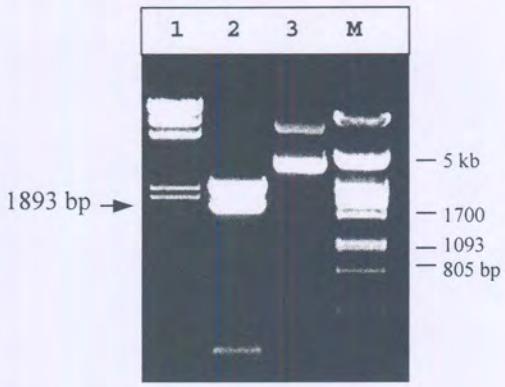


Figure 5.8 ***Pst*I restriction digestion of pAppRTL2.** Lane 1: 250 ng λDNA/ *Hind*III marker; lane 2: 350 ng *Pst*I digested pAppRTL2; lane 3: 250 ng undigested pAppRTL2; M: λDNA/ *Pst*I marker.

5.3.2.2 Preparation of DIG-labelled apple *pgip1* probe

The apple *pgip1* gene was labelled with DIG in a PCR reaction to use it as a non-radioactively labelled probe during Southern blot hybridisation. The DIG-labelled apple *pgip1* probe had a higher molecular weight compared to the unlabelled PCR product (compare lanes 1 and 2 of Figure 5.9). This is expected, due to incorporation of DIG-dUTP during the PCR process.

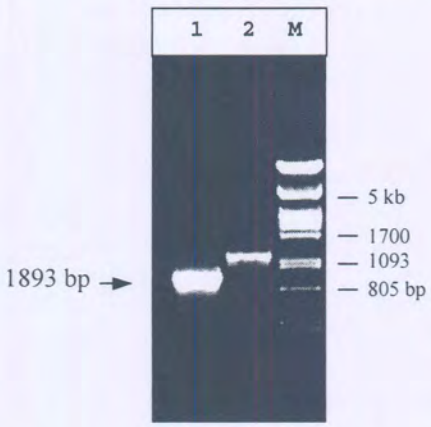


Figure 5.9 **DIG-labelled apple *pgip1* PCR product.** Lane 1: unlabelled apple *pgip1* PCR product; lane 2: DIG-labelled apple *pgip1* PCR product; M: λDNA/ *Pst*I marker.

5.3.2.3 Restriction digestion of potato genomic DNA

Before Southern blotting of the six chosen transgenic potato lines, the gDNA needed to be digested with the appropriate restriction enzymes and the fragments separated by agarose gel electrophoresis. Usually two restriction enzymes are chosen for each transgenic line. One that cuts on both sides of the

transgene (between the T-borders of the transformation vector) is selected to determine the number of copies of the transgene inserted into the plant genome. The other restriction enzyme is selected so that it doesn't have a recognition sequence between the T-borders, or cuts only once between the T-borders, so that it would cut randomly in the genome. From this the number of insertion events can be determined, since the transgene specific probe will hybridise to differently sized fragments.

Small samples of gDNA were first digested with restriction enzymes before large-scale digestions were carried out for the Southern blot. Four hundred nanogram (Figure 5.10) or 600 ng (Figure 5.11) samples of potato gDNA, isolated using the Dellaporta *et al.* (1983) method, were digested with 1.2 and 1.8U restriction enzyme, respectively. This corresponds to 3U enzyme per µg gDNA, which is the situation during large-scale digestion for Southern blot. Digestion was checked by agarose electrophoresis on 1% or 0.8% agarose gels. Undigested gDNA was loaded onto the gel to compare it with the digested samples. Complete digestion is characterised by a smear of fragments and an absence of high molecular weight gDNA.

Small-scale restriction digests indicated that *Pvu*I and *Pst*I, which both cut on both sides of the apple *pgip1* gene in both the A and B lines, digested the gDNA very poorly (Figure 5.10, lanes 4 and 5, respectively). Samples digested with these enzymes looked just like the undigested gDNA (Figure 5.10, lane 7). *Nco*I and *Bam*HI digested better, but digestion was still not complete (Figure 5.10, lanes 2 and 3, respectively). *Nsi*I digestion was the best of all the enzymes (Figure 5.10, lanes 1 and 6), with only a little high molecular weight gDNA remaining.

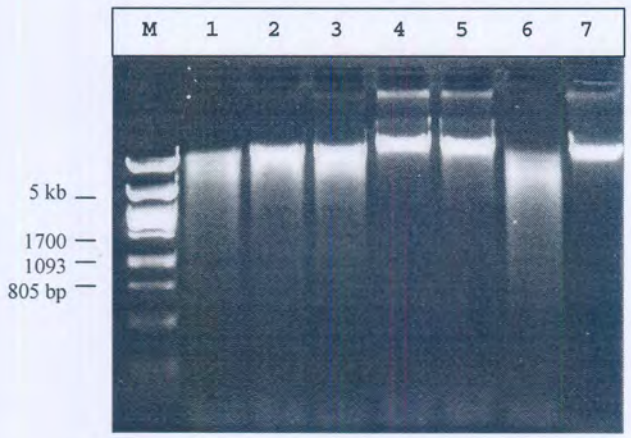


Figure 5.10 **Restriction digestion of 400 ng potato gDNA.** M: λDNA/ *Pst*I marker; lanes 1 to 5: 400 ng potato gDNA restriction digested with 1.2U of *Nsi*I, *Nco*I, *Bam*HI, *Pvu*I and *Pst*I, respectively; lane 6: 400 ng potato gDNA restriction digested with 3U of *Nsi*I; lane 7: undigested potato gDNA.

Upon repeating the restriction digestion with 600 ng gDNA, the same results were obtained (Figure 5.11). Digestion with *Nco*I and *Bam*HI was again not complete (Figure 5.11; lanes 3 to 5), with *Nsi*I

again giving the best smear of frag[illegible] 11, lane 2). Even increasing the *Bam*HI quantity to 5U did not improve digestion (Figure 5.11, lane 5).

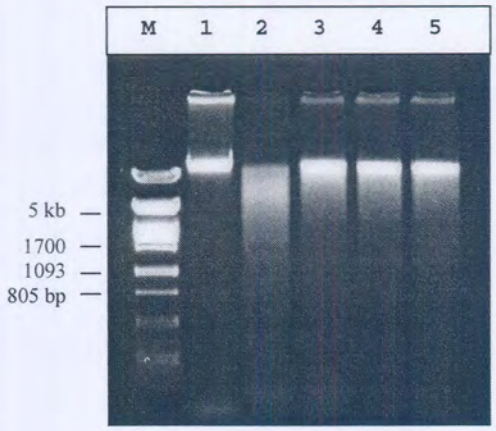


Figure 5.11 **Restriction digestion of 600 ng potato gDNA.** M: λDNA/ *Pst*I marker; lane 1: undigested potato gDNA; lanes 2 to 4: 600 ng potato gDNA restriction digested with 1.8U of *Nsi*I, *Nco*I and *Bam*HI, respectively; lane 5: 600 ng potato gDNA restriction digested with 5U of *Bam*HI.

Even though digestion with *Nco*I and *Bam*HI never seemed to be complete, large-scale digestion of gDNA were carried out for Southern blot. Eleven microgram of potato gDNA from transgenic lines A5, A6, A9, B10, B11, B13 and untransformed BP1 were digested overnight at 37°C with 33U each of *Nsi*I and either *Nco*I or *Bam*HI. Five hundred nanogram samples were checked for complete digestion by agarose gel electrophoresis. After the addition of 20U more of enzymes *Nsi*I and *Nco*I and 50U of *Bam*HI to the large-scale digestions and the incubation repeated, another 500 ng was checked. Figure 5.12 shows the digestion products after the second incubation. Digestion with *Nsi*I was good (Figure 5.12, lanes 5, 7, 9, 11, 13, 15 and 17), yielding a smear of fragments. *Nco*I digested samples contained more undigested gDNA than the *Nsi*I digestion (Figure 5.12, lanes 12, 14 and 16), while *Bam*HI digestion was poor (Figure 5.12, lanes 6, 8 and 10). Another 30U *Nco*I and 60U *Bam*HI was added and the tubes incubated overnight, before precipitating the digested gDNA and dissolving the pellet in 30 µl 1× TE (pH 8.0).

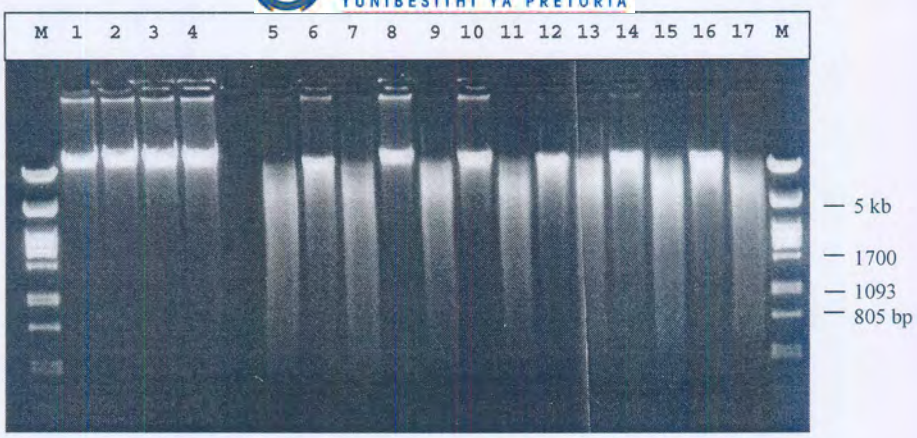


Figure 5.12 **Agarose gel to check if large-scale digestion of potato gDNA is complete.** Five hundred nanogram samples of potato gDNA digested with *Nsi*I, *Nco*I or *Bam*HI. M: λDNA/ *Pst*I marker; lanes 1 to 4: undigested gDNA from lines A9, B10, B13 and BP1, respectively; lanes 5, 7 and 9: *Nsi*I digestion of lines A5, A6 and A9 gDNA, respectively; lanes 6, 8 and 10: *Bam*HI digestion of lines A5, A6 and A9 gDNA, respectively; lanes 11, 13 and 15: *Nsi*I digestion of lines B10, B11 and B13 gDNA, respectively; lanes 12, 14 and 16: *Nco*I digestion of lines B10, B11 and B13 gDNA, respectively; lane 17: *Nsi*I digestion of BP1 gDNA.

The digested samples were electrophoresed overnight on a large gel at a low voltage (40 V) (Figure 5.13). The following were also loaded on the gel: DIG-labelled λDNA/ *Hind*III (Figure 5.13, lane 1), *Nsi*I digested untransformed BP1 gDNA (lane 20) and *Nsi*I digested untransformed BP1 gDNA spiked with 1, 10 and 20 copies of the apple *pgip1* gene (lanes 3, 4 and 5, respectively). After electrophoresis, the separated fragments were visualised by ethidium bromide staining of the gel (Figure 5.13). The contents of the agarose gel lanes are listed in Table 5.1.

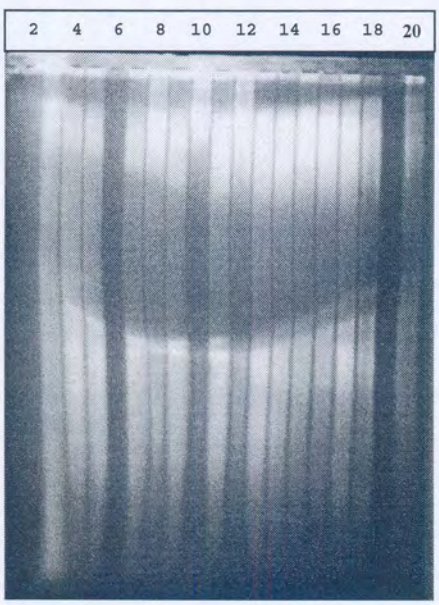


Figure 5.13 **Large-scale digestion of six transgenic potato lines for Southern blot.** Lanes were loaded as indicated in Table 5.1.

All the digestions except *Bam*HI (Figure 5.13: lanes 8, 10 and 12, especially lane 10) were complete, leading to long smears of digested gDNA in each lane. A region of the gel didn't stain well with ethidium bromide.

Table 5.1 **Lanes of agarose gel for Southern blot of six apple *pgip1* transgenic potato lines.**

Lane	DNA	Digestion by restriction enzyme
1	DIG-λDNA/ *Hind*III (60 ng)	
2		
3	Untransformed BP1	*Nsi*I digested + 1 copy apple *pgip1*
4	"	" + 10 copy apple *pgip1*
5	"	" + 20 copy apple *pgip1*
6		
7	A5	*Nsi*I
8	"	*Bam*HI
9	A6	*Nsi*I
10	"	*Bam*HI
11	A9	*Nsi*I
12	"	*Bam*HI
13	B10	*Nsi*I
14	"	*Nco*I
15	B11	*Nsi*I
16	"	*Nco*I
17	B13	*Nsi*I
18	"	*Nco*I
19		
20	Untransformed BP1	*Nsi*I digested

5.3.2.4 Southern blotting, hybridisation and detection of DIG-labelled probe

The gel containing separated fragments was blotted onto a nylon membrane using standard protocols. It was hybridised with the apple *pgip1* gene labelled with DIG. Figure 5.14 shows the result of the detection of chemiluminescence after 1 hour of exposure to an X-ray film.

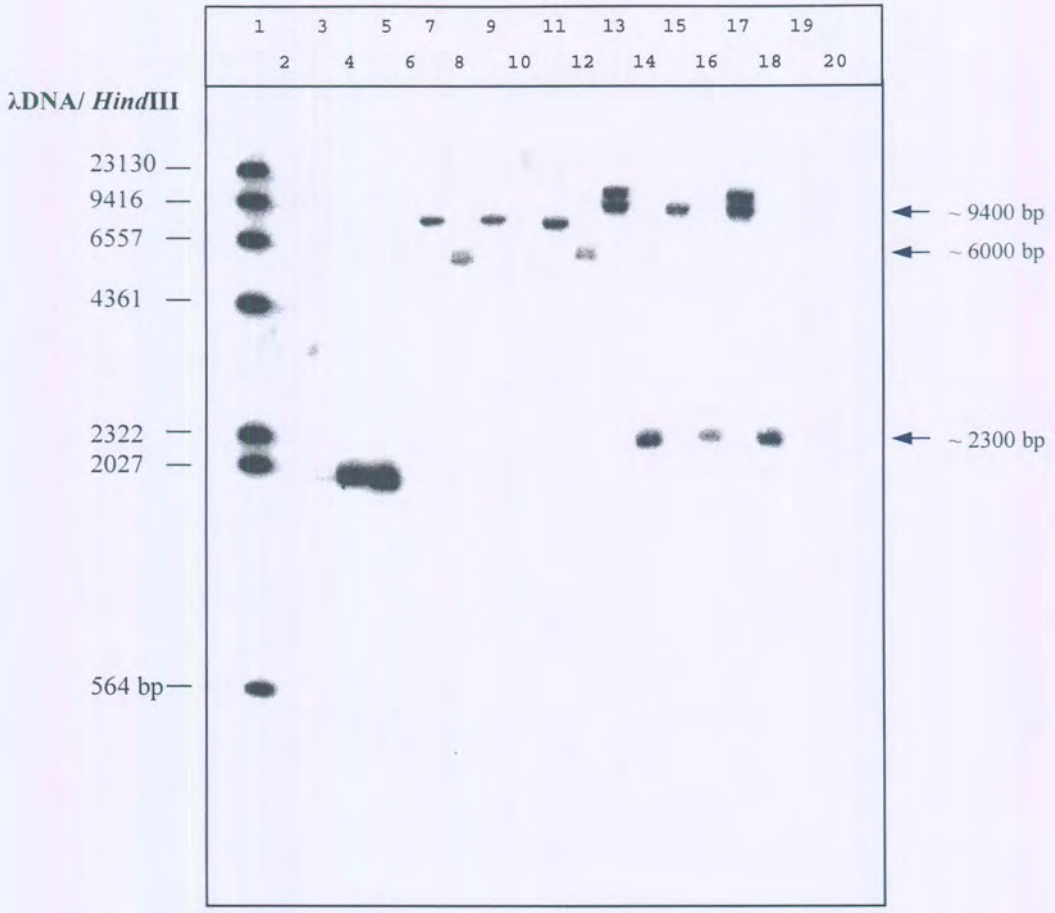


Figure 5.14 **Southern blot of six apple *pgip1* transgenic potato lines.** Contents of lanes are indicated in Table 5.1.

Using the non-radioactive DIG hybridisation and detection method, very low background signal was obtained. Klenow end-labelling of λDNA/ *Hind*III with DIG was successful, since 60 ng of labelled λDNA/ *Hind*III was sufficient for detection after electrophoresis and blotting to a membrane during Southern blot (Figure 5.14, lane 1). Sharp bands formed where the apple *pgip1* probe hybridised to the membrane and the anti-DIG antibody/ alkaline phosphatase conjugate subsequently bound. One copy of the apple *pgip1* gene spiked into 10 µg *Nsi*I digested untransformed BP1 gDNA (Figure 5.14, lane 3) was successfully detected. A fragment of 1893 bp was expected from the *Pst*I digestion of pAppRTL2. The fragment that hybridises to the apple *pgip1* probe consists of the CaMV e35S promoter, TEV leader and the apple *pgip1* gene.

*Nsi*I digestion of the apple *pgip1* transgenic potato A-lines (A5, A6 and A9) each yielded a single hybridising fragment of approximately 8000 bp (Figure 5.14, lanes 7, 9 and 11, respectively). *Bam*HI digestion of lines A5 and A9 yielded a fragment of approximately 6000 bp (lanes 8 and 12,

respectively), with lane A6 only giving a signal in the undigested large molecular weight region (lane 10). Line A9 also contained a smear of undigested gDNA in the *Nsi*I and *Bam*HI digested lanes (lanes 11 and 12, respectively). *Bam*HI digestion of the potato A-lines are expected to give a *pgip1*-hybridising fragment of 1943 bp (see Figure 5.15 and Discussion).

*Nsi*I digestion of the apple *pgip1* transgenic potato B-lines (B10 and B13) each yielded double hybridising fragments of approximately 9400 bp and larger than 9400 bp (Figure 5.14, lanes 13 and 17, respectively). Line B11 only gave a single fragment of approximately 9400 bp (lane 15). *Nco*I digestion of all three B-lines yielded fragments of approximately 2300 bp (lanes 14, 16 and 18, respectively). The intensity of the *Nco*I-fragment of line B11 (lane 16) was approximately half of that of the *Nco*I-fragments of lines B10 and B13 (lanes 14 and 18). *Nco*I digestion of the potato B-lines are expected to give a *pgip1*-hybridising fragment of 2356 bp (see Figure 5.15 and Discussion).

The lane containing *Nsi*I-digested untransformed BP1 gDNA (Figure 5.14, lane 20) was completely clear. No signal was obtained for the apple *pgip1* probe hybridising to a fragment. This means that there wasn't a sequence in potato gDNA that was sufficiently homologous to the apple *pgip1* probe to form a stable hybrid during these hybridisation conditions.

5.3.3 Restriction digestion of the pCAMBIA2300-ap*pgip1*A and pCAMBIA2300-ap*pgip1*B plasmids

The transgenic potato lines were generated by transforming potato cv. BP1 with the pCAMBIA2300-ap*pgip1*A and pCAMBIA2300-ap*pgip1*B constructs (**Appendix C**). These constructs were digested with *Bam*HI and *Nco*I, to verify the expected sizes of the excised inserts that will hybridise with the apple *pgip1* probe during Southern blot. The sizes of the obtained fragments were as expected. Digesting pCAMBIA2300-ap*pgip1*A with *Bam*HI released a fragment of 1943 bp, that contains the apple *pgip1* gene (Figure 5.15; lane 2). Digestion with *Nco*I released a fragment of 2010 bp that does not contain the gene (lane 3). *Bam*HI digestion of pCAMBIA2300-ap*pgip1*B released a 265 bp fragment (lane 5), while *Nco*I released a fragment of 2356 bp that is expected to hybridise with the *pgip1* probe (lane 6).

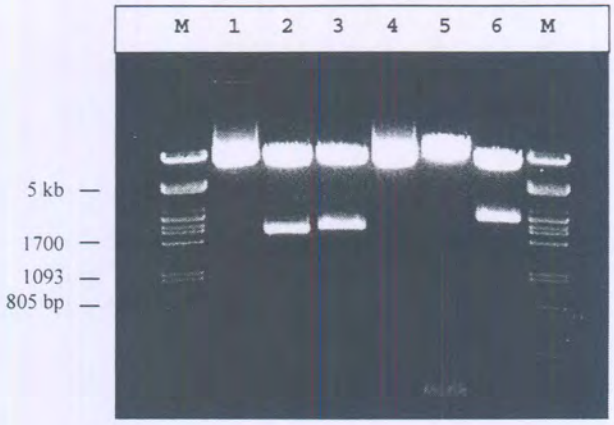


Figure 5.15 **Restriction digestion of pCAMBIA2300-ap*pgip1*A and pCAMBIA2300-ap*pgip1*B plasmids.** M: λDNA/ *Pst*I marker; lane 1: undigested pCAMBIA2300-ap*pgip1*A plasmid; lanes 2 and 3: *Bam*HI and *Nco*I digestion of pCAMBIA2300-ap*pgip1*A plasmid, respectively; lane 4: undigested pCAMBIA2300-ap*pgip1*B plasmid; lanes 5 and 6: *Bam*HI and *Nco*I digestion of pCAMBIA2300-ap*pgip1*B plasmid, respectively.

5.3.4 PCR of the six potato lines to verify the construct used for transformation

The pCAMBIA2300-ap*pgip1*A and pCAMBIA2300-ap*pgip1*B constructs differ from each other in the orientation of the CaMV e35S promoter-apple *pgip1* cassette. To verify that the six chosen potato lines were transformed with the appropriate pCAMBIA2300-appgip construct, PCR was performed utilising a vector specific primer and an apple *pgip1* specific primer. Combinations of U19F and AP-PGIP-R or AP-PGIP-L2 were used to determine the orientation of the cassette.

Amplification products of approximately 2000 bp were obtained with the pCAMBIA2300-ap*pgip1*B plasmid and the potato B-lines, using the U19F and AP-PGIP-R primer combination (Figure 5.16, lanes 5 to 8). The U19F and AP-PGIP-L2 primer combination yielded amplification products of approximately 1200 bp for the pCAMBIA2300-ap*pgip1*A plasmid, the potato A-lines and pCAMBIA2300-ap*pgip1*B plasmid (Figure 5.16, lanes 10 to 14). The negative dH_2O controls were clear. The expected amplification products are described in the Discussion.

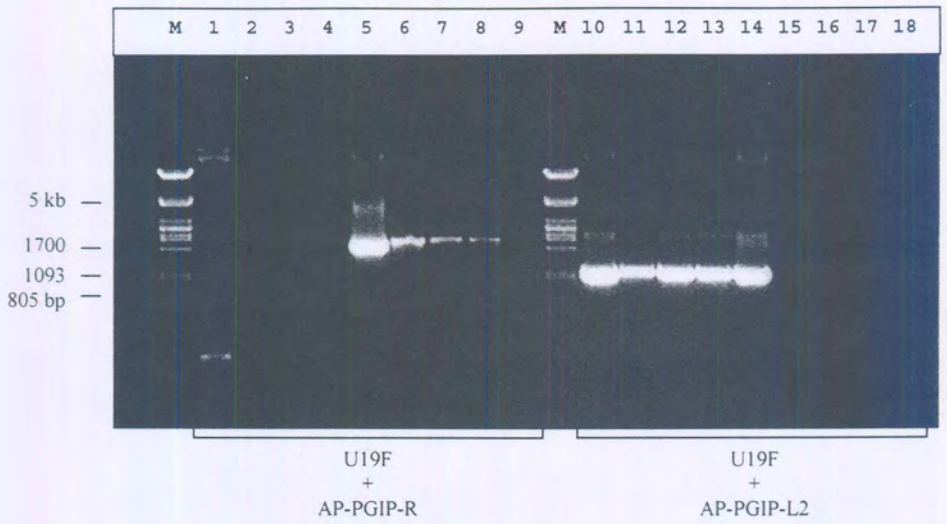


Figure 5.16 **PCR of six potato lines using U19F and AP-PGIP primers.** gDNA of the six transgenic potato lines and the pCAMBIA2300-ap*pgip1*A and pCAMBIA2300-ap*pgip1*B plasmids were used as templates for the PCR reactions. M: λDNA/ *Pst*I marker; lanes 1 to 9: U19F and AP-PGIP-R primer combination; lanes 10 to 18: U19F and AP-PGIP-L2 primer combination. The templates used for PCR were the following: lanes 1 and 10: pCAMBIA2300-ap*pgip1*A plasmid; lanes 2 and 11: A5 gDNA; lanes 3 and 12: A6 gDNA; lanes 4 and 13: A9 gDNA; lanes 5 and 14: pCAMBIA2300-ap*pgip1*B plasmid; lanes 6 and 15: B10 gDNA; lanes 7 and 16: B11 gDNA; lanes 8 and 17: B13 gDNA; lanes 9 and 18: dH_2O as negative control.

5.4 Discussion

5.4.1 PCR of putative apple *pgip1* transgenic potato *in vitro* plants

The PCR results from the reaction containing template gDNA isolated from *in vitro* potato plants indicate that there are seven transformants that contain the *nptII* gene but not the apple *pgip1* gene (Figures 5.1 and 5.2, lanes 3, 5, 14, 17, 18, 22 and 30 corresponding to the potato lines A2, A4, A13, B1, B2, B6 and B17, respectively). This would require the transgenic plant to lose one of the two genes found between the T-borders of the transformation construct that were transferred simultaneously to the genome during *Agrobacterium*-mediated transformation. This is a possible but not very likely event to occur, so the reasonable explanation is that the PCR was not successful in amplifying the apple *pgip1* gene in all the reactions. The fact that line A4 didn't show amplification products for either the apple *pgip1* or *nptII* primer sets may indicate that the template was not sufficient for the amplification reaction or the plant is not transgenic. The 22 lines out of 29 that did prove to be apple *pgip1* PCR-positive were selected for PGIP extraction and PG-inhibition assays (Chapter 6). Twenty of these lines were chosen for a glasshouse trial to screen for enhanced resistance to *Verticillium dahliae* (Chapter 7).

5.4.2 PCR to verify the presence of the apple *pgip1* gene in the glasshouse transgenic material

Genomic DNA was isolated from glasshouse leaf material of the 20 putative transgenic potato lines that were chosen for the glasshouse trial. Two different methods were used, the one being a small-scale CTAB method on 14 lines, and the other a large-scale Dellaporta method on six lines and untransformed BP1. gDNA from the small-scale isolated DNA contained a bright smear of contaminating RNA (Figure 5.4), because the isolation procedure did not include an RNase A step. The RNA may have negatively affected the PCR when using this DNA as template (Figure 5.3). gDNA prepared by the large-scale isolation method also seemed to have inhibitors of PCR. After clean-up and reprecipitation, DNA from both these extraction methods could be used successfully in amplifying the *nptII* and apple *pgip1* transgenes in the transgenic potato lines (Figures 5.6 and 5.7). The clean-up was therefore successful in removing the RNA (Figure 5.5) and other contaminants that previously inhibited the PCR.

5.4.3 Restriction digestion of potato genomic DNA for Southern blot

The potato A lines are transformed with the pCAMBIA-ap*pgip1*A construct, while the B lines are transformed with the pCAMBIA-ap*pgip1*B construct. To determine the number of insertion events of the transgene into the potato genome, the restriction enzyme *Nsi*I was chosen to digest gDNA for

Southern blot. It doesn't have a recognition site between the T-borders of pCAMBIA-ap*pgip1*A and pCAMBIA-ap*pgip1*B. It will therefore cut the gDNA randomly and the apple *pgip1* gene will reside on different sized fragments. The size of the T-DNA in these constructs is 4606 bp. so this is the minimum expected size of an *Nsi*I fragment. The number of fragments hybridising with the apple *pgip1* probe during Southern blot will indicate the number of insertion events that took place during transformation.

To determine the copy number of the transgene that was inserted into the potato genome, a restriction enzyme that cuts on both sides of the transgene but still between the T-borders was required. *Pvu*I and *Pst*I can excise the apple *pgip1* transgene from both transformation constructs, but they were both either inhibited by a contaminating agent present in the gDNA preparation, or are rare cutters of potato gDNA (Figure 5.10, lanes 4 and 5, respectively). They were therefore not useful in restriction digestion of gDNA for Southern blot. A different enzyme therefore needed to be selected that can excise the apple *pgip1* gene from both types of transgenic potato lines. Since the A and B transgenic potato lines were generated by transformation with constructs containing the gene in opposite orientations, two different enzymes had to be selected for the two types of transgenic lines. *Bam*HI was chosen to excise the apple *pgip1* gene from the A lines and *Nco*I from the B lines. Digestions with these enzymes were never as complete as with *Nsi*I (Figures 5.10 and 5.11).

A possible reason for the poor digestion of gDNA with *Bam*HI is the enzyme's sensitivity to methylation. The recognition sequence of *Bam*HI is GGATCC, and methylation at the first cytosine will lead to inhibition of cleavage. The sequence contains a recognition sequence (underlined) of the m-Ecodam1 methylase, which will lead to methylation of the adenines and cytosines if a fragment containing this sequence is propagated recombinantly in *E. coli*. Although this cannot directly be responsible for poor digestion due to methylation, because the gDNA was harvested from plant material and not *E. coli*, a similar mechanism may exist in the plant cell that will lead to methylated bases and inhibition of cleavage. *Nco*I (CCATGG) and *Nsi*I (ATGCAT) don't contain this methylase-recognition site.

5.4.4 Southern blot hybridisation of selected apple *pgip1* transgenic potato lines

Even though restriction digestion of small samples of potato gDNA with *Nco*I and *Bam*HI didn't seem to be complete, it was continued with the large-scale digestion for Southern blot. More restriction enzyme was added when digestion seemed to be incomplete (Figure 5.12). After overnight electrophoresis to separate the digested fragments, long smears were seen in all lanes except the lanes digested with *Bam*HI (Figure 5.13, especially lane 10). Even though extra units of this restriction enzyme were added and the reactions incubated for longer times, *Bam*HI seemed unable to completely

digest the potato gDNA. Apart from the reason stated above, another possible reason for this could be that its recognition sequence might occur at a low frequency in the genome, and that this enzyme is therefore a rare cutter. The recognition sequence for *Bam*HI is very GC rich, but so is *Nco*I's, and *Nco*I digested more completely than *Bam*HI, so this cannot be a possible explanation for the low restriction digestion success with *Bam*HI. The uneven ethidium bromide staining of the gel (Figure 5.13) might be due to deformation of the gel caused by heating during overnight electrophoresis.

Before Southern blotting, the gel was soaked in 0.25 N HCl. This caused partial hydrolysis of the DNA to smaller fragments (~1 kb), which was more efficiently transferred from the gel to the membrane before the gel dehydrated too much for the DNA to escape from the gel. The HCl partially depurinated the DNA, after which the phosphodiester backbone at the site of depurination was broken with the exposure to a strong base during the alkaline denaturation step. Another function of the denaturation solution (containing 0.4 N NaOH) was to denature the DNA to make it single stranded and accessible for the probe. Neutralising the gel to a pH below 9 before blotting is especially important when using nitrocellulose membranes, but nylon membranes will tolerate a higher pH.

Prehybridisation prepares the membrane for probe hybridisation by blocking all the non-specific nucleic acid-binding sites on the membrane. This reduces the background. Denatured salmon testes DNA was included in both the prehybridisation and hybridisation solution, to reduce non-specific DNA-DNA binding between the probe and the immobilised gDNA. Double-stranded DNA probes need to be denatured by heating in a boiling water bath for 10 min, after which it is chilled directly on ice. The DIG-detection kit gave a very clean Southern blot with very low background. The fragments hybridising with the DIG-labelled apple *pgip1* probe gave clear and sharp bands. It was possible to detect 1 copy of apple *pgip1* gene spiked into 10 µg *Nsi*I digested untransformed BP1 gDNA. The sensitivity of detection of the DIG-system was therefore high enough to detect single copy insertions of the transgene into the genome of transgenic potato lines.

5.4.5 Fragment sizes expected during the Southern blot of apple *pgip1* transgenic potato genomic DNA

The apple *pgip1* probe is expected to hybridise to a fragment 1943 bp in size for the *Bam*HI digested A-lines and 2356 bp for the *Nco*I digested B-lines during Southern blot (refer to **Appendix C**). These restriction enzymes were chosen to cut on both sides of the apple *pgip1* gene in the respective transformation constructs. The copy number can be determined by comparing the hybridising intensities of the resulting fragments to untransformed samples spiked with a known number of copies. The presence of these restriction sites in the constructs used for transformation was verified by restriction digestion analysis (Figure 5.15).

*Nsi*I digestion was used to determine the number of insertion events of the transgene into the potato genome. It is expected to cut the genome randomly so that fragments of different sizes will contain the apple *pgip1* transgene. These fragments were then separated by agarose gel electrophoresis and blotted onto a membrane for hybridisation to a DIG-labelled apple *pgip1* probe. The number of fragments hybridising with the apple *pgip1* probe during Southern blot will indicate the number of insertion events that took place during transformation.

The results of the Southern blot of gDNA restriction digested with *Nsi*I were interesting (Figure 5.14). All three potato lines transformed with the pCAMBIA2300-ap*pgip1*A construct (the A-lines) contained a single band of approximately the same size (approximately 8000 bp, Figure 5.14, lanes 7, 9 and 11). The fact that all three lines contain the same sized fragment capable of hybridising to the probe, means that the transgenic lines are either clones of each other, or that the transgene is located by chance on similarly sized DNA restriction fragments in the separate transgenic lines. From the single hybridising fragment of each line, it can be concluded that only one insertion event of the transgene took place during transformation.

Southern blot of *Bam*HI digested gDNA from the A-lines gave unexpected results. A fragment of 1943 bp was expected, but fragments of approximately 6000 bp were obtained for lines A5 and A9 (Figure 5.14, lanes 8 and 12, respectively). If all three A-lines were clones of the same transgenic event as indicated by the *Nsi*I digestion, it is expected that line A6 should also show a fragment of 6000 bp. gDNA from line A6 was poorly digested with *Bam*HI (Figure 5.13, lane 10), which may explain the absence of an excised hybridising fragment, and the probe hybridising to the undigested large molecular weight gDNA.

Possible reasons why a larger than expected fragment was obtained for Southern blotting of *Bam*HI digested samples, might be partial digestion by the enzyme, or that the *Bam*HI site on the one side of the transgene sustained a mutation before or during the transformation process and was therefore not present in the plant genome. The *Bam*HI recognition site was, however, present in the pCAMBIA2300-ap*pgip1*A construct that was used for the transformation of the A-lines. *Bam*HI digestion of this construct lead to the excision of the expected 1943 bp fragment (Figure 5.15, lane 2).

Another possible reason for the unexpectedly large hybridising fragment may be that the A-lines were not really transformed with pCAMBIA2300-ap*pgip1*A, but are in fact clones of a transgenic B-line transformed with the pCAMBIA2300-ap*pgip1*B construct. A mix-up during the subculturing of transgenic *in vitro* potato plants may have lead to the mixing of transgenic lines. *Bam*HI will then cut only on one side of the transgene construct (see map of pCAMBIA-2300-ap*pgip1*B in **Appendix C**),

with the second site residing in the adjacent nucleotides of the potato genome. Since insertion of the T-DNA into the genome is random, digestion with *Bam*HI will result in fragments of any size. If lines A5 and A9 are not clones of the same transformation event, it is a coincidence that the gene is located in similarly sized *Bam*HI fragments.

The possibility of the A-lines being transformed with the pCAMBIA2300-ap*pgip1*B construct was investigated with PCR. The results indicated that the A-lines are definitely transformed with the pCAMBIA2300-ap*pgip1*A construct and the B-lines with the pCAMBIA2300-ap*pgip1*B construct (Figure 5.16 and **Discussion** section 5.4.6). The Southern blot results of the *Bam*HI digested A-lines are therefore difficult to interpret unless it is assumed that the incomplete digestion caused the absence of the expected hybridising fragment. It can further be deduced that a single insertion event of the transgene occurred into all three A-lines, and that they are possibly clones from the same transformation event.

*Nsi*I digestion of the B-lines yielded two fragments that hybridised with the probe in line B10 and B13 (one approximately 9400 bp and the other larger than 9400 bp, Figure 5.14, lanes 13 and 17, respectively), and a single fragment (approximately 9400 bp) for line B11 (Figure 5.14, lane 15). From this it can be deduced that line B10 and B13 contains two insertion events of the transgene, and line B11 only one. For the same reason as stated above, lines B10 and B13 may be clones of the same transgenic event.

A fragment of 2356 bp was expected for the *Nco*I digested B-lines, and the Southern blot results correlated very well with this. Fragments of approximately 2300 bp were obtained for lines B10, B11 and B13 (Figure 5.14, lanes 14, 16 and 18, respectively), with the intensity of the fragment of line B11 half of that of lines B10 and B13. This correlates to the number of insertion events as was determined by *Nsi*I digestion, with lines B10 and B13 having two insertion events each and line B11 only one. The slight differences observed in size for the fragments in lanes 14, 16 and 18 might be accounted for by the unevenness of the large gel during separation of the digested fragments by overnight electrophoresis.

The intensities of the *Nco*I fragments were higher than the spiked 1 copy (Figure 5.14, lane 3). This may mean that more than one copy was inserted in tandem, and that lines B10 and B13 contain double the number of copies of lane B11. Otherwise, it can be explained by an overestimation of the plasmid concentration that was used for preparing the apple *pgip1* spike, or an underestimation of the gDNA that was digested and separated for the Southern blot. The lanes containing the spiked copy numbers can then not be used to estimate the copy number of the transgene in a potato line with absolute certainty.

From the *Nsi*I and *Nco*I Southern blot results, it can be concluded that potato line B11 contains a single insertion event and one copy of the apple *pgip1* transgene. The other two selected lines, B10 and B13, each had a double insertion event and have double copies of the transgene. The possibility exist that they are clones of each other.

The apple *pgip1* probe didn't hybridise to the untransformed potato gDNA. It can be speculated that the apple *pgip1* gene sequence is sufficiently different from the endogenous potato *pgip* gene sequence so that hybridisation couldn't occur during the conditions used for this experiment. Although a potato PGIP has been discovered recently from the Spunta cultivar (Machinandiarena *et al.*, 2001), nothing is yet known about its sequence.

5.4.6 PCR of the six potato lines to verify the construct used for transformation

PCR with the vector-specific primer U19F and the apple *pgip1* primers was performed to verify that the six chosen potato lines were transformed with the appropriate pCAMBIA2300-appgip constructs. The annealing site of the U19F primer lies between the right T-border and the CaMV e35S promoter-apple *pgip1* cassette in the pCAMBIA2300-appgip constructs (**Appendix C**). Using this primer in combination with AP-PGIP-L2 is expected to give an amplification product of 1290 bp with only the pCAMBIA2300-ap*pgip1*A construct and the transgenic potato A-lines. U19F in combination with AP-PGIP-R should only amplify a 1958 bp fragment from pCAMBIA2300-ap*pgip1*B and the B-lines.

The results obtained in Figure 5.16 corresponds very well with the expected, except that the pCAMBIA2300-ap*pgip1*B construct also produced an amplification product of ~ 1200 bp with the U19F and AP-PGIP-L2 primers (Figure 5.16, lane 14). The reason for this is unclear, since the two primers are oriented in the same direction on the plasmid map. The only possible explanation is that the pCAMBIA2300-ap*pgip1*B plasmid preparation was contaminated with the pCAMBIA2300-ap*pgip1*A plasmid. The transformed potato B-lines did not give this amplification product (Figure 5.16, lanes 15 to 17). A fragment of the expected size was amplified from the B-lines using the U19F and AP-PGIP-R primer combination (Figure 5.16, lanes 6 to 8), so it can be concluded that they are transformed with the pCAMBIA2300-ap*pgip1*B construct. The A-lines yielded the expected 1290 bp fragments only with the U19F and AP-PGIP-L2 primers (Figure 5.16, lanes 11 to 13), and nothing with the AP-PGIP-R primer combination (Figure 5.16, lanes 2 to 4). It can therefore be concluded that the A-lines are transformed with the pCAMBIA2300-ap*pgip1*A construct.

This chapter reported on the molecular characterisation of transgenic BP1 potato lines containing the apple *pgip1* transgene. Using PCR, the apple *pgip1* gene was shown to be present in 22 of the 29

kanamycin resistant *in vitro* transgenic lines (Figure 5.1). PCR was, however, successful in amplifying the *nptII* gene from all 29 lines (Figure 5.2). It is possible that all 29 lines contain the apple *pgip1* gene and that the PCR was just not successful for all lines. PCR on gDNA from 20 selected lines grown in a glasshouse still showed the presence of the apple *pgip1* transgene (Figure 5.6) and the *nptII* gene (Figure 5.7). A Southern blot of gDNA from six selected transgenic lines indicated the presence of single or double copies of the transgene in the genomic DNA (Figure 5.14). The possibility is presented that most of the lines are not individual transformation events. The three A-lines are possibly from the same clone, and the three B-lines are from two other different clones.

The analysis of transgene expression in the transgenic potato lines will be discussed in Chapter 6. Putative transgenics containing the apple *pgip1* gene were chosen for crude protein extractions. The extracts were used to test for inhibitory activity towards *V. dahliae* endopolygalacturonases.

CHAPTER 6

Expression studies of apple PGIP1 in transgenic potato and inhibition studies with *V. dahliae* PG

6.1 Introduction

Transgenic potato cv. BP1 lines have been generated with the apple *pgip1* gene expression driven by the constitutive enhanced CaMV 35S (e35S) promoter. The previous chapter described the molecular characterisation of the putative transgenic lines to assess the presence of the transgene. This chapter deals with the PG-inhibiting activities of PGIP extracts prepared from these lines as well as positive control transgenic plants and negative control untransformed plants. The aim was to determine whether apple PGIP1, expressed transgenically in potato, is able to inhibit the PGs secreted by *Verticillium dahliae in vitro*.

The role of fungal endoPGs in pathogenicity has been reviewed in the literature review. They have been shown to activate plant-defence responses by releasing oligogalacturonides from the plant cell walls. EndoPG can, however, also rapidly degrade the oligogalacturonides to inactive oligomers, too short to possess elicitor activity (Cervone *et al.*, 1989; De Lorenzo *et al.*, 1994). According to the hypothesis of Cervone *et al.* (1989), PGIP might affect the activity of the endoPG so that the oligogalacturonides created by the PG remain stable for longer. It does not inhibit fungal endoPG completely, so that the residual activity is sufficient to form elicitor-active oligogalacturonides, but limited enough to only slowly depolymerise the active molecules to molecules too short for elicitor activity. PGIP thus allows plants to convert endoPG, a virulence factor of pathogens, into a factor that elicits plant defence mechanisms (an avirulence factor).

Because *V. dahliae* causes the devastating disease *Verticillium*-wilt of potato, and resistance breeding against this disease is complex, a strategy involving the transgenic manipulation of potato was proposed. The apple *pgip1* gene has been isolated previously at ARC-Roodeplaat and transformed into LA Burley tobacco (Arendse and Berger, unpublished). Crude extracts from LA Burley: *pgip1* #8 transgenic plants were able to inhibit PGs isolated from *V. dahliae* grown on pectin medium. Apple PGIP1 was purified to homogeneity and the N-terminal sequence determined to be identical to the published sequence (Oelofse *et al.*, manuscript in preparation). This purified apple PGIP1 also had an inhibiting activity towards *V. dahliae* PGs, so apple PGIP1 was seen as a possible candidate to confer fungal resistance against this fungus to susceptible plants.

Expression of functional PGIP in bacteria and yeast has proved unsuccessful to date (Berger and others, unpublished). Several examples exist in which PGIP was functionally expressed in heterologous plants. One method is through stable genetic transformation. Bean PGIP1 expressed in tomato was still effective against *A. niger* and *S. maydis* PG (Berger *et al.*, 2000). Tomato was also transformed with bean *pgip1* (Desiderio *et al.*, 1997) and pear *pgip* (Powell *et al.*, 2000) with successful results. Another method of obtaining functional PGIP is by transiently infecting *Nicotiana benthamiana* with a modified potato virus X (PVX) containing the PGIP gene (Desiderio *et al.*, 1997; Leckie *et al.*, 1999). PGIPs expressed this way retained their ability to inhibit specific fungal PGs. Only in the case of transgenic tomato containing pear PGIP were *in vivo* experiments performed, and the plants showed increased resistance to *B. cinerea* (Powell *et al.*, 2000).

Reducing sugar assay to determine PG activity

This chapter will report on the various assays to determine the PG-inhibiting activity of PGIP extracts prepared from transgenic potato lines. The first assay, the agarose diffusion assay, was introduced in Chapter 4. The principle of the second assay will be discussed here.

Acid hydrazides react with reducing carbohydrates in alkaline solutions to give yellow anions. PAHBAH (*p*-hydroxybenzoic acid hydrazide) forms intensely yellow anions with reducing sugars when the reaction is carried out under alkaline conditions (Lever, 1972; York *et al.*, 1985). The absorption of these yellow anions can therefore be used for the colorimetric determination of carbohydrates. PAHBAH shows low reagent blank values, and colour development at 100°C reaches a maximum after 5 min and remains stable for at least 5 min. It was shown that derivative formation is linear over a wide range of glucose concentrations (0 µg/ml to 5 mg/ml), so PAHBAH can be used in a highly sensitive assay (Lever, 1972). In the reducing sugar assay for polygalacturonase activity, the PG degrades the substrate polygalacturonic acid to produce reducing sugars. The product of the reaction of the reducing sugars and PAHBAH is proportional to the amount of reducing sugars present, and can be quantified spectrophotometrically.

This chapter describes the preparation of polygalacturonases from *V. dahliae* grown on pectin as a carbon source. It also reports on the preparation of extracts containing polygalacturonase-inhibiting activity from apple *pgip1* transgenic potato and tobacco plants, from *in vitro* as well as glasshouse material. The hypothesis is that the transgenically expressed apple PGIP1 will inhibit the endopolygalacturonases from *V. dahliae*. The inhibitory activity of the extracts against *V. dahliae* PGs is presented.

6.2 Materials and Methods

6.2.1 *V. dahliae* PG isolation

6.2.1.1 Fungal isolate and growth media

V. dahliae was isolated from infected potato of the cultivar Lady Rosetta by A. McLeod (ARC-Roodeplaat). It was collected in 1998 from the Worcester area (South Africa) and stored in the collection of C. Millard at ARC-Roodeplaat with the number 61. It was plated and maintained on potato dextrose agar (PDA) plates containing 0.1 g streptomycin sulphate, dissolved in 10 ml ethanol per 1 litre PDA, to inhibit bacterial growth. Fresh cultures were initiated by transferring a plug of mycelia from one plate to fresh plates and incubating at 25°C for 12 h light and 12 h darkness.

6.2.1.2 Media for polygalacturonase (PG) production by *V. dahliae*

The *V. dahliae* fungal isolate was inoculated into Czapex-dox containing 100 µg/ml ampicillin to inhibit bacterial growth. The culture was incubated at 27°C with shaking for three days, after which pieces of mycelium were used to inoculate a number of flasks containing pectin medium. The pectin medium was prepared by adding 0.25 g pectin (Sigma P-9135 (St Louis, MO, USA), washed with 0.1 N HCl in 70% ethanol and dried) to 24 ml of citrate/ phosphate buffer (pH 6.0), and autoclaving before the addition of the sterile inorganic salt solutions. The buffer is prepared from 17.9 ml 0.1 M citric acid and 32.1 ml 0.2 M Na_2HPO_4 per 100 ml. The following volumes of sterile salt solutions were added to each 24 ml pectin medium-containing flask: 50 µl of 1 M $MgSO_4$, 250 µl of 0.001% $MnSO_4.H_2O$, 625 µl of 1 M KNO_3, 250 µl of 0.01% $ZnSO_4.7H_2O$, 250 µl of 0.0015% $CuSO_4.5H_2O$ and 250 µl of 0.01% $FeSO_4.7H_2O$. Ampicillin was added to a final concentration of 100 µg/ml.

6.2.1.3 Growth of *V. dahliae* for PG production

The flasks containing the inoculated pectin medium were incubated at 27°C with shaking at 100 rpm. One flask was harvested per day for 13 days, the culture filtrated through Whatman #1 filter paper (Whatman International) and the filtrate stored at -20°C.

6.2.1.4 Ammonium sulphate (AS) precipitation of the *V. dahliae* filtrates

V. dahliae filtrates from different harvest days were pooled and subjected to AS precipitation in order to remove the medium derived pectin, which interferes with the reducing sugar assay. The filtrate was centrifuged at 9900×g (Beckman rotor JA-14) for 20 minutes at 10°C, and the supernatant filter-sterilised consecutively through 0.45 µm and 0.22 µm filters. The exact volume of sterilised supernatant was determined. The amount of AS, to give a final AS concentration of 85% (55.9 g AS per 100 ml supernatant), was calculated. The samples were maintained at 4°C at all times. The AS

was added in four aliquots, dissolving it completely by mixing gently each time. The samples were left at 4°C overnight with gentle shaking. Samples were subsequently centrifuged at 15300×g (Beckman rotor JA-20) for 40 minutes at 4°C. The supernatant was decanted and the pellet drip-dried inverted on absorbent paper. The pellets were resuspended in 20 mM sodium acetate buffer (pH 4.7), a twentieth of the original sterilised supernatant volume, and stored in aliquots at -20°C.

6.2.2 Preparation of PGIP extracts from plant material

The method was adapted from Desiderio *et al.* (1997). Crude PGIP extracts were prepared from transgenic and untransformed leaf and root material. Apple *pgip1* transgenic potato lines and a positive control apple *pgip1* transformed tobacco line (called LA Burley: *pgip1* #8) were the transgenic lines.

Leaves were collected either from *in vitro* plantlets or from plants grown in the glasshouse, and stored at -70°C. The leaf material was ground to fine powder in liquid nitrogen using a mortar and pestle. Two volumes of 1 M NaCl, 20 mM NaAc buffer (pH 4.7) were added to the leaf material and the extracts shaken for 2 hours at 4°C. Extracts were subsequently centrifuged at 6500×g for 20 minutes at 4°C. The pellets were discarded and the supernatants dialysed extensively against 20 mM NaAc buffer (pH 4.7) at 4°C. A 12000 molecular weight cut-off dialysis membrane (Sigma D-9277) was used. Extracts were recovered from the dialysis tubes, centrifuged at 6500×g for 20 minutes at 4°C, and the supernatants stored at -20°C.

For a quicker PGIP extraction method, a small amount of plant material was ground directly in a 1.5 ml Eppendorf tube using carborundum powder (400 grit) and an Ultra Turrox. It was extracted with the same buffer as described above and used in the agarose diffusion assay without being dialysed. PGIP extracts were prepared from glasshouse-grown leaf material and 300 - 400 mg roots of *in vitro* grown potato lines using this quicker method.

To compare the inhibiting activity of dialysed PGIP extracts and extracts prepared using the quick method, samples of these extracts were dialysed by placing 100 µl onto a membrane with 0.025 µm pores (Osmonics) and floating it on 20 mM NaAc buffer (pH 4.7) at 4°C for an hour. Drops were recovered and the amount used in the ADA adjusted to compensate for the increase in volume that occurred during dialysis.

6.2.3 Assay for PGIP activity

6.2.3.1 Agarose diffusion assay (ADA)

Sixty-five millimetre diameter Petri dishes containing 10 ml of the agarose diffusion assay medium were prepared as described in Chapter 4. The wells were filled with 20 µl of *V. dahliae* culture filtrates. For PG:PGIP inhibition studies, 15 µl of *V. dahliae* PG was incubated with 15 µl of either 20 mM NaAc buffer (pH 4.7) or various PGIP extracts. Samples of PGIP extracts were boiled for 10 minutes and cooled on ice. As a positive control, purified apple PGIP1 (provided by D. Oelofse (ARC-Roodeplaat), unpublished) was used. The reactions were incubated and the plates stained as described before (Chapter 4).

A modified agarose diffusion assay was employed in which the assay medium consisted of 0.8% agarose and 0.5% PGA in 100 mM NaAc buffer (pH 4.7). The *V. dahliae* PG was incubated with the PGIP extracts as before, but the plates were developed with 6 N HCl instead of ruthenium red (Cervone *et al.*, unpublished method).

6.2.3.2 Reducing sugar assay

Release of reducing sugars by fungal polygalacturonase activity was measured by the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (Lever, 1972; York *et al.*, 1985). The reducing sugar assay was used to determine the linear trend for *V. dahliae* PG activity as well as the inhibition of *V. dahliae* PGs by transgenic tobacco and potato PGIP extracts.

6.2.3.2.1 Quick PAHBAH assay of inhibition of *V. dahliae* PGs by PGIP extracts

Dialysed PGIP extracts from apple *pgip1* transformed *in vitro* potato leaf material were used in these experiments. PGIP extracts from LA Burley: *pgip1* #8 tobacco and HPLC purified apple PGIP1 served as positive controls, and PGIP extracts from non-transformed tobacco and potato leaves were used as negative controls.

The *V. dahliae* PG was used at a 1 in 5 dilution with 20 mM NaAc buffer (pH 4.7). Two sets of Eppendorf tubes were prepared for each sample to be analysed for the PGIP:PG interaction (T_0 and T_{30}). Seven hundred and fifty microlitres substrate [0.025% PGA in 50 mM NaAc buffer, pH 4.7] was added to each of the Eppendorf tubes. The PG (30 µl) was mixed with either 20 mM NaAc buffer (30 µl) or PGIP extract (30 µl) and incubated at 25°C for 20 minutes. Seven hundred and fifty microlitres PAHBAH reagent was added to one set of Eppendorf tubes (T_0). The PAHBAH reagent was made fresh each time by mixing 1 volume of 5% PAHBAH in 0.5 M HCl with 4 volumes of 0.5 M NaOH to give a final PAHBAH concentration of 1%. Twenty-five microlitres of the PGIP:PG mix was added to this set of Eppendorf tubes (T_0). Twenty-five microlitres of the PGIP:PG mix was added to the

other set of Eppendorf tubes (T_{30}). These were left to incubate at 30°C for 30 minutes. After the 30 minutes incubation period, 750 µl PAHBAH reagent was added to the T_{30} Eppendorf tubes. All the Eppendorf tubes were boiled for 10 minutes, cooled and the A_{410nm} values obtained spectrophotometrically. The spectrophotometer was blanked with dH_2O, and the T_0 values subtracted from the T_{30} values. Percentage inhibition of PG by PGIP was calculated relative to the PG+NaAc buffer value (100% PG activity; 0% inhibition).

6.2.3.2.2 Linear range of *V. dahliae* PG activity

In this experiment undiluted, 1+1, 1+4, 1+9, 1+14 and 1+19 dilutions of the *V. dahliae* PG extract (AS precipitated) were used. The *V. dahliae* PG extract was diluted with 20 mM NaAc buffer (pH 4.7). Reactions were run in triplicate and samples were taken at six different time points (t=0', t=20', t=40', t=60', t=80' and t=100'). The PG (40 µl) was mixed with 20 mM NaAc buffer (40 µl) and incubated for 20 minutes at 25°C before the assay. A 72 µl aliquot of this sample was then added on ice to 108 µl of 0.42% PGA (in a citric acid/ sodium phosphate buffer, pH 4.6) to give a final PGA concentration of 0.25%. Immediately, a t=0' sample of 25 µl was removed into an Eppendorf safe lock tube and placed in a boiling water bath for 10 minutes. After boiling, the sample was kept on ice. The rest of the reaction mixture was placed at 30°C for the total time course of the reaction (up to 100 minutes). Twenty five microlitre samples were removed to a boiling water bath for 10 minutes at t=20', t=40', t=60', t=80' and t=100' and then kept on ice. The condensate on the tube lids was sedimented by a quick spin, and then the volume was increased to a total of 1 ml by the addition of 225 µl dH_2O and 750 µl 1% PAHBAH reagent. The PAHBAH reagent was made fresh each time by mixing 1 volume of 5% PAHBAH in 0.5 M HCl with 4 volumes of 0.5 M NaOH to give a final PAHBAH concentration of 1%. The samples were boiled for 10 minutes, cooled and the absorbance of each was read at 410 nm. The spectrophotometer was blanked with dH_2O. The average and standard deviation of each triplicate sample was calculated, and a graph containing error bars of polygalacturonase activity (A_{410nm} values) against time plotted for each PG dilution. Linear regression was applied to all graphs by calculating the R^2 value using Microsoft Excel. The R^2 value is an indicator that ranges in value from 0 to 1. It reveals how closely the estimated values for the trendline correspond to the actual data. A trendline is most reliable when its R^2 value is at or near 1.

6.2.3.2.3 Reducing sugar assay of inhibition of *V. dahliae* PGs by PGIP extracts

The method used for the determination of the linear trend for *V. dahliae* PG activity was followed, but here the 20 mM NaAc buffer was replaced in certain instances with dialysed apple *pgip1* transgenic tobacco and potato PGIP extracts. The PGs were mixed with equal volumes of PGIP extracts and incubated at 25°C for 20 minutes before the assay. Then the PG:PGIP reactions were mixed with the PGA substrate and incubated at 30°C for the appropriate time period. The reaction volume was scaled down since only two time points were needed. The average percentage activity relative to PG activity

in the presence of NaAc buffer as well as the standard deviation was calculated for each triplicate sample.

6.2.4 Protein concentration determination

The protein concentrations of PG and PGIP extracts were determined using the Bio-Rad protein assay kit (Hercules, CA, USA). The dye reagent concentrate consists of Coomassie Brilliant Blue G-250 dye, phosphoric acid and methanol. The Bio-Rad protein assay is based on the method of Bradford, in which a different colour change of the dye occurs in response to different concentrations of protein (Bradford, 1976). The absorbance maximum of Coomassie Brilliant Blue G-250, in an acidic solution, shifts from 465 nm to 595 nm when binding of the dye to protein occurs. The dye binds primarily to basic and aromatic amino acid residues, especially arginine. The relative measurement of a sample's protein concentration is obtained by comparison to a standard curve. Bovine serum albumin (BSA) was used as a protein standard.

For the BSA standard series, tubes containing 800 µl of BSA solution with concentrations of 0, 1.2, 3, 5 and 10 µg/ml were prepared in triplicate. Triplicate tubes containing 50 µl of each sample and 750 µl of dH_2O were also prepared. Two hundred microlitres of the dye reagent concentrate was added to each tube and the tubes were vortexed. After 10 min incubation at room temperature, the absorbance at 595 nm was measured. The blank value was subtracted from all the absorbance values. A standard curve was plotted with the absorbance values of the BSA standard series, and linear regression applied to the points falling within the linear range. The concentrations of the diluted samples (50 µl in 800 µl) were determined by comparison of its absorbance value to the standard curve. The concentrations of the undiluted samples were calculated by dividing these values by their dilution factor. The average protein concentration and standard deviation were calculated for each of the triplicate samples.

6.3 Results

6.3.1 *V. dahliae* PG isolation

V. dahliae produced polygalacturonase (PG) activity when grown in liquid culture on pectin as the sole carbon source. Enzyme activity was assessed using an agarose diffusion assay (ADA; Taylor and Secor, 1988). The wells were filled with 20 µl of *V. dahliae* culture filtrates of the different harvest days. Extracellular PG activity reached a maximum after 5 days of growth, with decreasing but substantial activity in the following days (Figure 6.1).

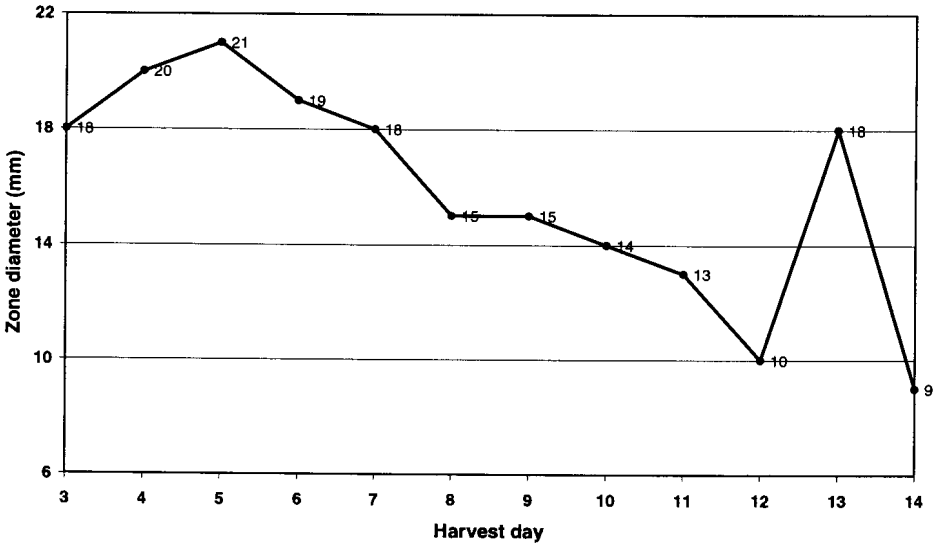


Figure 6.1 **Agarose diffusion assay (ADA) of *V. dahliae* culture supernatants.** Culture supernatants from different harvest days were assayed using the agarose diffusion assay and the zone diameters measured in mm.

6.3.2 ADA to determine the efficiency of AS precipitation

The PG activity produced was separated from the pectin in the growth media by AS precipitation of three separate pools of culture supernatants. Pool #1 consisted of culture supernatants of day 3 and 4, pool #2 included days 6 to 9 and pool #3 days 10 to 13. Culture supernatant of day 5 was not included in the AS precipitation since it was used up before then. The efficiency of concentrating the PG activity by AS precipitation and its activity after being precipitated was assessed by agarose diffusion of the resuspended pellets of each pool. The assay was carried out by the addition of 20 µl of the *V. dahliae* culture supernatant, before or after AS precipitation, to each well, and incubation at 27°C overnight. After incubation, the zones were visualised as described before. The diameters of the resulting clear zones of activity were measured in millimetres (Figure 6.2). The larger the cleared zone in the solidified pectin medium, the more PG activity is present. Unhydrolysed substrate is

stained by ruthenium red. Activity was higher in the post-precipitation pellet fraction than in the sterilised culture filtrate before AS precipitation. Very little PG activity was present in the post-precipitation supernatant. It should be taken into consideration that the diameter of the well in the agarose diffusion plate is 6 mm. The zone diameters were corrected with 6 mm (Figure 6.2). Figure 6.3 shows a representative ADA plate indicating the PG activity before and after precipitation.

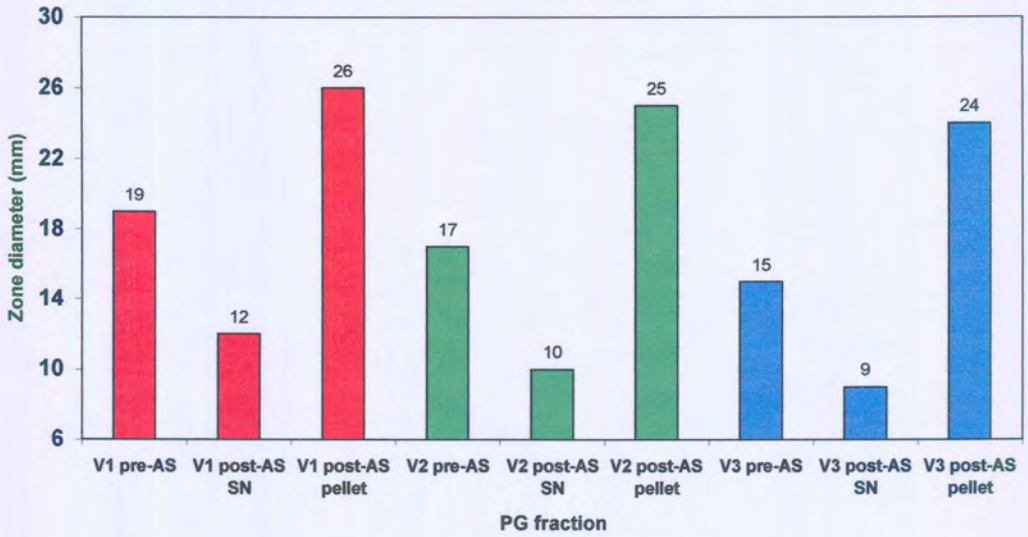


Figure 6.2 **ADA of pools of *V. dahliae* PGs before and after ammonium sulphate precipitation.** The explanations of the codes used in the figure are as follows: *V1 to V3*: pool 1 to 3; *Pre-AS*: Sterilised culture filtrate before ammonium sulphate precipitation; *Post-AS SN*: Supernatant decanted from precipitated PG pellet after centrifugation of the ammonium sulphate precipitation; *Post-AS pellet*: Pellet obtained from centrifugation of ammonium sulphate precipitated PGs and resuspended in a twentieth volume 20 mM NaAc buffer (pH 4.7).

Figure 6.3 **ADA plate of *V. dahliae* PGs from pool 2 before and after ammonium sulphate precipitation.** Well 1: Pre-AS culture filtrate; 2: Post-AS supernatant; 3: Post-AS pellet resuspended in 20 mM NaAc buffer (pH 4.7).

It was concluded that ammonium sulphate successfully precipitated and concentrated the *V. dahliae* PGs. The PGs also retained their activity after being precipitated, as was assessed by the agarose diffusion assay.

6.3.3 ADA to test PGIP inhibition of precipitated PG

Each pool of precipitated PG was tested for inhibition by purified apple PGIP1 (provided by Oelofse, unpublished). Fifteen microlitres PG of each pool was incubated with either 15 µl 20mM NaAc buffer (pH 4.7) or 3 µl purified apple PGIP plus 12 µl 20 mM NaAc buffer (pH 4.7) as described before. Figure 6.4 represents the results from the agarose diffusion assay, with the first bar of each of the respective three pools representing PG activity in the presence of NaAc buffer alone, and the second bar the activity in the presence of purified apple PGIP1.

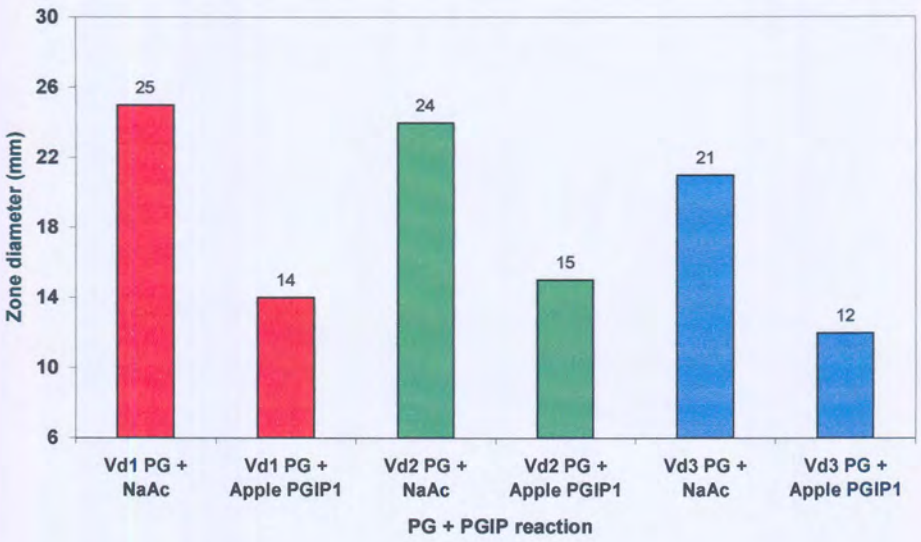


Figure 6.4 **ADA of apple PGIP1 inhibition of *V. dahliae* PGs isolated from different pools.** Vd1 to Vd3 PG: PG precipitated from pool 1 to 3. NaAc: buffer used for 100% activity of PG.

From the reduction in zone size in the presence of the apple PGIP1 of each of the three pools, it was concluded that all three pools' PG activity were inhibited by apple PGIP1. The PGs precipitated from the different pools were combined, and stored in aliquots at -20°C. Activity of the PGs was retained after thawing, since these stored PGs were used for all the subsequent studies.

6.3.4 Assay for PGIP activity

The PG-inhibiting activity of the crude PGIP extracts, prepared from the transgenic tobacco and potato material, was assessed. Different assays were performed, depending on whether qualitative or quantitative inhibition results were required. The ADA and quick PAHBAH assay give qualitative inhibition results. These were employed while preparing the *V. dahliae* PGs and when the PGIP extracts were quickly screened. The assays were performed singly to reduce the amount of sample required. The reducing sugar assay, performed with replicates, gives quantitative results.

6.3.4.1 Agarose diffusion assay (ADA)

6.3.4.1.1 Quick PGIP extraction (without dialysis) and ADA

In an effort to simplify the PGIP extraction for ADA purposes, the following experiment was performed. PGIP extracts were prepared as before, but samples were not dialysed. They were compared against dialysed samples in an ADA to test whether the inhibiting activity was still present.

The PGIP extracts prepared from transgenic potato lines using the quick extraction method, which doesn't include dialysis, yielded the same inhibiting effect on *V. dahliae* PG than the corresponding dialysed extracts (Figure 6.5). All the transgenic potato extracts tested showed successful inhibition with the formation of similarly sized cleared zones. Non-dialysed samples' zones were yellow compared to the colourless zones of the dialysed samples. Extracts from untransformed BP1 potato showed inhibiting activity nearly identical to its dialysed extract, with both causing very little zone inhibition.

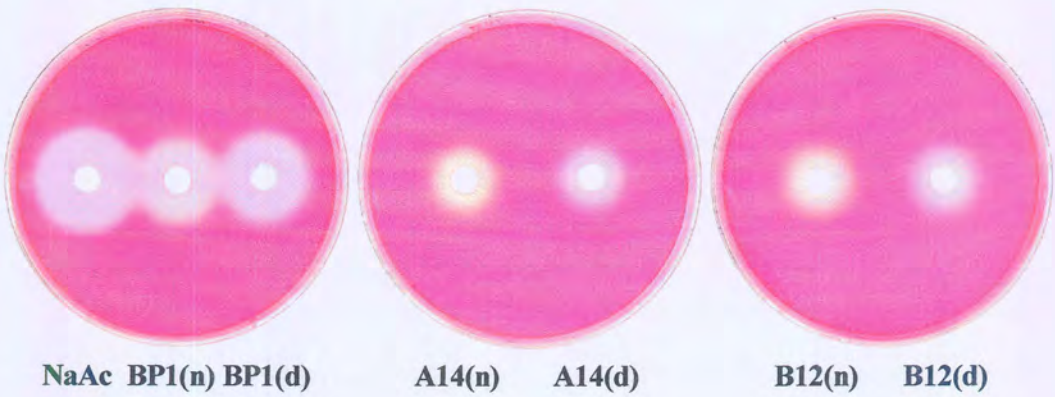


Figure 6.5 **ADA with *V. dahliae* PGs to compare the inhibiting activity of dialysed PGIP extracts with extracts that have not been dialysed.** n: non-dialysed; d: dialysed. The *V. dahliae* PGs were incubated with NaAc buffer alone, and with extracts prepared from untransformed BP1 potato and transgenic potato lines A14 and B12.

6.3.4.1.2 Inhibition of *V. dahliae* PG by the apple *pgip1* transgenic tobacco and potato PGIP extracts using ADA

ADA was performed on dialysed PGIP extracts from *in vitro* transgenic potato and tobacco leaf material (Figure 6.6) and on non-dialysed glasshouse potato leaf material using a modified method. PGIP expression in the roots of *in vitro* transgenic potato lines was also assayed.

6.3.4.1.2.1 ADA of *V. dahliae* PG activity with PGIP extracts from *in vitro* leaf material

As represented in Figure 6.6, all transgenic potato lines except line B16 caused approximately 43 - 48% reduction in zone diameter (11 - 12 mm) compared to *V. dahliae* PG incubated with NaAc buffer (21 mm). The boiled extracts of lines A12, B7, B13 and B18 (indicated with a (b)) didn't inhibit the PG, and resulted in zones with the same diameters as PG incubated with NaAc buffer. Untransformed BP1 (BP1 -) inhibited *V. dahliae* PG by only 10% (zone diameter of 19 mm), with this inhibitory activity being lost when the extract was boiled (BP1 - (b)). Unexpectedly, HPLC purified apple PGIP1 didn't inhibit *V. dahliae* PG substantially (zone diameter of 18 mm), but LA Burley: *pgip1* #8 inhibited it well (12 mm). Untransformed LA Burley (LA Burley -) and all the boiled extracts didn't inhibit zone formation. Reactions were not performed with replicates, since this was the first quick screen for PGIP activity.

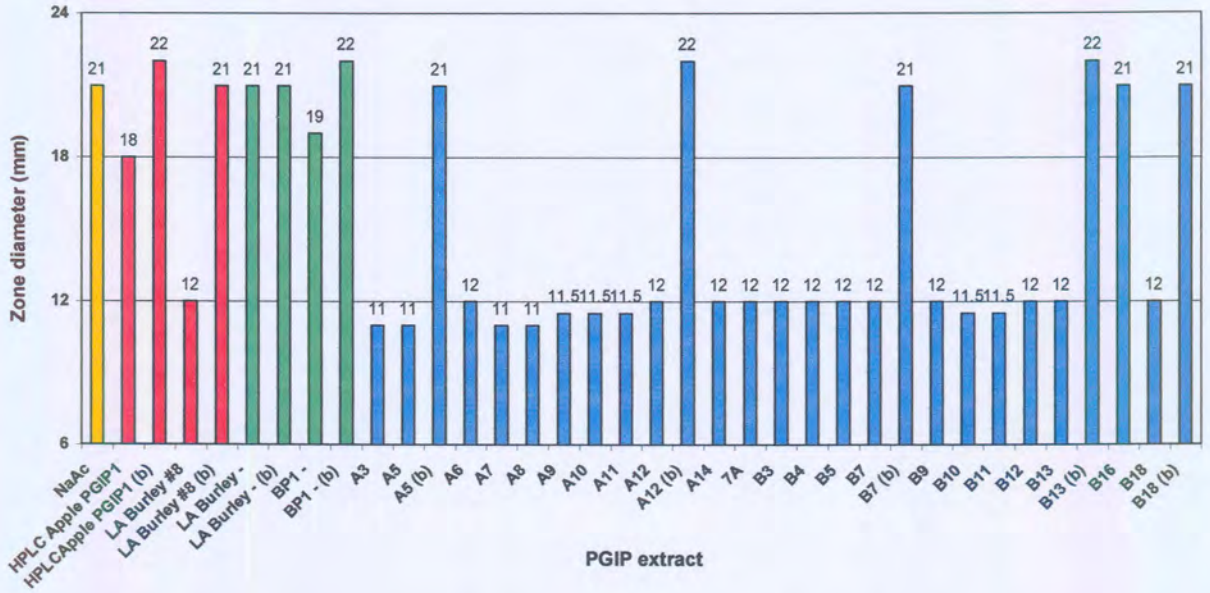


Figure 6.6 **ADA of *V. dahliae* PG activity with PGIP extracts from *in vitro* leaf material.** (b): PGIP extract boiled.

6.3.4.1.2.2 Modified ADA of *V. dahliae* PG activity with PGIP extracts from glasshouse-grown leaf material

A modified agarose diffusion assay was employed with PGIP extracts prepared from leaf material of

glasshouse-grown transgenic potato lines and LA Burley: *pgip1* #8. Inhibiting activity was present in the glasshouse-grown leaf material of all the transgenic potato lines except line B16. This result agrees with the *in vitro* results. Zone diameters for the inhibited *V. dahliae* PG ranged from 8 - 9 mm, while the zones from the uninhibited PG were 14 mm (figure not shown). The glasshouse material from LA Burley: *pgip1* #8 tobacco and HPLC purified apple PGIP1 were also able to inhibit the PG successfully. Extracts that were unable to inhibit the PG were NaAc buffer, untransformed BP1 potato and the transgenic potato line B16.

6.3.4.1.2.3 ADA of *V. dahliae* PG activity with PGIP extracts from *in vitro* transgenic potato root material

PGIP extracts from *in vitro* transgenic potato roots contained an active PGIP since it inhibited the *V. dahliae* PG in the agarose diffusion assay. The zone diameter of the *V. dahliae* PG in the presence of NaAc buffer alone is 22 mm, compared to the zone sizes of 14 - 15 mm for all lines except B16. This corresponds to the results obtained with PGIP extracts prepared from *in vitro* and glasshouse leaf material. HPLC purified apple PGIP1 also caused a zone reduction in this range, with a diameter of 14 mm. Untransformed BP1 caused a zone of 21 mm diameter, and transgenic potato line B16 a zone of 20 mm. Figure 6.7 shows representative ADA plates of the results obtained.

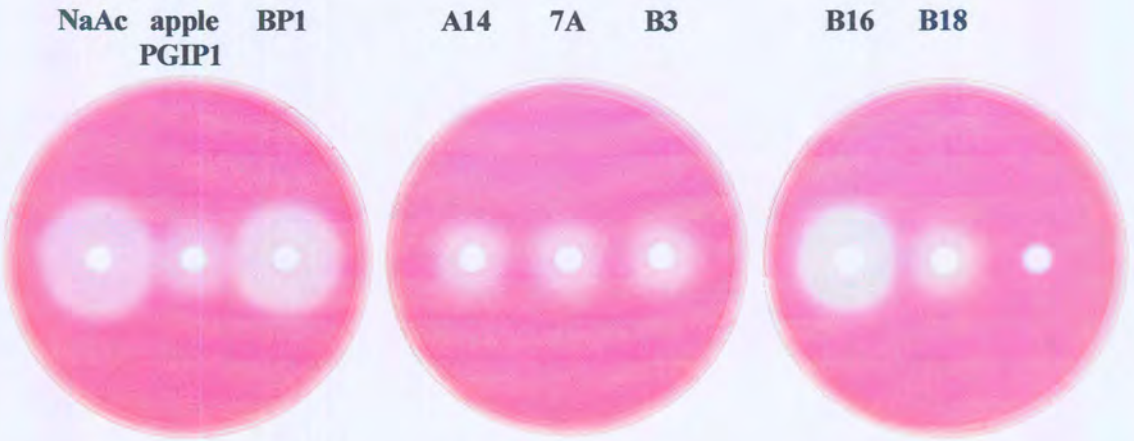


Figure 6.7 **ADA with *V. dahliae* PGs and PGIP extracts prepared from *in vitro* transgenic potato root material.** *V. dahliae* PG in the presence of NaAc buffer, HPLC purified apple PGIP1 or PGIP extract from the indicated potato line.

6.3.4.2 Reducing sugar assay

6.3.4.2.1 Inhibition of *V. dahliae* PGs by the apple *pgip1* transgenic tobacco and potato PGIP extracts using the Quick PAHBAH assay protocol

The quick PAHBAH reducing sugar assay was used to screen for PG-inhibiting activity in extracts

made from apple *pgip1* transgenic tobacco and potato *in vitro* plants. The results are represented by a bar graph in Figure 6.8. The activity of *V. dahliae* PG in the presence of the various PGIP extracts is plotted as a percentage of its activity in NaAc buffer alone. This assay was not performed with replicates, but was repeated with replicates in the reducing sugar assay.

All putative transgenic *in vitro* potato lines except line B16 contained PGIP activity that was able to decrease *V. dahlia* PG activity to 24 - 38%. This is comparable to the positive controls. Extracts from LA Burley: *pgip1* #8 (tobacco transformed with apple *pgip1* and used as a positive control transgenic plant) reduced PG activity to 26%, and HPLC purified apple PGIP1 to 24%. Extracts from untransformed LA Burley (LA Burley -), untransformed BP1 potato (BP1 -), and line B16 caused a PG activity of higher than 100% (130, 126 and 122% respectively).

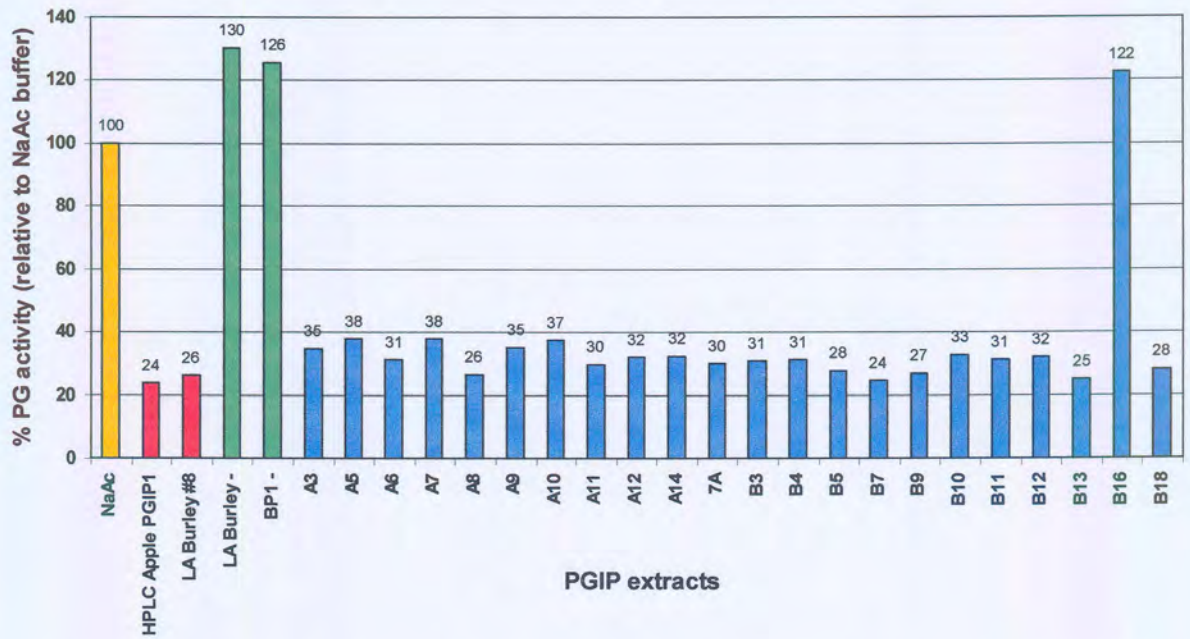


Figure 6.8 **Quick PAHBAH assay of *V. dahliae* PG activity with PGIP extracts from *in vitro* transgenic material.**

6.3.4.2.2 Linear range of *V. dahliae* PG activity

Different dilutions of *V. dahliae* PG were incubated with a fixed PGA substrate concentration for various periods. The aim was to determine the PG dilution at which there was a linear increase in release of reducing sugars. Figure 6.9 represents the activity of *V. dahliae* PG (absorbance at 410 nm) against time. The averages of the triplicate samples were determined and plotted together with the standard deviation. Regression analysis showed that there was a linear increase in the release of reducing sugars by the *V. dahliae* PG from 0 to 100 min, when the PG was diluted 1+4 or more times (Figure 6.9). $R^2 = 0.9776$ for the 1+4 dilution, and the R^2 values for the 1+9, 1+14 and 1+19 dilutions were higher than 0.99 (R^2 calculated using Microsoft Excel). This means that the fitted line accounted

for more than 97.7% of the variance in the data. The activity of the undiluted and 1+1 diluted PG enzyme plateaued after 60 min of incubation.

This data enabled the selection of the 30 min time point for the PGIP inhibition studies. It was within the linear range of PG activity for the 1+4 and higher dilutions. The 1+4 dilution of PG was chosen to yield an absorbance difference of 0.2 - 0.3 between the time points t_{30} and t_0.

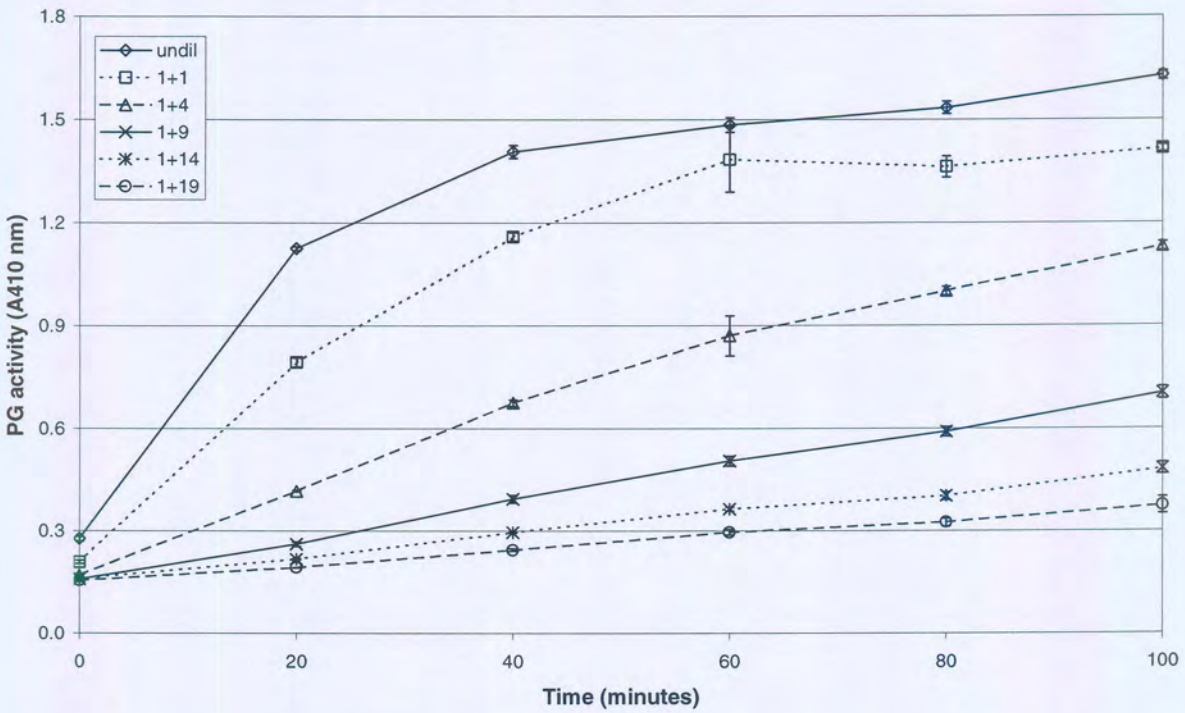


Figure 6.9 **Determination of time points at which different dilutions of *V. dahliae* PG exhibit a linear increase in activity in the reducing sugar assay.** *V. dahliae* PG activity is represented by the mean values of three replicate reactions, and the standard deviations are plotted as vertical bars.

6.3.4.2.3 Inhibition of *V. dahliae* PGs by the apple *pgip1* transgenic tobacco and potato PGIP extracts

The dialysed PGIP extracts from the *in vitro* apple *pgip1* transgenic potato and tobacco lines were used in a reducing sugar assay against endoPGs from *V. dahliae* to test their inhibitory activities. The results obtained are represented in Figure 6.10. The *V. dahliae* PG activity in NaAc buffer at 30 minutes was set at 100% to compare the inhibitory effects of the different PGIP extracts. The activities of the test reactions were set as a percentage of the control reaction (100%). The activities of the reactions are indicated within the respective columns. Each column represents the mean of triplicate samples, and a vertical bar indicates the standard deviation. All lines except B16 decreased the *V. dahliae* PG activity to 7 - 18%, indicating the presence of an active PG inhibitor. This correlates well with the results from the Quick PAHBAH and agarose diffusion assays. Inhibition

was, however, not abolished in line B16, which correlates to the results obtained in the other two assays, since the PGs still retained 74% of their activity in the presence of this PGIP extract.

Inhibition was heat denaturable, since the boiled samples (HPLC purified apple PGIP1, and extracts from LA Burley: *pgip1* #8, BP1 and A12) allowed the PG activity to return to 100% and above. *V. dahliae* PG showed only 89% activity in the presence of untransformed BP1 extract. This result is comparable to the results obtained from the ADA. The apparent inhibiting activity in the untransformed potato extract was lost when the extract was boiled.

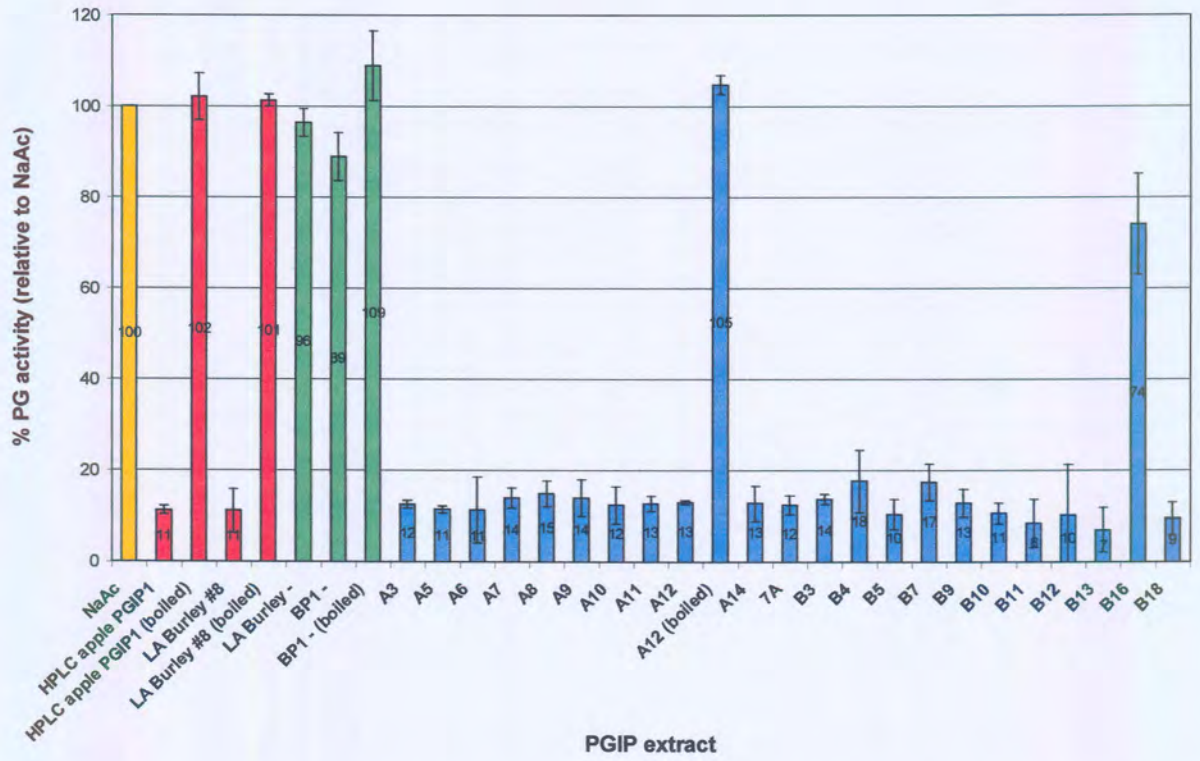


Figure 6.10 **Reducing sugar assay of *V. dahliae* PG activity with PGIP extracts from *in vitro* material.** *V. dahliae* PG activities are represented by the mean values of three replicate reactions, and the standard deviations are plotted as vertical bars.

Statistical analysis was performed on the positive and negative control reducing sugar assay data. Analysis of variance (ANOVA) indicated the least significant difference of means at the 1% level of significance was 10.15 percent PG activity. The Fisher's protected least significant difference test was performed by M. Smith (ARC Biometry unit) using the statistical program GenStat (2000). Table 6.1 summarises the percent PG activity in the presence of extract from the indicated source, and their statistical analysis. The results are expressed as a percentage relative to the PG activity in the presence of sodium acetate buffer (20 mM, pH 4.7). Different letters in the column labelled SDI (significant difference indicator) indicates activities significantly different from each other. For example,

transgenic tobacco (LA Burley: *pgip1* #8) (SDI = a) causes significantly more inhibition of *V. dahliae* PGs than NaAc buffer and untransformed tobacco (LA Burley -) (SDI = b). The protein concentrations were determined as described in the next section and presented in Table 6.2.

Table 6.1 **Apple PGIP1 causes inhibition of *V. dahliae* PGs.** PGIP extracts causing significant different PG inhibiting activities are indicated with different letters.

***V. dahliae* PG**[1]	**Source of PGIP**	**Treatment of PGIP**[5]	**Mean PG activity (%)**[6]	**SDI**[7]
+	NaAc buffer	-	100.0	b
+	Transgenic tobacco[2]	-	11.1 ± 4.7	a
+	Untransformed tobacco[3]	-	96.5 ± 3.1	b
+	Transgenic tobacco[2]	Boiled	101.3 ± 1.3	b
+	Purified PGIP1[4]	-	11.2 ± 0.9	a
+	Purified PGIP1[4]	Boiled	102.1 ± 5.1	b

The PG activity was determined by the reducing sugar assay and is shown as the mean of three separate reactions. The PG:PGIP mixtures were incubated for 20 min at 25°C prior to addition of the substrate PGA, and incubation for a further 30 min at 30°C.

[1] *V. dahliae* PG (0.56 µg crude PG extract) was mixed with the PGIP from the indicated sources.

[2] Transgenic tobacco = LA Burley: *pgip1* #8 (0.21 µg crude PGIP extract).

[3] Untransformed tobacco = LA Burley - (0.35 µg crude PGIP extract).

[4] Purified PGIP1 = HPLC purified apple PGIP1.

[5] Where indicated the extracts had been boiled for 10 min and cooled prior to mixing with the PG.

[6] The PG activity is presented as a percentage of the activity obtained in the presence of sodium acetate buffer (20 mM, pH 4.7).

[7] SDI = Significant difference indicator. PGIP sources with different lower case letters had significantly different PG activity % from one another at the 1% confidence level using Fisher's protected least significant difference test (F-test).

Purified and transgenic tobacco extracts caused a significant reduction in *V. dahliae* PG activity (from 100% down to 11%). When these samples were boiled, activity returned to normal (101% and 102%, respectively). As already stated above, this indicated that the inhibitor was heat denaturable.

6.3.5 Protein concentration determination of PG and dialysed PGIP extracts

The protein concentrations of *V. dahliae* PG and the dialysed PGIP extracts, prepared from *in vitro* plants, were measured to determine the amount of protein that was used in the inhibition assays. The Bio-Rad protein assay kit was used. The standard deviations for the triplicate samples of the standard

curve, as well as those of the samples, were very small. The BSA standard protein concentration curve was approximately linear between the 0 and 5 µg/ml protein concentrations (Figure 6.11). Linear regression between these points yielded the equation y = 0.0605 x, with the regression line accounting for 99.45% of the variance in the data (the R^2 value). The protein concentrations of the samples were calculated by comparing their absorbancies to the standard curve. The mean protein concentrations of triplicate samples, as well as their standard deviations, are presented in Table 6.2.

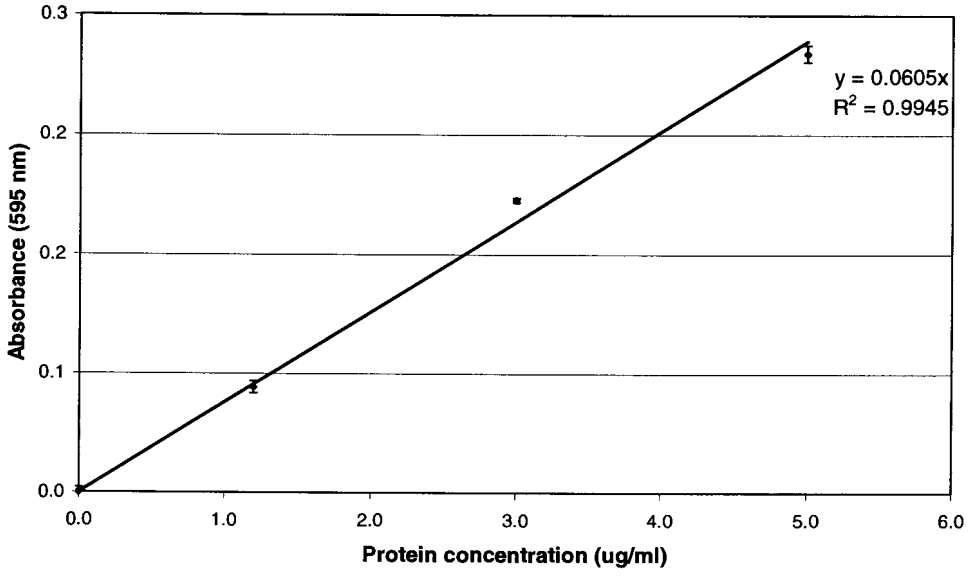


Figure 6.11 **Standard curve for the Bio-Rad protein assay using BSA as protein standard.** The average absorbance at 595 nm of each triplicate sample are plotted against protein concentration (µg/ml). The standard deviations are presented by vertical bars. A regression line is fitted to the points.

A twofold difference in protein concentrations of the potato PGIP extracts was observed between the potato lines (Table 6.2). The concentrations ranged from 47 to 111 µg/ml. The tobacco plants' PGIP extracts had much lower protein concentrations. It was 14 µg/ml for positive control transgenic LA Burley: *pgip1* #8 tobacco and 23 µg/ml for untransformed LA Burley tobacco. The protein concentration of the PGIP extract from LA Burley: *pgip1* #8 was not measured in triplicate, because an insufficient amount of this sample was available. The protein concentration of the *V. dahliae* PG, after AS precipitation of the fungal culture supernatant, was 186 µg/ml.

Table 6.2 **Protein concentrations of PGIP and PG extracts.**

Protein sample[1]	Mean protein concentration (µg/ml)[6]
A3	101 ± 3
A5	57 ± 2
A6	63 ± 2
A7	80 ± 2
A8	63 ± 1
A9	49 ± 2
A10	88 ± 1
A11	80 ± 2
A12	108 ± 4
A14	90 ± 1
7A	90 ± 5
B3	47 ± 1
B4	48 ± 3
B5	69 ± 3
B7	66 ± 1
B9	70 ± 3
B10	55 ± 2
B11	51 ± 2
B12	65 ± 0
B13	94 ± 2
B16	72 ± 5
B18	83 ± 2
BP1-[2]	111 ± 7
Transgenic tobacco[3]	14
Untransformed tobacco[4]	23 ± 1
V. dahliae PG[5]	186 ± 5

[1] Protein concentrations of the crude PGIP extracts prepared from the indicated transgenic potato lines and tobacco controls or PG from *V. dahliae*.

[2] BP1- = untransformed BP1 potato.

[3] Transgenic tobacco = apple *pgip1* transgenic tobacco positive control (LA Burley: *pgip1* #8).

[4] Untransformed tobacco = LA Burley -.

[5] *V. dahliae* PG = crude PG extract after AS precipitation as described in this chapter.

[6] The values represent the means of triplicate samples, and the standard deviations are indicated.

6.3.6 *V. dahliae* PG activity per microgram crude PGIP extract

The percentage activity of *V. dahliae* PGs in the presence of transgenic potato lines PGIP extracts, as determined using the reducing sugar assay, was calculated per microgram crude PGIP extract prepared from each line. The protein concentration data of Table 6.2 was used to calculate the amount of microgram protein present when fifteen microlitres of extract was used in each assay. The percentage PG activity per microgram crude PGIP extract was expressed relative to the activity in the presence of untransformed BP1 potato extract (BP1- = 100%). Statistical analysis was performed using Genstat (2000) as described before. Table 6.3 summarises the percent PG activity per µg of the indicated

PGIP extract, and their statistical analysis. Analysis of variance (ANOVA) indicated the least significant difference of means at the 1% level of significance was 18.97 percent PG activity.

Table 6.3 **Inhibition of *V. dahliae* PG by crude PGIP extracts from transgenic potato transformed with the *pgip1* gene.**

V. dahliae PG[1]	Source of PGIP	Treatment of PGIP[2]	Mean PG activity (%) per µg crude PGIP extract relative to untransformed BP1[3]	SDI[4]
+	BP1 -	-	100 ± 0	b
+	BP1 -	Boiled	123 ± 9	a
+	A3	-	15 ± 1	e f g
+	A5	-	25 ± 2	c d e f g
+	A6	-	22 ± 14	d e f g
+	A7	-	20 ± 3	d e f g
+	A8	-	27 ± 5	c d e f g
+	A9	-	33 ± 10	c d e
+	A10	-	17 ± 5	d e f g
+	A11	-	19 ± 2	d e f g
+	A12	-	14 ± 0	f g
+	A12	Boiled	117 ± 2	a b
+	A14	-	17 ± 5	d e f g
+	7A	-	16 ± 3	d e f g
+	B3	-	34 ± 3	c d
+	B4	-	42 ± 16	c
+	B5	-	17 ± 5	d e f g
+	B7	-	30 ± 7	c d e f
+	B9	-	21 ± 5	d e f g
+	B10	-	22 ± 5	d e f g
+	B11	-	20 ± 12	d e f g
+	B12	-	19 ± 20	d e f g
+	B13	-	9 ± 6	g
+	B16	-	123 ± 19	a
+	B18	-	14 ± 5	f g

V. dahliae PGs were produced from growth on pectin. PG activity was determined by the reducing sugar assay. For each reaction, the PG was mixed with the PGIP extracts from the different transgenic lines for 20 min at 25°C prior to addition of the substrate PGA and incubation for 30 min at 30°C. The amount of reducing sugars released was assessed using the PAHBAH method.

[1] *V. dahliae* PG (0.56 µg crude PG extract) was mixed with the PGIP extracts from leaves of potato transformed with the *pgip1* gene.

[2] Where indicated the extracts had been boiled for 10 min and cooled prior to mixing with the PG.

[3] 100% PG activity represents 837 pmoles reducing sugars released min^{-1} µg PGIP $extract^{-1}$. The PG activity is presented as a percentage of the activity obtained in the presence of untransformed BP1 extract. Values are the means of three separate reactions and standard deviations are indicated.

[4] SDI = Significant difference indicator. PGIP sources with different lower case letters had significantly different % PG activity from one another at the 1% confidence level using Fisher's protected least significant difference test (F-test).

Different letters in the column labelled SDI (significant difference indicator) indicates activities significantly different from each other. The untransformed potato (BP1-), transgenic potato line B16 and boiled extracts of BP1 and A12 had SDI's of a or b (100 - 123%). They therefore differed significantly from the rest of the transgenic potato lines which had SDI's of c,d, e, f and/or g (9 - 42%). An active inhibitor was thus present in all the lines except line B16. The PGIP extracts from the transgenic potato lines dramatically reduced the PG activity from 100% in the presence of untransformed BP1 extract to between 9% (line B13) and 42% (line B4). Line B16 and the boiled extracts caused an activity of higher than 100%.

6.4 Discussion

6.4.1 *V. dahliae* PG isolation and AS precipitation

The agarose diffusion assay was employed on the *V. dahliae* culture filtrates from different growth days to determine the fraction with the highest PG activity, so that these fractions could be pooled and the PGs precipitated (Figure 6.1). During the agarose diffusion assay, ruthenium red reacts with the unhydrolysed substrate to form cleared zones of PG activity. The highest PG activity leads to the development of zones with the largest diameter. Activity was high throughout a number of collection days, so it was precipitated in three pools. Maximal PG activity was obtained after 5 days of growth, which is very different from that found by James *et al* (2001), which was after 18 days. Possible reasons for this may include that a different isolate (a pathogenic isolate from infected cotton stems vs. the potato pathogenic isolate used in this study) and different culture media composition were used.

A possible reason for the high PG activity at day 13 (Figure 6.1) is perhaps an unequal amount of mycelium distributed to the flasks during inoculation with Czapex-dox fungal culture. This would then lead to faster fungal growth and more PG secretion into the medium. Another reason may be that the fungus secretes different PG's at different stages of growth, and perhaps the PG profile was at a peak on this day.

Ammonium sulphate precipitation was employed to remove pectin in the growth medium from the PG that was being isolated. Because the activity of PG in each pool after precipitation was high (Figure 6.2), and apple PGIP1 could successfully inhibit PG from all three pools (Figure 6.4), it was decided to combine the three pools of precipitated PG. This lead to the production of a large amount of *V. dahliae* PGs of a uniform concentration, which was advantageous to use in subsequent PG activity assays.

PG expression in culture on pectin medium and *in vivo* during infection of a plant is not necessarily the same. Even under different media conditions PG expression is not the same. For example, six PG enzymes of *B. cinerea* were differentially expressed when cultured on two different liquid culture media, the one supplemented with glucose and the other with polygalacturonic acid as the sole carbon source (Wubben *et al.*, 1999). Yao *et al* (1995) demonstrated that this fungus secretes different PGs *in vivo* than when it is cultured *in vitro*. He showed that apple PGIP was able to inhibit four out of five PGs secreted by *B. cinerea* in liquid culture, but was completely unable to inhibit PGs produced on fruit inoculated with this fungus. A PG from *Penicillium expansum* was only expressed in the invasion and colonisation of apple fruit, and not in fungal mycelia grown on apple pectin medium (Yao *et al.*, 1996).

Because PGIP's interaction with fungal PGs is highly specific, it is hoped that the apple PGIP1 in transgenic potato will still be able to inhibit fungal invasion of *V. dahliae in vivo*.

6.4.2 Quick PGIP extraction and ADA

Dialysed and non-dialysed PGIP extracts yielded the same inhibiting activity of *V. dahliae* PG in an agarose diffusion assay (Figure 6.5). The fact that dialysis doesn't have an influence on the inhibiting activity of PGIP extracts prepared from transgenic potato lines, makes screening of large numbers of transgenic plants for PGIP activity simpler and less time-consuming. The causal agent of the yellowing of the zones around wells containing non-dialysed PGIP extracts might be proteins that normally precipitate during dialysis and are subsequently removed by centrifugation before using the extract in an ADA. These undialysed PGIP extracts are suspected to be unsuitable for reducing sugar assays, since the NaCl in the extraction buffer (1 M NaCl, 20 mM NaAc buffer, pH 4.7) will interfere with the PG:PGIP interaction. In the ADA, NaCl perhaps diffuses away and doesn't influence the interaction.

6.4.3 Assay for PGIP activity

Three assays were performed to assess the PG-inhibiting activity of the crude PGIP extracts prepared from the transgenic tobacco and potato material. The different assays were performed when quick qualitative or quantitative inhibition results were required.

The qualitative assays did not have replicates, and included the ADA and quick PAHBAH assays. They were used for preparative purposes without using too much of the sample. The agarose diffusion assay is usually employed to rapidly screen many transgenic plants for the expression of PGIP. Dialysis of the PGIP extracts is not necessary, for the possible reason as stated above. The ADA represents the inhibiting activity in the form of decreased cleared zones in a medium containing polygalacturonic acid as substrate. The quick PAHBAH assay is based on the same principles as the reducing sugar assay, but it is faster and only a rough indication of PGIP-activity since the reactions are not performed in triplicate. Quantitative inhibition results are obtained when the reducing sugar assay is performed with replicates.

6.4.3.1 Agarose diffusion assay (ADA)

The agarose diffusion assay was performed on PGIP extracts prepared from leaves of *in vitro* and glasshouse transgenic potato and tobacco, and *in vitro* roots of transgenic potato lines.

6.4.3.1.1 ADA of *V. dahliae* PG activity with PGIP extracts from *in vitro* and glasshouse-grown leaf material

Boiling of PGIP extracts prepared from *in vitro* transgenic leaf material abolished their inhibiting activity (Figure 6.6, samples with a (b)). This indicated that the inhibitor is a protein that can be heat-denatured. The fact that the HPLC purified apple PGIP1 didn't inhibit *V. dahliae* PG substantially (zone diameter of 18 mm compared to 21 mm of PG + NaAc buffer) might be because too little of the purified inhibitor was used in the ADA. During subsequent ADA experiments, much better inhibition was observed when 5 µl instead of 2 µl of the purified PGIP1 was used. The stoichiometry of the PG:PGIP inhibition might have been more optimal using more purified PGIP1.

The small percentage of zone reduction that occurred with the untransformed BP1 potato extract (BP1-), indicated a low level of endogenous PG inhibiting activity active against *V. dahliae* PGs. A PGIP has been discovered in potato from the cultivar Spunta (Machinandiarena, 2001). It showed a broad inhibitory activity against crude PG preparations from the fungi *Aspergillus niger*, *Fusarium moniliforme* and *F. solani*. It was cell wall bound since the sodium chloride extract of the potato leaves contained most of the inhibitory activity. It was induced in the leaves by wounding, salicylic acid and the incompatible interaction with the potato pathogen *Phytophthora infestans*. Potato thus seems able to use PGIP as a defence mechanism against fungal pathogens. Extracts prepared from the BP1 cultivar contained a very small amount of inhibitory activity against *V. dahliae* PG, which was lost by heat denaturing. Thus, the cultivar BP1 could also contain an endogenous PGIP, which is not very effective in inhibiting *V. dahliae* PG since it possibly has different PG specificities.

The modified agarose diffusion assay, using HCl instead of ruthenium red for zone visualisation, leads to cleared zones forming within minutes in the opaque agarose plate. Results are obtained much faster, but it is not so graphical as the ruthenium red staining. PGIP extracts from glasshouse leaves of transgenic potato and tobacco were assayed using the modified ADA (data presented in **Results**, figure not shown). It gave similar inhibition results as those obtained with the extracts prepared from the *in vitro* plants, indicating that the *pgip1* gene is expressed also under glasshouse conditions.

6.4.3.1.2 ADA of *V. dahliae* PG activity with PGIP extracts from *in vitro* transgenic potato root material

Very low levels of PGIP have been reported in the roots of *Phaseolus vulgaris*, with the levels increasing in the stems during plant growth (Salvi *et al.*, 1990). If this expression pattern is universal to all plants, PGIP might not be present to protect plants from pathogens invading through the roots. The only plant in which PGIP has been characterised in the roots to date, is lupin (Costa *et al.*, 1997).

The CaMV 35S promoter is commonly used as a promoter to drive transgene expression in plants. It is a constitutive promoter, active in most cell types. Its activity in roots is presented here by a few examples. The CaMV 35S promoter gave strong expression of the GUS reporter gene in all organs of *A. thaliana*, except the hypocotyl (Holtorf *et al.*, 1995). Levels of expression were, however, approximately threefold higher in the leaves than in the roots. The meristematic (root tip) and elongation regions of rice stained strongly in rice plants transformed with CaMV 35S-*gus* (Mazithulela *et al.*, 2000). This promoter (and its enhanced duplicated derivative) was also active in expressing GFP in various tissue types of grape and cotton, including the root (Li *et al.*, 2001; Sunilkumar *et al.*, 2002).

The ADA showed successful inhibition of *V. dahliae* PG by PGIP extracts prepared from *in vitro* transgenic potato roots (Figure 6.7). No substantial inhibition was obtained with the extract prepared from untransformed BP1 potato roots. This indicates the successful expression of the apple *pgip1* transgene under control of the enhanced CaMV 35S (e35S) promoter in this tissue type. This result corresponds to the publications on the CaMV 35S promoter, and is important in the overall aim of the project, which is to confer enhanced resistance to potato against *Verticillium*-wilt. The pathogen enters its host through the roots, and if PGIP1 can be expressed at the site of entry, the possibility for protecting the plant is much higher. Using the CaMV e35S promoter, PGIP is expressed constitutively and is not dependent on the natural tissue specific expression pattern of PGIP. Thus, PGIP was able to accumulate in the roots of transgenic potato plants.

6.4.3.2 Reducing sugar assay

6.4.3.2.1 Quick PAHBAH assay

It is expected that extracts from BP1, which is susceptible to *V. dahliae*, will not inhibit *V. dahliae* PGs. Inhibition of *V. dahliae* PG by extracts prepared from apple *pgip1* transgenic potato lines, but not from control untransformed plants, indicated the presence of a compound capable of inhibition only found in the transgenics (Figure 6.8). It may be concluded that it is the apple PGIP1 that is being functionally expressed.

The apparent increase in PG activity of samples incubated with PGIP extracts from untransformed LA Burley (LA Burley -), untransformed BP1 potato (BP1 -), and line B16 (130, 126 and 122% activity, respectively) is unlikely to be due to activation of the *V. dahliae* PG enzyme (Figure 6.8). Rather, other enzymes present in the crude plant extracts can be responsible for the increased absorbance at 410 nm. These enzymes could be plant PGs or plant cellulases that release sugars from the cell wall. The sugars then react with the colour reagent (PAHBAH), to cause a 22 - 30% higher absorbance at 410 nm than PG incubated with NaAc buffer alone. This inherent enzyme activity observed in the

untransformed plants and line B16 will also be present in the transgenic lines. These enzymes are not inhibited by the transgenic PGIP and cause background absorbance in the assay. Therefore, if the 22 - 30% background activity is deducted from the PG activity in the presence of the extracts prepared from the transgenic lines (ranging between 24 and 38%), the PGs show 0 - 16% activity. Thus, these extracts show 84 to 100% inhibition of *V. dahliae* PGs. Even without the correction for this background, all except one (line B16) of the transgenic potato lines showed very good inhibiting activity against *V. dahliae* PG *in vitro*.

The quick PAHBAH assay gave a good indication of the relative expression of transgenic PGIP in various PGIP transgenic potato lines. It can therefore be used for the quick screening of high numbers of transgenic lines.

6.4.3.2.2 Linear range of *V. dahliae* PG activity

The reducing sugar assay was employed to determine the PG activity over time. This enabled the determination of the time points between which *V. dahliae* PG activity has a linear trend (Figure 6.9). The time points chosen for the inhibition assays with PGIP extracts were t=0` and t=30`, when the *V. dahliae* PG was used at a 1 in 5 dilution. This yielded an absorbance difference at 410 nm of 0.2 - 0.3, which was considered to be sufficient for determining inhibiting activity of PGIP extracts in the subsequent inhibition experiments.

6.4.3.2.3 Reducing sugar assay of inhibition of *V. dahliae* PGs by PGIP extracts

Using the reducing sugar assay, the extracts from *in vitro* apple *pgip1* transgenic tobacco and all the transgenic potato lines except B16 were shown to contain an active PG inhibitor (Figure 6.10). It inhibited 82 - 93% of the *V. dahliae* PG activity present in the fungal culture supernatant. Boiling abolished the inhibitory effect of the extracts (columns labelled with the description (boiled)), indicating that the inhibitor is a protein that can be heat-denatured. The activity of PG increased to 101 - 109% in the presence of the boiled samples (HPLC purified apple PGIP1, and extracts from LA Burley: *pgip1* #8, BP1 and A12). This may be due to the stabilising effect that the heat-denatured PGIP had on the fungal PGs. When the PG is stabilised, it probably remains active for longer over the assay period and more sugars are released to react with the PAHBAH reagent.

ANOVA indicated statistically significant differences in PG activity percentages, at the 1% level of significance, between the positive and negative controls. The data could be grouped into two groups, significantly different from each other (Table 6.1). The first group included the positive controls (LA Burley: *pgip1* #8 and HPLC purified apple PGIP1) (remaining PG activity of 11%). The second group contained the untransformed LA Burley control, NaAc buffer and the boiled PGIP extracts (96 - 102% PG activity). The first group had the significance indicator (a) and the second group (b). It was

concluded that the PGIP extracts from the transgenic LA Burley: *pgip1* #8 tobacco line and purified PGIP1 caused a significantly different PG activity from the rest of the control reactions, so that the null hypothesis of no difference was rejected.

6.4.4 Protein concentration determination of PG and dialysed PGIP extracts

For the ADA, 15 µl of AS precipitated *V. dahliae* PG was incubated with 15 µl NaAc buffer or PGIP extract, and 25 µl of this mix was loaded onto the ADA plate. Thus 12.5 µl of PG was loaded onto each plate during the ADA. Since the protein concentration of *V. dahliae* PG after AS precipitation was 186 µg/ml, 12.5 µl PG corresponded to 2.3 µg of protein. The AS precipitation of fungal culture supernatant is only a crude preparation method for fungal PGs, so there are many other contaminating proteins still present. Therefore, the protein concentration just gives a rough indication of the amount of protein used in assays, and not the amount of PG enzyme.

The protein concentrations between the different potato lines' PGIP extracts differed twofold, and differed greatly from the tobacco extracts (Table 6.2). Apart from the protein concentration, the amount of PGIP in each extract can also vary greatly, depending on the expression level in each transgenic line. A western blot can be used to quantify the PGIP in the crude extractions, if an antibody is available. A dilution series of the PGIP extract, and a purified PGIP standard with known concentration, are separated on a polyacrylamide gel, blotted to a membrane and hybridised to the PGIP-specific antibody. Quantification of the bound antibody will give a relative estimation of the PGIP content in the crude PGIP extract.

During the PG:PGIP inhibition assays (ADA and reducing sugar assays), equal volumes of PGIP extracts from the different lines were incubated with *V. dahliae* PG. The PG content of each of the reactions were constant. Since the PGIP content of each extract could differ because of the two reasons as stated above (different protein concentrations and expression levels), the levels of PG inhibiting activity could differ between the lines. The ratio between PG and PGIP in the PG:PGIP interaction would be different between the different lines, causing the activity of the PG to be more or less inhibited by the co-incubated PGIP extract.

To try and compensate for this difference in PGIP levels between the different transgenic potato lines, the % PG activity was normalised with the amount of protein present in the crude PGIP extract. Table 6.3 presents the percentage PG activity per microgram crude PGIP extract. It was further expressed as a relative percentage to the activity in the presence of untransformed BP1 potato extract (BP1- = 100%). All lines except B16 decreased the *V. dahliae* PG activity to between 9% (line B13) and 42% (line B4). Untransformed potato (BP1-), transgenic potato line B16 and boiled extracts of BP1 and

A12 caused PG activities of 100 - 123%. Statistical analysis of the data revealed that the differences between these PG activities were significant. An active inhibitor was thus present in all the lines except line B16. This correlates well with the results from the Quick PAHBAH and agarose diffusion assays.

6.4.5 Combined results of the inhibition assays

Results from all three inhibition assays corresponded very well with each other. The reducing sugar assay and the quick PAHBAH assay both indicated more than 80% inhibition of *V. dahliae* PG (or 60% inhibition per µg crude PGIP extract), when equal volumes of PGIP extract and 1 in 5 diluted PG were incubated together. Boiled PGIP extracts were not active in inhibiting the PGs, indicating that the inhibitor was a heat-denaturable protein. All three methods indicated the absence or reduced inhibiting activity in putative transgenic potato line B16. Since the apple *pgip1* gene was detected using PCR (Chapter 5), the gene might either not be expressed, or expressed to produce a non-functional protein. Non-expression may occur as result of the positioning in the genome (close to gene silencers or in a region of inactive heterochromatin) or a mutation in the e35S promoter region preceding the transgene. An inactive, non-functional protein may be the product of a point mutation at a critical site, or an insertion or deletion mutation that causes a shift in the reading frame that leads to a truncated protein. RT-PCR can be employed to test whether transcription of the transgene actually takes place in this plant, and western blot can determine the presence of a translated protein.

An argument for the hypothesis that apple PGIP1 in the extracts is causing the inhibition of *V. dahliae* PGs, is that 21 out of the 22 tested transgenic potato lines contained inhibiting activity. It can therefore be safely said that inhibition of PG by transformed plant extracts was not due to somaclonal variation (random mutations introduced during the transformation process) that caused an alteration in their metabolic composition. For example, an event that would lead to reduced PG activity in the PG:PGIP inhibition assays, would be the increased expression of a protease that degrades the PG. The chance of a somaclonal event like this happening in all the lines tested is very small.

6.4.6 Conclusion

In conclusion, all except one of the PGIP extracts prepared from leaves and roots of apple *pgip1* transgenic potato lines showed inhibitory activity *in vitro* against crude PG preparations from *V. dahliae*. This indicates an advantageous situation where it is possible that the apple *pgip1* gene can confer enhanced resistance to transgenic plants against this fungus in the field. The next chapter will report on the effect this transgene had on the resistance of potatoes when grown in a glasshouse in the presence of this fungus.

CHAPTER 7

Glasshouse trial of potato for increased resistance to *V. dahliae*

7.1 Introduction

The literature on *Verticillium*-wilt of potato was reviewed in Chapter 2. This disease is caused by the soil-borne fungal pathogen *Verticillium dahliae*. *V. dahliae* causes symptoms of wilting and yellowing to appear on potatoes earlier than expected from natural senescence. It therefore causes yield reduction by shortening the growing period. Developing genetically stable resistant or tolerant cultivars was proposed to be the best means of controlling this disease (Tsror and Nachmias, 1995). Due to its involvement in the potato early dying complex, the development of potato cultivars highly resistant to *Verticillium* is also critical for the management of potato early dying disease (Wheeler *et al.* 1994).

Due to the challenges of breeding for resistance to this disease, it was proposed that the transformation of potato with an antifungal gene could confer resistance against this fungus to susceptible plants. Preliminary studies indicated the apple *pgip1* gene to be a possible candidate. Chapter 6 provided evidence that apple PGIP1, purified to homogeneity, was able to inhibit *V. dahliae* PGs *in vitro*. Several apple *pgip1* transgenic potato lines were also able to express active apple PGIP1. According to the hypothesis of Cervone *et al.* (1989), the interaction of PGIP in the plant with fungal polygalacturonases could lead to the accumulation of oligogalacturonides, elicitors of plant defence responses.

Examples of transgenic plants with enhanced resistance to fungal pathogens

Several examples exist in which chitinase genes were transformed into plants to confer resistance against fungal attack. In the first example, transgenic manipulation of tobacco and potato yielded enhanced resistance against several foliar pathogens and the soil-borne pathogen *Rhizoctonia solani*. A chitinase gene from a biocontrol fungus, *Trichoderma harzianum*, was the successful gene (Lorito *et al.*, 1998). Another example includes transgenic tomato plants that have been generated with improved resistance to *V. dahliae* race 2 (Tabaeizadeh *et al.*, 1999). An acidic endochitinase gene (pcht28) from the wild tomato *Lycopersicon chilense* was transformed into tomato (*L. esculentum* cv. Starfire). The CaMV 35S promoter was used to drive expression of the transgene. Foliar disease symptoms, the extent (cm) of vascular discoloration in the above-ground stem and a vascular discoloration index (vascular discoloration / plant height × 100) were measured to evaluate the

response of plants to *V. dahliae* race 2 in the greenhouse. The transgenic R1 and R2 progeny demonstrated a significantly higher level of tolerance to the *V. dahliae* race 2 compared to nontransgenic plants. These plants developed less necrotic areas than nontransgenic plants, and showed an overall improved resistance. Since no genetic source for resistance to *V. dahliae* race 2 has yet been identified for tomato, these results represent an important source of genetic resistance to this fungal pathogen.

Transgenic potato lines containing the apple *pgip1* gene under control of the constitutive CaMV 35S promoter were generated, and their molecular characterisation was reported in Chapter 5. The hypothesis of this chapter is that the apple *pgip1* transgene will confer enhanced resistance against *V. dahliae* to the transgenic potato lines compared to the untransformed BP1 control. The aim of this chapter was therefore to screen these transgenic potato lines in a glasshouse trial for enhanced resistance to *V. dahliae*. To test the response due to the transgene, the transgenic lines and untransformed control were planted in a glasshouse in *V. dahliae* inoculated soil. Symptom and colonisation measurements were made, and used in statistical analysis to test for the significance of differences between transgenic and untransformed lines.

7.2 Materials and Methods

7.2.1 Planting of tubers in the glasshouse

Apple *pgip1* transgenic and untransformed BP1 potato *in vitro* propagated plantlets were grown in a glasshouse to produce minitubers. Ten plants each of 20 transgenic lines and untransformed BP1 potato were planted into 15 cm diameter pots containing a sterile mixture (tindalization at 105°C for 3 alternative days) of sandy soil (7% clay) and vermiculite (3:1, v/v). The pots were placed in a glasshouse of which the temperature was regulated at 25°C. The plantlets were covered with transparent plastic cups to harden them off from the *in vitro* conditions. The pots were watered three times a day (7:30, 13:15 & 17:00) for two minutes with an automatic micro-irrigation system.

Three days later the cups were removed from the plantlets. The growing plants were tied up to stakes to support their vertical growth. Potato minitubers were harvested from the pots when the plants had senesced. The harvested minitubers were treated with Rindite (**Appendix A**) two weeks prior to planting to stimulate node development.

Rindite-treated minitubers of all the transgenic lines and untransformed BP1 potato were planted in a randomised block design (Samuels, 1989). There were nine replicates of each of the 20 transgenic lines and 18 replicates of untransformed BP1 (in two groups, called BP1A and BP1B, respectively). They were planted in pots containing *V. dahliae* infected soil (termed "inoculated" soil from here on) and uninoculated control soil. The inoculum density for *V. dahliae* was 62 microsclerotia gram^{-1} soil. Pots filled with sand/ vermiculite (prepared as before) were inoculated with the inoculum by placing 10 g of inoculated vermiculite into a hollow of each pot and mixing it into the soil. Fertiliser (1 g of 2:3:2 (22) N: P: K) was applied at planting to each pot. Plants were grown in the glasshouse with conditions as described for the *in vitro* plantlets.

7.2.2 Preparation of *V. dahliae* microsclerotia inoculum

V. dahliae microsclerotia inoculum was produced by C. Millard (ARC-Roodeplaat). The source of fungus was the same as section 6.2.1.1. A suspension comprising 200 ml V-8 juice (tomato and vegetable juice blend; Campbell soup company, Camden, NJ, USA) and 800 ml distilled water was added at a rate of 175 ml per flask to 1 litre Erlenmeyer flasks each, containing 500 ml vermiculite. Flasks were plugged with cotton wool, capped with aluminium foil, and autoclaved at 121°C for 30 minutes. After cooling, each flask was inoculated with a 5 mm diameter mycelial disc from a 10-day-old potato-dextrose agar culture of *V. dahliae* (isolates 61 and 77) and the flasks were incubated at 25°C for 28 days (Denner, 1997). The vermiculite was then air-dried for 14 days. Microsclerotia

produced by the various isolates on the vermiculite were pooled and the composite inoculum was incorporated at 10 g vermiculite per 1900 g of the sterile soil mixture, to a density of 62 microsclerotia gram^{-1} soil.

Microsclerotia in soil was enumerated according to the method of Harris *et al.*, (1983). Ten subsamples of soil of 10 gram each was suspended in 100 ml distilled water in an Erlenmeyer flask. The suspension was blended in a mixer for 1 minute. The suspension was washed through 90- and 25 µm mesh sieves (20 cm diameter) with tap water, and the material on the 25 µm sieve was recovered into the original flask, and resuspended in 100 ml 0.1% wateragar. The suspension was shaken thoroughly before withdrawing 1 ml samples of soil suspension. These samples were plated onto three plates of modified soil extract agar (MSEA). Plates were incubated at 25°C for 4 weeks in the dark. The soil was removed by washing with tap water. Using a dissecting microscope, plates were observed for colonies of *V. dahliae* at 25× magnification. The number of microsclerotia per gram of soil was determined as follows: average number of colonies of the 3 plates / (10 g of soil / 100 ml of 0.1% wateragar).

7.2.3 Visual assessment of *V. dahliae* symptoms

The first visual assessment of *V. dahliae* disease symptoms was performed nine weeks after planting of the tubers. It was performed twice weekly until 16 weeks. The earliest symptoms of typical potato senescence include yellowing and wilting of the bottom leaves, which spread upwards until it reaches the top of the plant. Ultimately the whole plant dies and becomes dried-out.

Visual assessments of disease symptoms were performed using a 5-point scale of Robinson *et al.* (1957) and Isaac and Harrison (1968). The stems were divided into three equal regions and class values assigned to each plant according to the following scale:

1 = no symptoms of yellowing/ wilting
2 = single yellow leaf or symptoms up to the bottom third of the plant
3 = symptoms up to the middle third
4 = symptoms up to the top third or the whole plant symptomatic
5 = the whole plant wilted, dried out and completely dead.

7.2.4 Plating out of stem sections onto potato dextrose agar (PDA)

At the end of the growth stage, stem sections were collected and screened for the presence of *V. dahliae* stem colonisation. Stem sections were collected weekly from week 10 to 16, as plants reached the final stage of infection (scale number 5) and became completely dried-out. Stem isolations were

made from the remaining plants 16 weeks after planting of the tubers. Segments 50 mm long were taken from the stem base of plants, surface-disinfected in 1% sodium hypochlorite for 5 min, and then rinsed in sterile water. Stem sections were allowed to air-dry on paper towel. Under sterile conditions, the stem section was vertically split in half, one half divided into five sections and the pieces plated onto PDA plates amended with 100 µg/ml streptomycin sulphate (0.1 g suspended in 10 ml ethanol per litre of PDA medium). The plates were incubated in a growth room at 25°C at 12 h light and 12 h darkness for 3 - 5 days. The plates were microscopically examined to identify *V. dahlia* fungal cultures growing on the stems. The number of stems infected was scored.

7.2.5 Calculation of the disease index

Analysis of *Verticillium*-wilt resistance or susceptibility of the transgenic potato lines were based on visual assessments of the foliage symptoms typical for *Verticillium*, and the number of stalk sections harbouring *V. dahliae* when plated out onto PDA. A modification of the index of Corsini *et al.* (1988) was calculated for each replicate as follows:

$$\frac{(\text{wilt severity 1 - 5 scale}) \times (\text{individual showing wilt 0 / 1}) + (\text{individual stem colonised 0 / 1})}{\text{median time for symptoms to appear}} \times \frac{10}{1}$$

The values as they were on week 16 were used for the calculation, since this was the time when all the remaining stem sections were plated out onto PDA.

7.3 Results

7.3.1 Planting of tubers in the glasshouse

To produce minitubers for the glasshouse trial, *in vitro* propagated apple *pgip1* transgenic and untransformed BP1 potato lines were grown in a glasshouse. Hardening off of *in vitro* plantlets was very successful. None of the plantlets wilted or died after transplantation. Varying numbers of minitubers, ranging in size, were harvested from the glasshouse-grown plants. These tubers were planted for the glasshouse trial, after being treated with Rindite. Shoots developed from the tubers at differing times, even though only tubers with developing nodes were selected for planting.

7.3.2 Visual assessment of *V. dahliae* symptoms

The foliar symptoms of *Verticillium*-wilt were on a scale of 1 for no yellowing and wilting symptoms to 5 for the whole plant senesced and dried out. Figure 7.1 shows examples of plants from all the classes of the visual symptom scale. Symptoms typical to that published for *Verticillium*-wilt were obtained on the plants grown in inoculated soil (Millard and Denner, 2001). Natural senescence of the control plants displayed similar symptoms, but *Verticillium* symptoms appeared sooner on all the potato lines planted in inoculated soil, than the incidence of natural senescence. The median of time after planting for symptom expression was 10 weeks for plants grown in inoculated soil, and 12 weeks for control soil. There were large amounts of variability of symptom expression within the replicates of the same lines.

Figure 7.1 ***Verticillium*-wilt symptoms on a scale of 1 to 5.** Representative plants of each class of disease symptoms, from no yellow leaves (1) to the plant completely dried-out (5), are displayed.

7.3.3 Plating out of stem sections onto PDA

Seventy percent of all stem sections isolated from plants grown in *V. dahliae* inoculated soil lead to the formation of *V. dahliae* colonies on the PDA plates. This corresponded to 140 out of the total of 198 stem sections. Only 1%, corresponding to 2 stem sections, of the plants grown in control soil produced *V. dahliae* colonies. This low percentage can be ascribed to cross-contamination during harvesting of the stems, or during plating of the sections onto PDA plates. The data was used to calculate disease indices.

7.3.4 Calculation of the disease index

A disease index was calculated for each individual plant, including all the replicates of all the lines, and all the plants planted in the inoculated and the control soil. For calculation of the disease index, the formula presented at section 7.2.5 was applied (Corsini *et al.*, 1988).

For the term "wilt severity 1 - 5 scale", the visual disease severity on week 16 after planting of the tubers was used, since this was the time when all the remaining stem sections were plated out onto PDA. It was on a scale of 1 to 5. For the next term in the equation, individuals showing any visual symptoms (scale 2 to 5) were given a 1, while those showing no symptoms were given a 0. The data obtained from the plating out of the stem sections onto PDA were used to determine the colonisation (1) or no colonisation (0) of an individual stem. Colonisation was a 1 if *V. dahliae* colonies could be identified microscopically. The median time (in weeks) for symptoms to appear was determined by arranging the weeks when symptoms started to appear for each individual plant in an increasing order, and choosing the middle value (if the number of samples (n) is odd), or midway between the two middle values (if n is even).

7.3.5 Statistical analysis of the disease index data

After calculation of disease indices for each individual plant, Fisher's protected least significant difference test (F-test) was applied separately to the disease indices of plants grown in each soil type (inoculated or control). Data were analysed by M. Smith (ARC Biometry unit) using the statistical program GenStat (2000). Data were tested for statistical significant differences between the disease indices of untransformed BP1 and the transgenic potato lines. The overall F test was significant at the 1% level of significance for both the inoculated and control groups. The null hypothesis of no difference between lines was therefore rejected. The least significant difference (lsd) of index values at the 1% level of significance was determined to be 1.0676 for the lines planted in inoculated soil. Lines planted in control soil had an lsd of 0.9386.

The following table summarises the index values of the different potato lines planted in inoculated soil. Table 7.2 has the data for the lines planted in control soil. The mean index values of the nine replicates of each line are sorted in a decreasing order, and different letters indicates indices significantly different from each other. For example in Table 7.1, line A3 (d) had a significantly lower disease index than all the lines from A11 to B12, including the untransformed BP1 lines (letters ranging from (a) to (a b c)). Line A3's index (4.444) differed by at least the lsd value (1.0676) from these lines.

Table 7.1 **Potato lines planted in inoculated soil with significantly different disease indices.** Disease indices are sorted in a decreasing order, and the potato lines with significant different indices are indicated with different letters (a - d).

Potato line	Mean disease index	Significant difference indicator
A11	6.000	a
B3	6.000	a
A12	5.889	a b
A9	5.889	a b
B18	5.889	a b
B9	5.889	a b
7A	5.778	a b
A5	5.778	a b
B11	5.778	a b
B5	5.778	a b
BP1A (untransformed)	5.778	a b
A6	5.667	a b c
A7	5.667	a b c
BP1B (untransformed)	5.667	a b c
B12	5.556	a b c
A8	5.000	a b c d
B16	4.889	b c d
A14	4.667	c d
A3	4.444	d
B13	4.333	d
B10	4.222	d
A10	4.111	d

Thus, the set of potato lines grown in inoculated soil that had significantly different disease index values from the rest (including the untransformed controls), were A10, B10, B13, A3, A14 and B16.

They are arranged with increasing indices. They do not have the significance difference indicator (a) and are therefore significantly different from the lines with the (a) indicator.

The Multiple t-distribution test procedure of Gupta and Panchapakesan (1979) was also applied to the disease index data of the inoculum block. The most resistant groups of lines, with a probability of 95% for the correct decision, were selected. Lines A10, B10, B13, A3, A14, B16 and A8 were the best lines in the inoculated soil at the 5% level. These were the same lines as indicated by the F-test, with only line A8 extra.

Table 7.2 summarises the index values of the different potato lines planted in control soil. The mean index values of the nine replicates of each line are sorted in a decreasing order, and different letters indicates indices significantly different from each other. For example in Table 7.2, line A8 (e f) had a significantly lower disease index than all the lines from A11 to BP1B (letters ranging from (a) to (a b c d)). Line A8's index (2.687) differs by at least the lsd value (0.9386) from these lines. The mean indices were lower than the disease indices calculated for the lines planted in the inoculated soil (Table 7.1). This was expected, due to the fact that *Verticillium*-wilt causes the earlier appearance of senescence symptoms.

The set of potato lines grown in control uninoculated soil that had significantly smaller disease index values from the rest (including the untransformed controls), were A3, B10, A8, B13, B16, A14 and 7A. They are arranged with increasing indices. They do not have the significance difference indicator (a) and are therefore significantly different from the lines with the (a) indicator.

The Multiple t-distribution test procedure of Gupta and Panchapakesan (1979) was also applied to the disease index data of the control block. The most resistant groups of lines, with a probability of 95% for the correct decision, were selected. Lines A3, B10, A8, B13, B16, A14, 7A and A10 were the best lines in control soil at the 5% level. These were the same lines as indicated by the F-test, with only line A10 extra.

symptom expression for these two lines was also approximately 2 weeks later than BP1 (Figure 7.3). It was 12.88 weeks for line A10 and 12.33 weeks for line A14, compared to the 10.33 weeks for BP1.

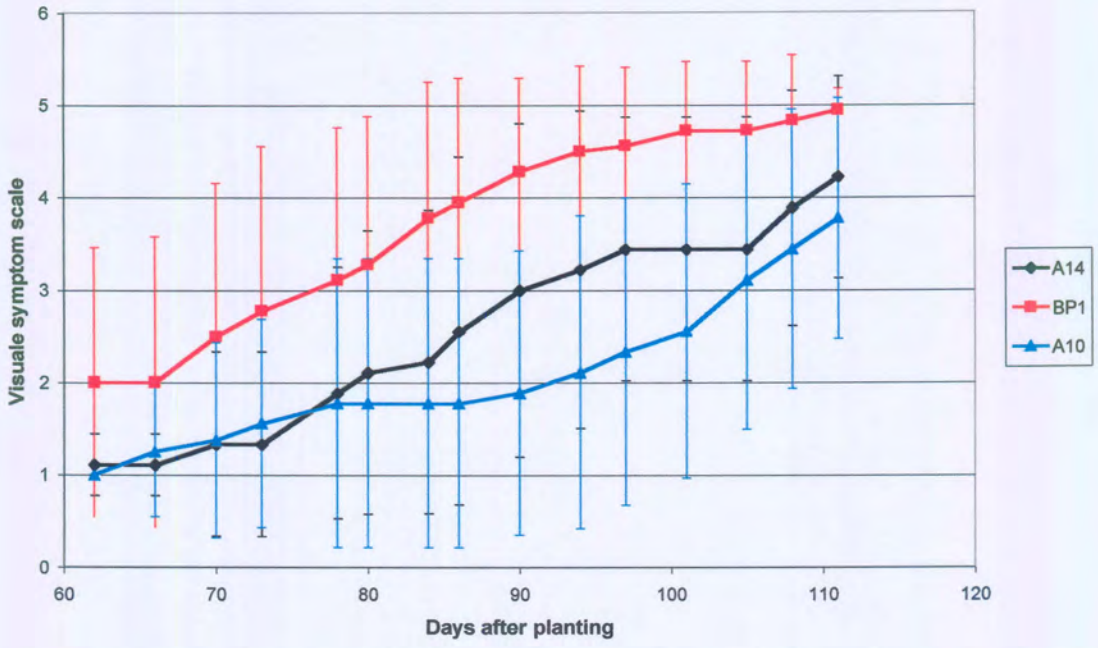


Figure 7.2 **Progression of visual disease symptoms over time for three potato lines.** The average visual disease symptom class for the nine replicates of each line is plotted against the number of days after planting of the tubers when the visual assessments were made.

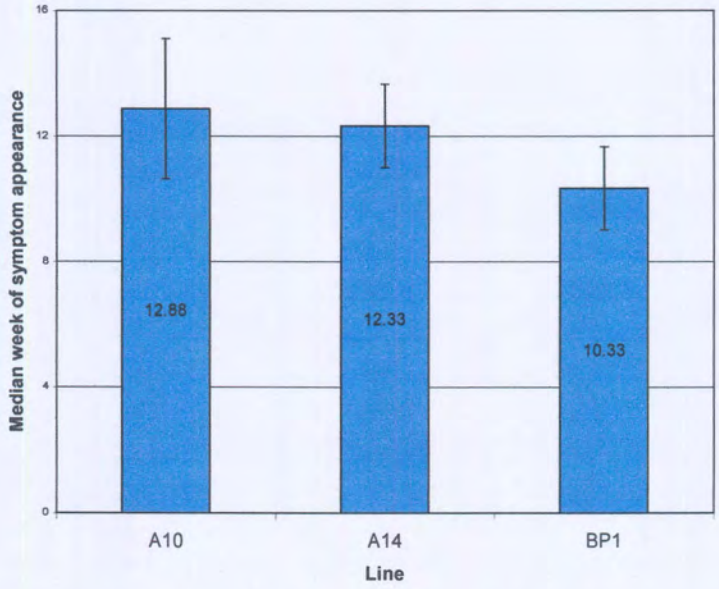


Figure 7.3 **Median time (in weeks) after planting of symptom development of three potato lines grown in inoculated soil.** The mean medians for nine replicates are presented, and their standard deviations are indicated by vertical bars.

Table 7.2 **Potato lines planted in control soil with significantly different disease indices.** Disease indices are sorted in a decreasing order, and the potato lines with significant different indices are indicated with different letters (a – f).

Potato line	Mean disease index	Significant difference indicator
A11	4.170	a
B18	4.170	a
A5	4.077	a b
A9	4.077	a b
B9	3.984	a b c
A7	3.983	a b c
BP1A (untransformed)	3.890	a b c
A6	3.798	a b c
B12	3.706	a b c d
B3	3.706	a b c d
BP1B (untransformed)	3.703	a b c d
B5	3.613	a b c d e
A12	3.521	a b c d e
B11	3.521	a b c d e
A10	3.242	a b c d e f
7A	3.151	b c d e f
A14	3.147	b c d e f
B16	3.057	c d e f
B13	2.778	d e f
A8	2.687	e f
B10	2.408	f
A3	2.318	f

7.3.6 Median week of symptom appearance

Two lines that differed significantly from untransformed BP1, when planted in the inoculated soil, were chosen. They were line A10 and A14. The average visual wilt symptom index for the nine replicates of each line was plotted against the number of days after planting (Figure 7.2). It was compared to the development of symptoms in untransformed BP1. Although there was a large amount of variation between the nine replicates of each line (standard deviations shown as vertical bars on the graphs), the overall trend in symptom development was evident. Lines A14 and A10 showed a delayed symptom development compared to BP1. Symptom development was more gradual for these two lines, compared to the more hyperbolic shape of the BP1 curve. The median time after planting of

7.4 Discussion

Symptoms of *Verticillium*-wilt are not easy to assess, since they show similarity to the general chlorosis and necrosis associated with natural senescence. However, *V. dahliae* tends to cause unilateral chlorosis and necrosis, stunting of growth, reduction in size of the root system and discoloration of vascular system (Nachmias *et al.*, 1990). Symptom expression can be biased due to various unrelated factors, such as insect damage or drought stress (Wheeler *et al.*, 1994). Therefore, when selecting cultivars for resistance, they are also judged by the degree of stem colonisation in addition to symptom expression. In general, there was agreement between the incidence of symptom expression and the degree of stem colonisation. It is expected that *Verticillium*-susceptible cultivars will show earlier senescence symptoms compared with resistant ones.

Table 7.1 and 7.2 summarised the disease index data for the potato lines grown in inoculated and control soil, respectively. Different letters (a - f) were used to indicate which lines' disease indices were significantly different from each other at the 1% significance level. An observed difference is statistically significant at the 1% level if it is large enough to justify rejection of the null hypothesis (H_0) at $\alpha = 0.01$ (Samuels, 1989). Therefore, the probability to reject a true null hypothesis (H_0) is 0.01 if H_0 is true. When a true H_0 is rejected, it is called a type I error. When choosing α, you are choosing the level of protection against type I error. Statistical significance simply indicates rejection of the null hypothesis (H_0) of no difference between the disease indices of the lines. It does not necessarily indicate a large or important effect. A significant correlation may be a weak one, but its significance means only that it cannot easily be dismissed as a chance pattern.

The results of Fisher's protected least significant difference test (F-test) and Gupta test on the disease indices of plants grown in inoculated or control soil, indicated the same lines to be significantly different from the rest. They were lines B10, B13, A3, A14 and B16. The reason why these lines fall in the significant different categories for both blocks is probably because they were slower growers, and due to physiological effects slower to produce *Verticillium*-wilt (inoculum block) or natural senescence (control block) symptoms. The phenomenon of slower growers giving the impression of *Verticillium*-resistance is a well-known factor when selecting for resistant cultivars under field conditions. This is because plants that stay in a non-tuberising juvenile condition do not become systemically infected with *Verticillium* and do not show wilt symptoms (Corsini and Pavek, 1996). Early screening for resistance to *Verticillium* tended to eliminate clones with acceptable tuber maturity characteristics. So to overcome this inefficiency when selecting for resistant cultivars, researchers suggested to rather select for yield and other agronomic traits while growing cultivars in *Verticillium* infested fields (Corsini and Pavek, 1996).

When using the F-test, the only potato line that was statistically different from a portion of the rest of the lines in the inoculated block, but not in the control block, was line A10. This line was, however, also included in the significant different group of the control block using the Gupta test. If it was indeed significantly resistant, and not due to physiological reasons because it was a slower grower, the increased resistance to *Verticillium*-wilt symptoms might have been due to the expression of apple PGIP1. A western blot quantifying PGIP1, and showing more PGIP1 in line A10 than the rest of the lines, would support the hypothesis that the resistance is due to the expression of PGIP1.

A comparison of the median time for symptom expression was made for three chosen lines grown in inoculated soil. Lines A14 and A10 showed a delayed symptom development compared to BP1 (Figure 7.2), and the median time after planting for symptom expression for these two lines were also approximately 2 weeks later than BP1 (Figure 7.3). This is significant in the breeding for *Verticillium*-resistant potato cultivars, since low yields are associated with earlier senescence. If the appearance of disease symptoms of plants grown in the presence of *V. dahliae* can be delayed, the growing time of a cultivar is extended and more tuber bulking can take place, leading to increased yield. These two lines are examples of the increased resistance obtained in the transgenic lines compared to the untransformed control.

As discussed above, the enhanced resistance may not solely be due to the apple *pgip1* transgene expression, but probably due to a physiological effect present in the transgenic lines. In both the inoculated soil and the uninoculated soil, the same five lines showed significant differences in the means of their disease indices compared to the rest of the lines, which included the untransformed BP1 controls. A few possible reasons exist why transformation of the potato lines could result in slower growth or other phenotypic changes. The first is that insertion events of the transgene could have disrupted essential plant genes, leading to altered growing behaviour. Alternatively, somaclonal variation could have taken place during the tissue culture regeneration of transformants, also leading to altered phenotypes. The third reason may be due to a direct effect of the apple PGIP1 itself. However, plant PGIPs are not expected to inhibit plant PGs (Federici *et al.*, 2001), so this interaction may not be responsible for the developmental differences.

Possible reasons why PGIP was not effective in the glasshouse trial

Even though significant differences in the disease indices of the different potato lines were obtained during the glasshouse trial, the differences were not strikingly large. There may be several reasons why apple PGIP1 was not effective in the glasshouse trial to confer enhanced resistance to *Verticillium*-wilt.

Previous chapters investigated the presence of the apple *pgip1* gene and PGIP1 expression product in the transgenic potato lines. The apple *pgip1* gene was shown with PCR to be present in all 20 lines used in this glasshouse trial (Chapter 5, Figure 5.6). It was confirmed by Southern blot for some lines (Figure 5.14). PGIP extracts prepared from *in vitro*-grown leaf material were shown to contain an inhibitor of *V. dahliae* PGs *in vitro*. The inhibition results of PGIP extracts prepared from *in vitro* grown potato lines were presented in Figures 6.6, 6.8 and 6.10. PGIP activity was retained in glasshouse-grown leaf material (Chapter 6, results reported but figure not shown). Active PGIP was also shown to be present in the roots of *in vitro* grown plants (Figure 6.7). Even though the gene and the active protein product were detected *in vitro*, it is not to say that PGIP1 will be effective in protecting the transgenic potato plants from *V. dahliae* infection *in vivo*. Fungi synthesises many PGs, and each has a different expression pattern *in planta* and *in vitro* (Wubben *et al.*, 1999). *V. dahliae* might have expressed a different set of PGs when infecting potato roots *in vivo* than when the fungus was cultured *in vitro* on pectin medium. PGIP from a specific plant can demonstrate differential inhibition of PGs secreted by a certain fungus under different growth conditions (Yao *et al.*, 1995). Apple PGIP was able to inhibit four out of five PGs secreted by *B. cinerea* in liquid culture, but was completely unable to inhibit PGs produced on fruit inoculated with this fungus (Yao *et al.*, 1995). This is an illustration of fungi secreting different PGs *in vivo* than when they are cultured *in vitro*. Apple PGIP1 expressed in the potato lines might not have been active in inhibiting the major *V. dahliae* PG secreted during infection and colonisation of the potato plants *in vivo*.

Another possible reason for the ineffectiveness of PGIP1 in this trial is because it is not known whether the transgenic plant accumulated a sufficient amount of heterologous PGIP to maximally inhibit the endoPGs in the infected plant tissues. The stoichiometry of PG:PGIP interaction is important in determining whether PGs are inhibited enough to cause the release of elicitor-active oligogalacturonides, while preventing their complete degradation to inactive monomers (according to the hypothesis by Cervone *et al.* (1989)).

In conclusion, the results indicated a significant difference in disease indices of a few transgenic potato lines compared to the untransformed control, but did not lead to visibly more resistant plants. The plants that were indicated to be more resistant in the inoculated soil, also showed significantly slower senescence symptoms from the rest in the control soil. This may be a physiological effect of slower growth and a prolonged juvenile phase, and therefore delayed senescence.

CHAPTER 8

Concluding Discussion

Verticillium-wilt is an important fungal disease of potatoes, causing great yield losses. The overall aim of this study was to evaluate polygalacturonase-inhibiting protein (PGIP)-mediated resistance against *V. dahliae*, the fungal pathogen causing *Verticillium*-wilt. Purified apple PGIP1 and PGIP extracts prepared from apple *pgip1* transgenic potato cv. BP1 lines were shown to be active *in vitro* against PGs secreted by this fungus when grown in liquid culture. Untransformed BP1 potato did not contain this active inhibitor. The results of a glasshouse trial, in which potato minitubers were planted into soil inoculated with *V. dahliae* microsclerotia were, however, not conclusive in proving that enhanced resistance compared to untransformed plants was obtained by the transformation with the apple *pgip1* gene.

A sub-aim of this study was to evaluate whether the pathogen-inducible *gst1* promoter from *Arabidopsis thaliana* (L.) Heynh could be used for the inducible expression of antifungal genes in *A. thaliana* and crops of importance. Transformation of *A. thaliana* was chosen since it is a simple process without any need for tissue culture, except when screening for kanamycin resistant seedlings. For this study, a construct containing the apple *pgip1* gene downstream of the *gst1* promoter was generated by various molecular techniques and subcloning steps. These were presented in Chapter 3. The appropriate part of the *gst1* promoter first had to be isolated using PCR, after which it was subcloned into the plant transformation vector pCAMBIA2300. The apple *pgip1* gene was inserted downstream of the *gst1* promoter in the form of an expression cassette. It was released by partial restriction enzyme digestion from a previous vector containing this gene. Nucleotide sequencing after each subcloning step consistently showed the expected nucleotide sequence.

The plant transformation constructs containing the apple *pgip1* gene under control of the *gst1* and enhanced CaMV 35S promoters were transformed into *A. thaliana* using the floral-dip method (presented in Chapter 4). The expression of active PGIP from these two promoters was compared by preparing PGIP extracts from transgenic lines and testing them for PG-inhibiting activity against *V. dahliae* PG. A gene encoding a reporter enzyme could have also been inserted downstream of the promoters to test their activities, since its expression could be more easily monitored. The hypothesis was that the *gst1* promoter would drive pathogen-inducible expression of the apple *pgip1* gene.

Studies confirmed the presence of a functional PGIP in the transgenic *A. thaliana* plants. Both constructs lead to the production of an active apple PGIP1. The expression levels could, however, not

CHAPTER 9

References

Abu-Goukh AA, Greve LC and Labavitch JM. (1983a). Purification and partial characterization of "Bartlett" pear fruit polygalacturonase inhibitors. *Physiological Plant Pathology* **23**, 111-122.

Abu-Goukh AA, Labavitch JM. (1983). The *in vivo* role of "Bartlett" pear fruit polygalacturonase inhibitors. *Physiological Plant Pathology* **23**, 123-135.

Abu-Goukh AA, Strand LL and Labavitch JM. (1983b). Development-related changes in decay susceptibility and polygalacturonase inhibitor content of "Bartlett" pear fruit. *Physiological Plant Pathology* **23**, 101-109.

Albersheim P and Anderson AJ. (1971). Proteins from plant cell walls inhibit polygalacturonases secreted by plant pathogens. *Proceedings of the National Academy of Sciences of the United States of America* **68**(8), 1815-1819.

An G. (1986). Development of plant promoter expression vectors and their use for analysis of differential activity of Nopaline Synthase promoter in transformed tobacco cells. *Plant Physiology* **81**, 86-91.

Anderson AJ and Albersheim P. (1972). Host-pathogen interactions. V. Comparison of the abilities of proteins isolated from three varieties of *Phaseolus vulgaris* to inhibit the endopolygalacturonases secreted by three races of *Colletotrichum lindemuthianum*. *Physiological Plant Pathology* **2**, 339-346.

Arendse MS, Dubery IA and Berger DK. (1999). Isolation by PCR-based methods of a plant antifungal polygalacturonase-inhibition protein gene. *Electronic Journal of Biotechnology* **2**(3), 152-159.

Arumuganathan K and Earle ED. (1991). Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* **9**(3), 208-218.

Ausubel FM, Katagiri F, Mindrinos M and Glazebrook J. (1995). Use of *Arabidopsis thaliana* defense-related mutants to dissect the plant response to pathogens. *Proceedings of the National Academy of Sciences of the United States of America* **92**(10), 4189-4196.

Berger DK, Oelofse D, Arendse MS, Du Plessis E and Dubery IA. (2000). Bean polygalacturonase inhibitor protein-1 (PGIP-1) inhibits polygalacturonases from *Stenocarpella maydis*. *Physiological and Molecular Plant Pathology* **57**, 5-14.

Berger DK (2000). Developing country profiles (potato production): South Africa, pp 46-49 in: Lizárraga C and Hollister A (eds), *Proceedings of the International workshop on "Transgenic potatoes for the benefit of resource-poor farmers in developing countries"*. International Potato Center (CIP) Press, Lima, Peru.

Bergmann CW, Ito Y, Singer D, Albersheim P, Darvill AG, Benhamou N, Nuss L, Salvi G, Cervone F and De Lorenzo G. (1994). Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. *The Plant Journal* **5**(5), 625-634.

Bidochka MJ, St Leger RJ, Stuart A and Gowanlock K. (1999). Nuclear rDNA phylogeny in the fungal genus *Verticillium* and its relationship to insect and plant virulence, extracellular proteases and carbohydrases. *Microbiology* **145**, 955-963.

Bradford MM. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Bradley DJ, Kjellbom P and Lamb CJ. (1992). Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant-cell wall protein: a novel, rapid defense response. *Cell* **70**, 21-30.

Brown AE and Adikaram NKB. (1982). The differential inhibition of pectic enzymes from *Glomerella cingulata* and *Botrytis cinerea* by a cell wall protein from *Capsicum annuum* fruit. *Phytopathologische Zeitschrift* **105**, 27-38.

Brown AE and Adikaram NKB. (1983). A role for pectinase and protease inhibitors in fungal rot development in tomato fruits. *Phytopathologische Zeitschrift* **106**, 239-251.

Brown AE. (1984). Relationship of endopolygalacturonase inhibitor activity to the rate of fungal rot development in apple fruits. *Phytopathologische Zeitschrift* **111**, 122-132.

Buchanan BB, Gruissem W and Jones RL. (eds). (2000). Pages 1114-1115 in: Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists, Courier Companies, Inc., USA.

Caprari C, Mattei B, Basile ML, Salvi G, Crescenzi V, De Lorenzo G, Cervone F. (1996). Mutagenesis of endopolygalacturonase from *Fusarium moniliforme*: histidine residue 234 is critical for enzymatic and macerating activities and not for binding to polygalacturonase-inhibiting protein (PGIP). *Molecular Plant-Microbe Interactions* **9**(7), 617-624.

Cassidy BG and Nelson RS. (1995). Differences in protection phenotypes in tobacco plants expressing coat protein genes from peanut stripe potyvirus with or without an engineered ATG. *Molecular Plant-Microbe Interactions* **8**(3), 357-365.

Cervone F, De Lorenzo G, Degrà L and Salvi G. (1987a). Elicitation of necrosis in *Vigna unguiculata* Walp. by homogeneous *Aspergillus niger* endo-polygalacturonase and by α-D-galacturonate oligomers. *Plant Physiology* **85**, 626-630.

Cervone F, De Lorenzo G, Degrà L, Salvi G and Bergami M. (1987b). Purification and characterization of a polygalacturonase-inhibiting protein from *Phaseolus vulgaris* L. *Plant Physiology* **85**, 631-637.

Cervone F, De Lorenzo G, Pressey R, Darvill AG and Albersheim P. (1990). Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? *Phytochemistry* **29**(2), 447-449.

Cervone F, Hahn MG, De Lorenzo G, Darvill, A and Albersheim P. (1989). Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. *Plant Physiology* **90**, 542-548.

Chimwamurombe PM, Botha A-M, Wingfield MJ and Wingfield BD. (2001). Molecular relatedness of the polygalacturonase-inhibiting protein genes in *Eucalyptus* species. *Theoretical and Applied Genetics* **102**, 645-650.

Clough SJ and Bent AF. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**(6), 735-743.

Corsini D and Pavek JJ. (1996). Agronomic performance of potato germplasm selected for high resistance to Verticillium wilt. *American Potato Journal* **73**, 249-260.

Corsini DL, Pavek JJ and Davis JR. (1988). *Verticillium* wilt resistance in noncultivated tuber-bearing *Solanum* species. *Plant Disease* **72**(2), 148-151.

Costa MMR, Costa J and Ricardo CPP. (1997). A *Lupinus albus* root glycoprotein homologous to the polygalacturonase inhibitor proteins. *Physiologia Plantarum* **99**, 263-270.

De Lorenzo G, Cervone F, Bellincampi D, Caprari C, Clark AJ, Desiderio A, Devoto A, Forrest R, Leckie F, Nuss L and Salvi G. (1994). Polygalacturonase, PGIP and oligogalacturonides in cell-cell communication. *Biochemical Society Transactions* **22**(2), 394-397.

De Lorenzo G, D'Ovidio R and Cervone F. (2001). The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annual Review of Phytopathology* **39**, 313-335.

De Lorenzo G, Ito Y, D'Ovidio R, Cervone F, Albersheim P and Darvill AG. (1990). Host-pathogen interactions. XXXVII. Abilities of the polygalacturonase-inhibiting proteins from four cultivars of *Phaseolus vulgaris* to inhibit the *endo*polygalacturonases from three races of *Colletotrichum lindemuthianum*. *Physiological and Molecular Plant Pathology* **36**, 421-435.

Degrá L, Salvi G, Mariotti D, De Lorenzo G and Cervone F. (1988). A polygalacturonase-inhibiting protein in alfalfa callus cultures. *Journal of Plant Physiology* **133**, 364-366.

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E and Ryals J. (1994). A central role of salicylic acid in plant disease resistance. *Science* **266**, 1247-1250.

Dellaporta SL, Wood J and Hicks JB. (1983). A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter* **1**(4), 19-21.

Delledonne M, Xia YJ, Dixon RA and Lamb C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**, 585-588.

Denner FDN. (1997). Black dot and silver scurf of potatoes in South Africa. PhD thesis, University of Pretoria.

Desfeux C, Clough SJ and Bent AF. (2000). Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the Arabidopsis floral-dip method. *Plant Physiology* **123**, 895-904.

Desiderio A, Acacri B, Leckie F, Mattei B, Salvi G, Tigelaar H, Van Roekel JSC, Baulcombe DC, Melchers LS, De Lorenzo G and Cervone F. (1997). Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. *Molecular Plant-Microbe Interactions* **10**(7), 852-860.

Devoto A, Clark AJ, Nuss L, Cervone F and De Lorenzo G. (1997). Developmental and pathogen-induced accumulation of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. *Planta* **202**, 284-292.

Dudler R, Hertig C, Rebmann G, Bull J and Mauch F. (1991). A pathogen-induced wheat gene encodes a protein homologous to glutathione-S-transferases. *Molecular Plant-Microbe Interactions* **4**(1), 14-18.

Durner J, Shah J and Klessig DF. (1997). Salicylic acid and disease resistance in plants. *Trends in Plant Science* **2**(7), 266-274.

English PD, Jurale JB and Albersheim P. (1971). Host-pathogen interactions. II. Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. *Plant Physiology* **47**, 1-6.

Favaron F, Castiglioni C and Lenna PD. (1993). Inhibition of some rot fungi polygalacturonases by *Allium cepa* L. and *Allium porrum* L. extracts. *Journal of Phytopathology* **139**, 201-206.

Favaron F, Castiglioni C, D'Ovidio R and Alghisi P. (1997). Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. *Physiological and Molecular Plant Pathology* **50**, 403-417.

Favaron F, D'Ovidio R, Porceddu E, Alghisi P. (1994). Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. *Planta* **195**(1), 80-87.

Favaron F. (2001). Gel detection of *Allium porrum* polygalacturonase-inhibiting protein reveals a high number of isoforms. *Physiological and Molecular Plant Pathology* **58**, 239-245.

Federici L, Caprari C, Mattei B, Savino C, Di Matteo A, De Lorenzo G, Cervone F, Tsernoglou D. (2001). Structural requirements of endopolygalacturonase for the interaction with PGIP (polygalacturonase-inhibiting protein). *Proceedings of the National Academy of Sciences of the United States of America* **98**(23), 13425-13430.

Feys BJF, Benedetti CE, Penfold CN and Turner JG. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* **6**, 751-759.

Fielding AH. (1981). Natural inhibitors of fungal polygalacturonases in infected fruit tissues. *Journal of General Microbiology* **123**, 377-381.

Flor HH. (1946). Genetics of pathogenicity in *Melampsora lini*. *Journal of Agricultural Research* **73**, 335-357.

Frediani M, Cremonini R, Salvi G, Caprari C, Desiderio A, D'Ovidio R, Cervone F and De Lorenzo G. (1993). Cytological localization of the *PGIP* genes in the embryo suspensor cells of *Phaseolus vulgaris* L. *Theoretical and Applied Genetics* **87**, 369-373.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H and Ryals J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* **261**, 754-756.

GenStat for Windows. 2000. Release 4.2. Fifth Edition. Oxford: VSN International.

Glazebrook J and Ausubel FM. (1994). Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 8955-8959.

Goodman HM, Ecker JR and Dean C. (1995). The genome of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 10831-10835.

Grant JJ and Loake GJ. (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiology* **124**, 21-29.

Grant JJ, Yun BW and Loake GJ. (2000). Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *The Plant Journal* **24**(5), 569-582.

Gupta SS and Panchapakesan S. (1979). Multiple decision procedures: theory and methodology of selecting and ranking populations. John Wiley & Sons, New York. 573 pp.

Guzmán P and Ecker JR. (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell* **2**, 513-523.

Hammond-Kosack KE and Jones JDG. (1997). Plant disease resistance genes. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 575-607.

Hanahan D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**(4), 557-580.

Harris DC, Yang JR and Ridout MS. (1993). The detection and estimation of *Verticillium dahliae* in naturally infested soil. *Plant Pathology* **42**, 238-250.

Hijmans RJ. (2001). Global distribution of the potato crop. *American Journal of Potato Research* **78**, 403-412.

Hoffman RM and Turner JG. (1982). Partial purification of proteins from pea leaflets that inhibit *Ascochyta pisi* endopolygalacturonase. *Physiological Plant Pathology* **20**, 173-187.

Hoffman RM and Turner JG. (1984). Occurrence and specificity of an endoplogalacturonase inhibitor in *Pisum sativum*. *Physiological Plant Pathology* **24**, 49-59.

Holtorf S, Apel K and Bohlmann H. (1995). Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*. *Plant Molecular Biology* **29**, 637-646.

Hooykaas PJJ and Schilperoort RA. (1992). *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology* **19**, 15-38.

Isaac I and Harrison JAC. (1968). The symptoms and causal agents of early-dying disease (*Verticillium* wilt) of potatoes. *Annals of Applied Biology* **61**, 231-244.

Itzhaki H, Maxson JM and Woodson WR. (1994). An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-*S*-transferase (*GST1*) gene. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 8925-8929.

James JT and Dubery IA. (2001). Inhibition of polygalacturonase from *Verticillium dahliae* by a polygalacturonase inhibiting protein from cotton. *Phytochemistry* **57**, 149-156.

Johnston DJ, Ramanathan V and Williamson B. (1993). A protein from immature raspberry fruits which inhibits endopolygalacturonases from *Botrytis cinerea* and other micro-organisms. *Journal of Experimental Botany* **44**(262), 971-976.

Joshi CP. (1987). An inspection of the domain between putative TATA box and translation start site in 79 plant genes. *Nucleic Acids Research* **15**(16), 6643-6654.

Karr AL and Albersheim P. (1970). Polysaccharide-degrading enzymes are unable to attack plant cell walls without prior action by a "wall-modifying enzyme". *Plant Physiology* **46**, 69-80.

Kawchuk LM, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, Kokko E, Byers R, Howard RJ, Fischer R and Prüfer D. (2001). Tomato *Ve* disease resistance genes encode cell surface-like receptors. *Proceedings of the National Academy of Sciences of the United States of America* **98**(11), 6511-6515.

Kobe B and Deisenhofer J. (1995). Proteins with leucine-rich repeats. *Current Opinion in Structural Biology* **5**, 409-416.

Koncz C and Schell J. (1986). The promoter of the T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Molecular and General Genetics* **204**, 383-396.

Kozak M. (1981). Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. *Nucleic Acids Research* **9**(20), 5233-5256.

Lafitte C, Barthe J-P, Gansel X, Dechamp-Guillaume G, Faucher C, Mazau D and Esquerré-Tugayé M-T. (1993). Differential induction by endopolygalacturonase of β-1,3-glucanases in *Phaseolus vulgaris* isoline susceptible and resistant to *Colletotrichum lindemuthianum* race β. *Molecular Plant-Microbe Interactions* **6**(5), 628-634.

Laugé R and De Wit PJGM. (1998). Fungal avirulence genes: structure and possible functions. *Fungal Genetics and Biology* **24**(3), 285-297.

Leckie F, Mattei B, Capodicasa C, Hemmings A, Nuss L, Aracri B, De Lorenzo G and Cervone F. (1999). The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid

substitution in the solvent-exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. *EMBO Journal* **18**(9), 2352-2363.

Lever M. (1972). A new reaction for colorimetric determination of carbohydrates. *Analytical Biochemistry* **47**, 273-279.

Li Z, Jayasankar S and Gray D. (2001). Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). *Plant Science* **160**, 877-887.

Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S and Scala F. (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 7860-7865.

Machinandiarena MF, Olivieri FP, Daleo GR and Oliva CR. (2001). Isolation and characterization of a polygalacturonase-inhibiting protein from potato leaves. Accumulation in response to salicylic acid, wounding and infection. *Plant Physiology and Biochemistry* **39**, 129-136.

Mattei B, Bernalda MS, Federici L, Roepstorff P, Cervone F and Boffi A. (2001). Secondary structure and post-translational modifications of the leucine-rich repeat protein PGIP (polygalacturonase-inhibiting protein) from *Phaseolus vulgaris*. *Biochemistry* **40**(2), 569-576.

Mazithulela G, Sudhakar D, Heckel T, Mehlo L, Christou P, Davies JW and Boulton MI. (2000). The maize streak virus coat protein transcription unit exhibits tissue-specific expression in transgenic rice. *Plant Science* **155**, 21-29.

McDowell JM and Dangl JL. (2000). Signal transduction in the plant immune response. *Trends in Biochemical Sciences* **25**(2), 79-82.

Meinke DW, Cherry M, Dean C, Rounsley SD and Koornneef M. (1998). *Arabidopsis thaliana* : a model plant for genome analysis. *Science* **282**, 678-682.

Millard C en Denner F. (2001). Die beheer van Verticillium-verwelk op aartappels in Suid-Afrika - 'n strategie. *CHIPS* (May-June), 40-45.

Müller M and Gessler C. (1993). A protein from apple leaves inhibits pectinolytic activity of *Venturia inaequalis in vitro*. Pages 68-71 in: *Mechanisms of Plant Defense Responses*. B. Fritig and M. Legrand , eds. Kluwer Academic Publishers.

Murray MG and Thompson WF. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**(19), 4321-4325.

Nachmias A, Buchner V, Tsror L, Burstein Y and Keen N. (1987). Differential phytotoxicity of peptides from culture fluids of *Verticillium dahliae* races 1 and 2 and their relationship to pathogenicity of the fungi on tomato. *Phytopathology* 77(3), 506-510.

Nachmias A, Orenstein J, Tal M and Goren M. (1990). Reactions to a *Verticillium dahliae* phytotoxin in tissue culture derived from susceptible and tolerant potato. *Plant Science* **68**, 123-130.

Nuss L, Mahé A, Clark AJ, Grisvard J, Dron M, Cervone F and De Lorenzo G. (1996). Differential accumulation of PGIP (polygalacturonase-inhibiting protein) mRNA in two near-isogenic lines of *Phaseolus vulgaris* L. upon infection with *Colletotrichum lindemuthianum*. *Physiological and Molecular Plant Pathology* **48**, 83-89.

Old RW and Primrose SB. (1994). In: Principles of gene manipulation: An introduction to genetic engineering. 5th Edition. Blackwell Scientific Publications.

Palmer T. (1995). Pages 148, 151, 243-245, 262-263 in: Understanding Enzymes. 4th Edition. Prentice Hall, Ellis Horwood, NY.

Parinov S and Sundaresan V. (2000). Functional genomics in *Arabidopsis*: large-scale insertional mutagenesis complements the genome sequencing project. *Current Opinion in Biotechnology* **11**, 157-161.

Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Métraux J-P, Manners JM and Broekaert WF. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell* **8**, 2309-2323.

Powell ALT, van Kan J, ten Have A, Visser J, Greve LC, Bennett AB and Labavitch JM. (2000). Transgenic expression of pear PGIP in tomato limits fungal colonization. *Molecular Plant-Microbe Interactions* **13**(9), 942-950.

Powelson ML and Rowe RC. (1993). Biology and management of early dying of potatoes. *Annual Review of Phytopathology* **31**, 111-126.

Powelson ML and Rowe RC. (1994). Potato early dying: causes and management tactics in the eastern and western United States. Pages 178-190 in: Advances in Potato Pest Biology and Management. G.W. Zehnder *et al.*, eds. St. Paul, MN: APS Press.

Pressey R. (1996). Polygalacturonase inhibitors in bean pods. *Phytochemistry* **42**(5), 1267-1270.

Ramanathan V, Simpson CG, Thow G, Iannetta PPM, McNicol RJ and Williamson B. (1997). cDNA cloning and expression of polygalacturonase-inhibiting proteins (PGIPs) from red raspberry (*Rubus idaeus*). *Journal of Experimental Botany* **48**(311), 1185-1193.

Robinson DB, Larson RH and Walker JC. (1957). *Verticillium* wilt of potato: in relation to symptoms, epidemiology and variability of the pathogen. *Research Bulletin* 202. University of Wisconsin, Madison.

Rommens CM and Kishore GM. (2000). Exploiting the full potential of disease-resistance genes for agricultural use. *Current Opinion in Biotechnology* **11**(2), 120-125.

Rowe RC. (1985). Potato early dying - a serious threat to the potato industry. *American Potato Journal* **62**, 157-161).

Salvi G, Giarrizzo F, De Lorenzo G and Cervone F. (1990). A polygalacturonase-inhibiting protein in the flowers of *Phaseolus vulgaris* L. *Plant Physiology* **136**, 513-518.

Sambrook J, Fritch EF and Maniatis T. (1989). Molecular Cloning. A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Samuels ML. (1989). Pages 210, 216-218, 228, 273, 276 and 473 in: Statistics for the life sciences. Dellen Publishing Company, Macmillan, Inc., NJ, USA.

Sharrock KR and Labavitch JM. (1994). Polygalacturonase inhibitors of Bartlett pear fruits: differential effects on *Botrytis cinerea* polygalacuronase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. *Physiological and Molecular Plant Pathology* **45**, 305-319.

Skare NH, Paus F and Raa J. (1975). Production of pectinase and cellulase by *Cladosporium cucumerinum* with dissolved carbohydrates and isolated cell walls of cucumber as carbon sources. *Physiologia Plantarum* **33**, 229-233.

Southern EM. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.

St Leger RJ, Joshi L and Roberts DW. (1997). Adaptation of proteases and carbohydrases of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. *Microbiology* **143**, 1983-1992.

Stotz HU, Bishop JG, Bergmann CW, Koch M, Albersheim P, Darvill AG and Labavitch JM. (2000). Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors. *Physiological and Molecular Plant Pathology* **56**, 117-130.

Stotz HU, Contos JJA, Powell ALT, Bennett AB, Labavitch JM. (1994). Structure and expression of an inhibitor of fungal polygalacturonases from tomato. *Plant Molecular Biology* **25**(4), 607-617.

Stotz HU, Powell ALT, Damon SE, Greve LC, Bennett AB and Labavitch JM. (1993). Molecular characterization of a polygalacturonase inhibitor from *Pyrus communis* L. cv. Bartlett. *Plant Physiology* **102**, 133-138.

Sunilkumar G, Mohr L, Lopata-Finch E, Emani C and Rathore KS. (2002). Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. *Plant Molecular Biology* **50**(3), 463-474.

Tabaeizadeh Z, Agharbaoui Z, Harrak H and Poysa V. (1999). Transgenic tomato plants expressing a *Lycopersicon chilense* chitinase gene demonstrate improved resistance to *Verticillium dahliae* race 2. *Plant Cell Reports* **19**, 197-202.

Taylor RJ and Secor GA. (1988). An improved diffusion assay for quantifying the polygalactruonase content of *Erwinia* culture filtrates. *Phytopathology* **78**(8), 1101-1103.

ten Have A, Mulder W, Visser J and van Kan JAL. (1998). The endopolygalacturonase gene *Bcpg*1 is required for full virulence of *Botrytis cinerea*. *Molecular Plant-Microbe Interactions* **11**(10), 1009-1016.

The Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**. 796-815.

Toubart P, Desiderio A, Salvi G, Cervone F, Daroda L and De Lorenzo G. (1992). Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L. *The Plant Journal* **2**(3), 367-373.

Tsror L and Nachmias A. (1995). Significance of the root system in *Verticillium* wilt tolerance in potato and resistance in tomato. *Israel Journal of Plant Science* **43**, 315-323.

Visser M. (1999). Genetic variation among *Verticillium dahliae* isolates using pathogenicity and AFLP analysis. MSc thesis. University of the Western Cape, South Africa.

Von Heijne G. (1985). Signal sequences: the limits of variation. *Journal of Molecular Biology* **184**, 99-105.

Wang KL-C, Li H and Ecker JR. (2002). Ethylene biosynthesis and signaling networks. *The Plant Cell* S131-S151.

Wheeler TA, Rowe RC, Riedel RM and Madden LV. (1994). Influence of cultivar resistance to *Verticillium* spp. on potato early dying. *American Potato Journal* **71**, 39-57.

Williams JS, Hall SA, Hawkesford MJ, Beale MH and Cooper RM. (2002). Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. *Plant Physiology* **128**, 150-159.

Williamson B, Johnston DJ, Ramanathan V and McNicol RJ. (1993). A polygalacturonase inhibitor from immature raspberry fruits: a possible new approach to grey mould control. *Acta Horticulturae* **352**, 601-606.

Wubben JP, Mulder W, ten Have A, van Kan JAL and Visser J. (1999). Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. *Applied and Environmental Microbiology* **65**, 1596-1602.

Xie D-X, Feys BF, James S, Nieto-Rostro M and Turner JG. (1998). *COI1*: An *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* **280**, 1091-1094.

Yang K-Y, Kim E-Y, Kim C-S, Guh J-O, Kim K-C and Cho B-H. (1998). Characterization of a glutathione S-transferase gene *ATGST 1* in *Arabidopsis thaliana*. *Plant Cell Reports* **17**, 700-704.

Yao C, Conway WS and Sams CE. (1995). Purification and characterization of a polygalacturonase-inhibiting protein from apple fruit. *Phytopathology* **85**(11), 1373-1377.

Yao C, Conway WS and Sams CE. (1996). Purification and characterization of a polygalacturonase produced by *Penicillium expansum* in apple fruit. *Phytopathology* **86**(11), 1160-1166.

Yao C, Conway WS, Ren R, Smith D, Ross GS and Sams CE. (1999). Gene encoding polygalacturonase inhibitor in apple fruit is developmentally regulated and activated by wounding and fungal infection. *Plant Molecular Biology* **39**, 1231-1241.

York WS, Darvill AG, McNeil M, Stevenson TT and Albersheim P. (1985). Isolation and characterization of plant cell walls and cell wall components. *Methods in Enzymology* **118**, 3-40.

Zupan J and Zambryski P. (1997). The *Agrobacterium* DNA transfer complex. *Critical Reviews in Plant Sciences* **16**(3), 279-295.

Appendices

APPENDIX A

Buffers, Solutions, Reagents and Culture media

Agarose diffusion assay (ADA) medium

1.0% (w/v)	Type II agarose (Sigma A-6877)
0.01% (w/v)	Polygalacturonic acid (PGA) (Sodium polypectate, Sigma P-1879)
0.5% (w/v)	Ammonium oxalate (Sigma A-8545)

For 100 ml of buffer with pH 4.6:

26.7 ml	0.1 M Citric acid
23.2 ml	0.2 M Na_2HPO_4

ADA (Modified)

0.8%	Agarose
0.5%	PGA
100 mM	Sodium acetate buffer (pH 4.7)

Develop plate with 6 N HCl for a few minutes until zones appear.

Antibiotic stocks

Ampicillin	100 mg in 1 ml dH_2O
Cefotaxime	250 mg in 1 ml dH_2O
Gentamycin	50 mg in 1 ml dH_2O
Kanamycin	50 mg in 1 ml dH_2O
Rifampicin	25 mg in 1 ml 100% methanol

All antibiotics dissolved in dH_2O were filter-sterilised through 0.2 µm sterile filters. Rifampicin was made fresh before use. Antibiotic stocks were stored in aliquots at -20°C.

Ca^{2+} /Mn^{2+} solution for the preparation of competent *E. coli* cells

40 mM	NaAc
100 mM	$CaCl_2$
70 mM	$MnCl_2.4H_2O$

Adjust to pH 5.5 with HCl, be careful not to over-acidify the solution as precipitation will occur. Filter-sterilise and store at 4°C.

Citrate / Phosphate buffer

For 100 ml of buffer with the following pH:

pH 4.6	pH 6.0	
26.7 ml	17.9 ml	0.1 M Citric acid
23.3 ml	32.1 ml	0.2 M Na_2HPO_4
50 ml	50 ml	dH_2O

2% CTAB DNA extraction buffer

2% (w/v)	CTAB (Hexadecyl trimethyl ammonium bromide)
1.4 M	NaCl
20 mM	EDTA
100 mM	Tris (pH 8.0)
0.2% (v/v)	β-mercaptoethanol, added just before use.

2% CTAB DNA extraction buffer with PVP

2% (w/v)	CTAB (Hexadecyl trimethyl ammonium bromide)
1.4 M	NaCl
20 mM	EDTA
100 mM	Tris (pH 8.0)
0.2% (v/v)	β-mercaptoethanol
1% (w/v)	PVP (Polyvinyl pyrrolidone) (Sigma PVP-40)

10% CTAB

0.7 M	NaCl
10% (w/v)	CTAB

Dellaporta DNA extraction buffer

100 mM	Tris (pH 8.0)
0.5 M	NaCl
50 mM	EDTA (pH 8.0)
0.07% (v/v)	β-mercaptoethanol

Denaturation solution

0.4 N	NaOH
0.6 M	NaCl

Depurinating solution

0.25 M HCl

DIG Blocking buffer

1% (w/v) Blocking reagent (Roche Diagnostics) in maleic acid buffer.

DIG Detection buffer

0.1 M Tris-HCl
0.1 M NaCl
pH 9.5

DIG Maleic acid buffer

0.1 M Maleic acid
0.15 M NaCl
pH 7.5

DIG Washing buffer

0.3% (v/v) Tween 20 in maleic acid buffer.

Ethidium bromide

Dissolve 10 mg ethidium bromide powder in 1 ml dH_2O. Cover tube with aluminium foil and store at 4°C.

0.1 N HCl/ 70% Ethanol

For 600 ml:

6 ml 10 N HCl
420 ml Ethanol
174 ml dH_2O

2× LB Medium for competent cells

Composition per l:

20 g Tryptone
10 g Yeast extract
1 g NaCl

Adjust the pH to 7.0 and autoclave. Before use, add $1/100^{th}$ volume 20% sterile glucose.

Luria-Bertani (LB) Medium

Composition per l:

10 g Tryptone

5 g Yeast extract

5 g NaCl

For LB agar, add 15 g Bacto-agar.

Minimal salts medium

Composition per 100 ml:

0.2 ml 1 M $MgSO_4.7H_2O$

1 ml 0.001% $MnSO_4.H_20$

2.5 ml 1 M KNO_3

1 ml 0.01% $ZnSO_4$

1 ml 0.0015% $CuSO_4$

1 ml 0.01% Fe $SO_4.7H_2O$

91.5 ml Citrate-PO_4 buffer (pH 6.0)

Add 1% (w/v) pectin (Sigma P-9135, washed with 0.1 N HCl/ 70% Ethanol and dried) to the citrate-phosphate buffer (pH 6.0) and autoclave. Add the filter-sterilised salts just before use.

Miniprep Solution I

25 mM Tris-HCl, pH 8.0

10 mM EDTA

50 mM Glucose

Miniprep Solution II

0.2 N NaOH

1% SDS

Miniprep Solution III

2.5 M Potassium acetate

2.5 M Acetic acid (MW = 60.05g/mol; 1 l = 1.05 kg ∴ 3.574 ml per 25 ml solution III)

pH 5.4 with Acetic acid

Modified soil extract agar (MSEA)

Composition per l:

a. Soil extract

Boil 1 kg of soil in 1 litre of water for 30 min. Filter through filter paper. Use 24 ml of filtrate per litre of MSEA medium.

b. Agar

12 g Agar nr. 3

2 g Poligalacturonic acid (PGA)

1.5 g KH_2PO_4

4.0 g K_2HPO_4

c. Salts

0.2 g KH_2PO_4

0.1 g KCl

0.1 g $MgSO_4.7H_2O$

0.002 g $FeSO_4.7H_2O$

0.4 g $NaNO_3$

Mix a, b and c. Add 1 ml Tergitol and 966 ml dH_2O. Stir while heating to dissolve. Autoclave for 20 min.

d. Antibiotics

0.006 g Biotin

0.06 g Chloramphenicol

0.06 g Tetracycline hydrochloride

0.06 g Streptomycin

Dissolve in 10 ml methanol. Filter-sterilise and add to cooled medium.

1× MS media

1× MS salts (M5519 from Sigma; or CN2230 from Highveld Biologicals)

3% (w/v) Sucrose

0.8% (w/v) Agar

Adjust pH to 5.9 before adding agar, autoclave. Add the appropriate antibiotics after cooling.

Neutralisation solution

0.5 M Tris (pH 7.5)

1.5 M NaCl

1% PAHBAH reagent

5% *p*-hydroxybenzoic acid hydrazide (PAHBAH) in 0.5 M HCl, store at -20°C.

Just before use, mix 1 volume of 5% PAHBAH in 0.5 M HCl with 4 volumes of 0.5 M NaOH to give a final PAHBAH concentration of 1%.

0.42% PGA (in a sodium phosphate/ citric acid buffer, pH 4.6)

For 10 ml:

42 mg PGA (Sodium polypectate, Sigma P-1879)

2.33 ml 0.2 M $NaHPO_4$

2.67 ml 0.1 M Citric acid

5 ml dH_2O

Aliquot and store at -20°C.

Potassium phosphate buffer pH 5.8

Per 100 ml of buffer:

0.85 ml 1 M K_2HPO_4

9.15 ml 1 M KH_2PO_4

Rindite

7 vol. 2-Chloro-ethanol

3 vol. 1,2 Dichloro-ethanol

1 vol. Carbon tetrachloride

Place 300 µl of this mixture per kg potatoes on a piece of cotton wool. Seal in plastic bag with potatoes for 48 hr. Remove potatoes from bag and place at 25°C.

RNase A (10 mg/ml)

Dissolve 10 mg RNAse A in 1 ml 1× TE buffer, pH 8.0. Heat to 100°C for 10 min. Allow to cool slowly to room temperature. Store at -20°C.

20× SSC

3 M NaCl

0.3 M Sodium citrate

pH 7.4

Stringency wash buffer I

2× SSC

0.1% SDS

Stringency wash buffer II

0.5× SSC

0.1% SDS

50× TAE

Composition per l:

242 g Tris hydroxy methyl aminoethane (Tris)

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

For 0.5× TAE: 20 ml 50× TAE and 1980 ml dH_2O.

1× TE buffer (pH 8.0)

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

1× TNE buffer

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

0.2 M NaCl

pH 7.4

5% X-gal

Dissolve 50 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in 1 ml 100% dimethylformamide (DMF). Store in dark at -20°C. Plate out 35 µl per petridish.

APPENDIX B

Primers used in this study

Primer	Length (bp)	Sequence 5' - 3'	T_m[a] (°C)	% GC
AP-PGIP-INVR	25	AGG TTC TTG AGT TGG CTG AGG AAG T	74	48
AP-PGIP-L2	23	GCA GCC ATG GAA CTC AAG TTC TC	70	52
AP-PGIP-R	30	CCC GGA TCC ATC TGC AGT TGT GGC CAT TAC	94	57
GSTreverse	38	AAA CTG CAG[b] CCA TGT CGA C[c]TG TTA ATA CTG TGT TTT TC	54[d]	39
NPTII-L	21	GAG GCT ATT CGG CTA TGA CTG	64	52
NPTII-R	21	ATC GGG AGC GGC GAT ACC GTA	68	62
pBI121 Seq.primer 2	20	GAC GCA CAA TCC CAC TAT CC	62	55
PUC/M13-40F	17	GTT TTC CCA GTC ACG AC	52	53
PUC/M13R	17	CAG GAA ACA GCT ATG AC	50	47
SK	20	CGC TCT AGA ACT AGT GGA TC	60	50
T3	24	GCG CGA AAT TAA CCC TCA CTA AAG	70	46
T7	20	TAA TAC GAC TCA CTA TAG GG	56	40
U19F	19	GTT TTC CCA GTC ACG ACG T	58	53

a – T_m calculated using formula: T_m (°C) = 4(GC) + 2(AT)

b – *Pst*I restriction enzyme recognition site

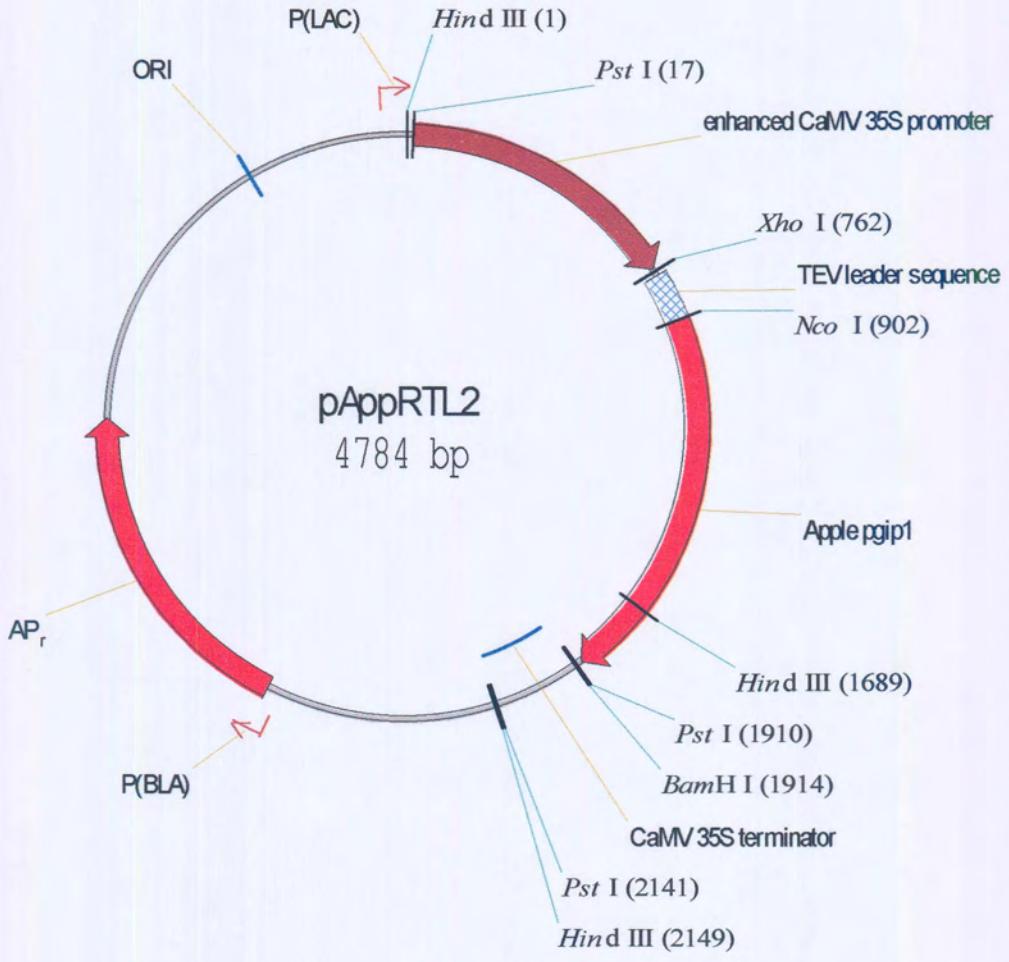
c – *Sal*I restriction enzyme recognition site

d – T_m calculated using *Primer Designer Version 3.0* (Scientific and Educational Software).

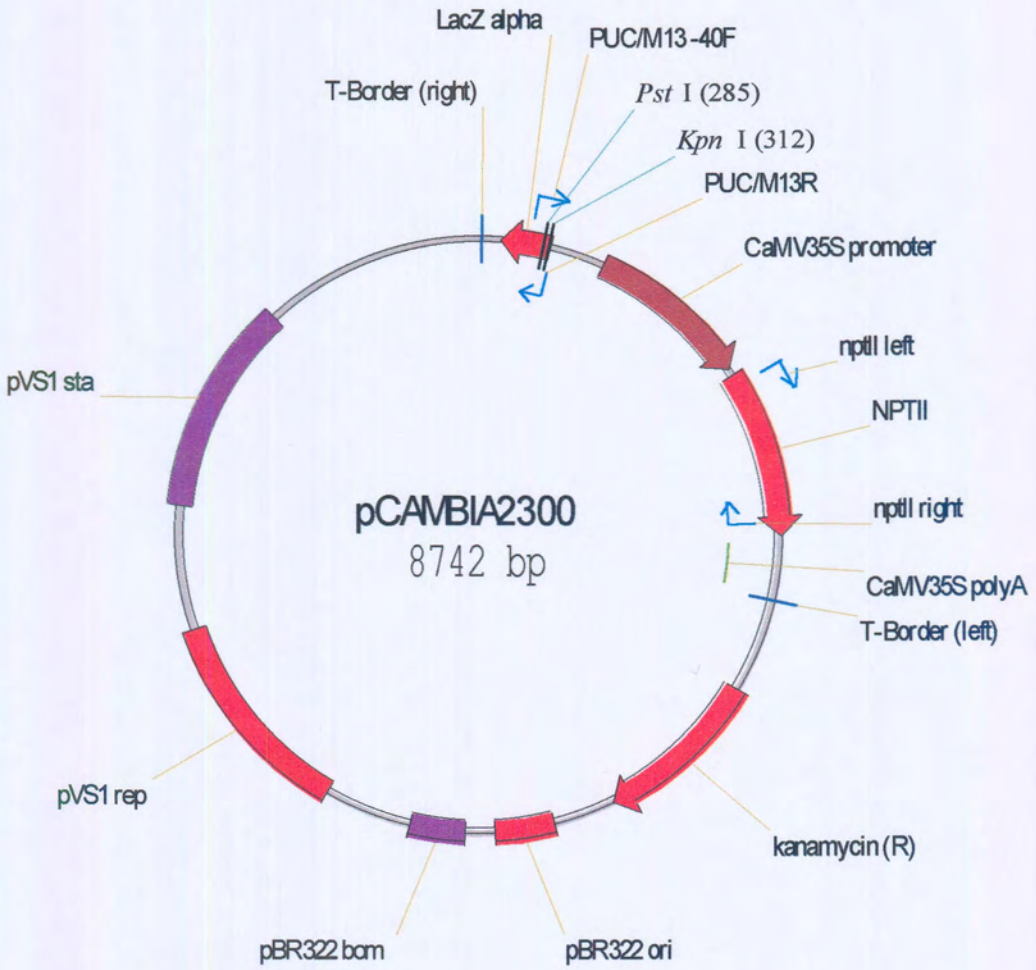
APPENDIX C

Plasmid maps

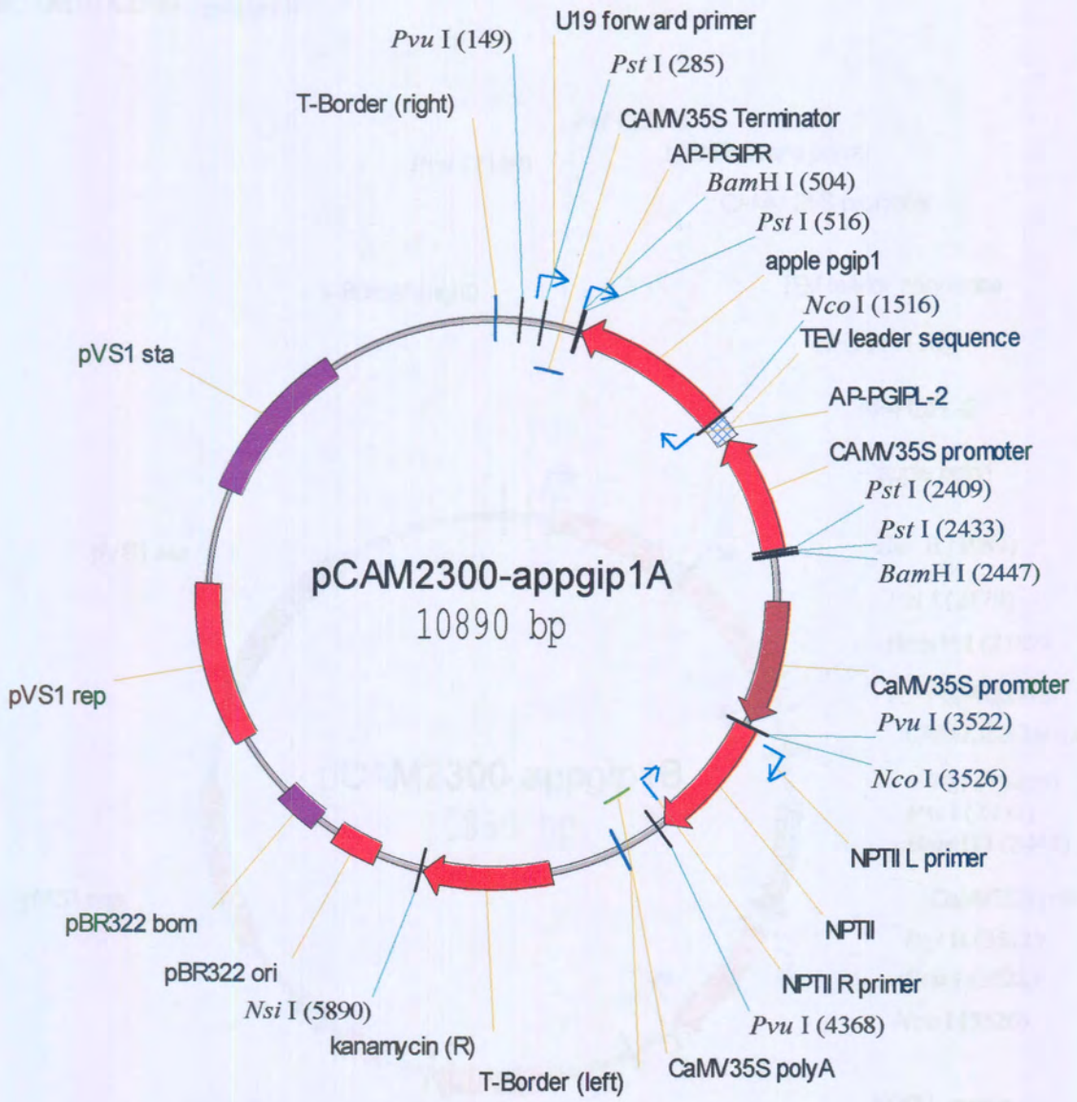
pAppRTL2



pCAMBIA2300



pCAMBIA2300-a*ppgip1*A



pCAMBIA2300-a*ppgip1*B