

## 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  $\text{Ca}^{2+}$ . The ammonium oxalate is included to bind and remove the  $\text{Ca}^{2+}$  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<sub>2</sub>O 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<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub>O. 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<sub>2</sub>O, were included. A positive control containing 5 ng *GST1*prom-*appgip1*-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<sup>R</sup>. 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-*appgip1*B and *GST1*prom-*appgip1*-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  $\mu$ l sterile 0.1% (w/v) agarose. The resuspended seeds were plated out onto MS selection plates [1 $\times$  MS salts (Sigma M5519 (St Louis, MO, USA) or Highveld Biologicals), 3% (w/v) sucrose, pH 5.9, 0.8% (w/v) agar, 50  $\mu$ g/ml kanamycin and 250  $\mu$ g/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<sup>R</sup> (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<sub>2</sub>, 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<sub>2</sub>O. 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<sub>2</sub>O, were included. A positive control containing 15 ng *GST1*prom-*appgip1*-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<sub>2</sub>O 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-*appgip1B* and *GST1*prom-*appgip1*-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). *KpnI* and *PstI* double-digestion of *GST1*prom-*appgip1*-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-*appgip1B* 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.

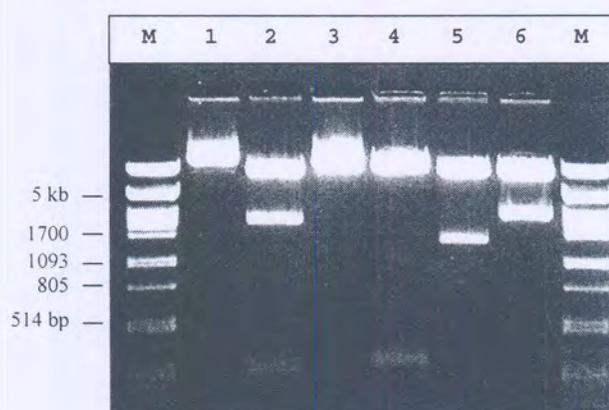


Figure 4.1 **Restriction analysis of constructs used for *A. thaliana* transformation.** M:  $\lambda$ DNA/ *PstI* marker: lanes 1 and 3: undigested *GST1*prom-*appgip1*-pCAMBIA#30 and pCAM2300-*appgip1B* plasmid, respectively: lane 2: *KpnI* and *PstI* double-digested *GST1*prom-*appgip1*-pCAMBIA#30 plasmid: lanes 4 to 6: pCAM2300-*appgip1B* 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-*apgip1*-pCAMBIA#30, pCAM2300-*apgip1B* 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<sub>2</sub>O negative controls (Figure 4.2, lanes 4 and 6). The apple *pgip1* primers yielded only 1 kb products for colonies transformed with *GST1*prom-*apgip1*-pCAMBIA#30 and pCAM2300-*apgip1B* and the positive control (Figure 4.2, lanes 7, 8 and 11). As expected, the apple *pgip1* gene was not present in the pCAMBIA2300 vector transformed colonies, the untransformed *A. tumefaciens* or the dH<sub>2</sub>O 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-*apgip1B* and *GST1*prom-*apgip1*-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-*appgip1*-pCAMBIA#30 construct. Similarly, the “e” is for pCAM2300-*appgip1*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-*appgip1*-pCAMBIA#30 construct (lines g14.2, g7-9, g9-13, Figure 4.3 lanes 1 to 3, respectively) and pCAM2300-*appgip1*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<sub>2</sub>O 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-*appgip1*-pCAMBIA#30 plasmid as template (Figure 4.3, lane 12).

A



B

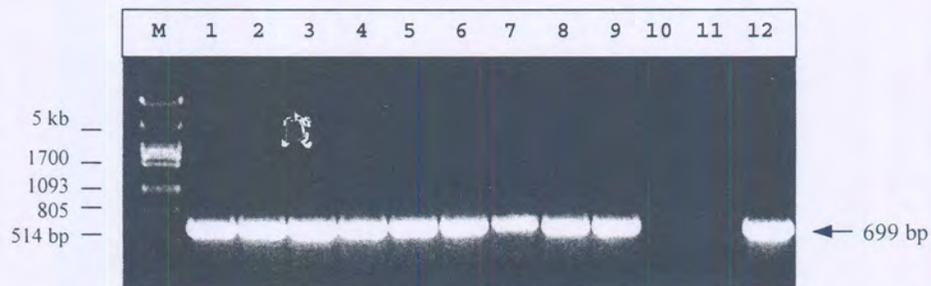


Figure 4.3 PCR analysis of *A. thaliana* Col-0 transformed with apple *pgip1* constructs, using AP-PGIP and NPTII primers. A: AP-PGIP PCR; B: NPTII PCR; For both A and B: M:  $\lambda$ DNA/*Pst*I marker; lanes 1 to 12: Amplification products of reactions containing the following as template: lanes 1 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-*appgip1*-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.

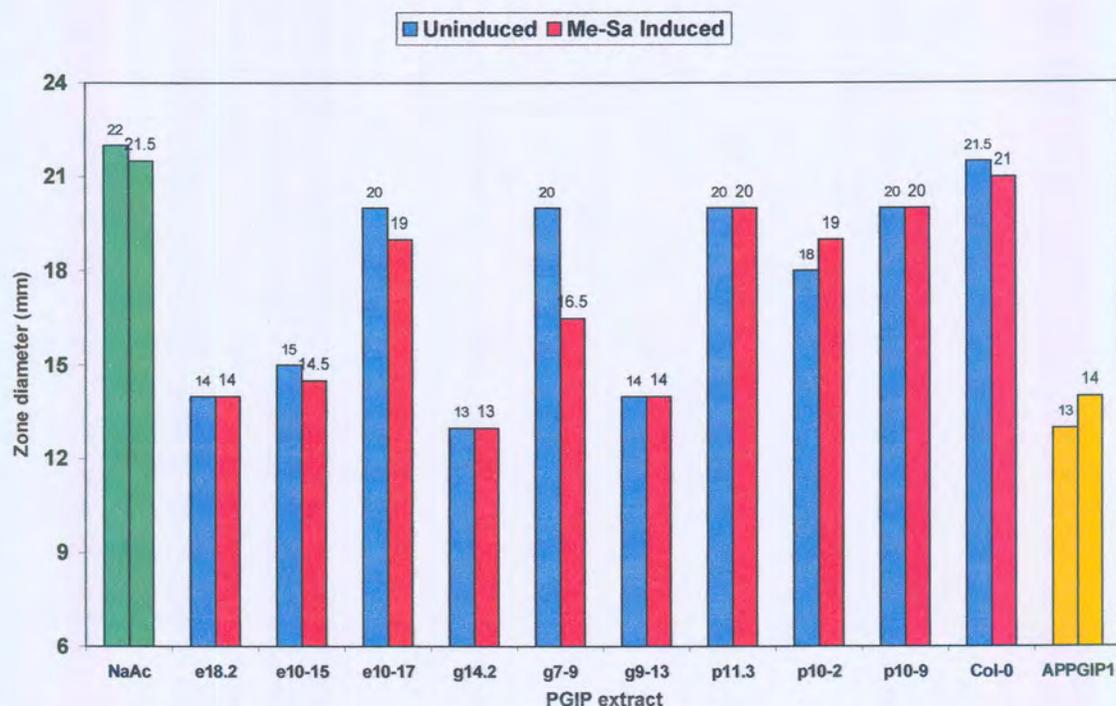


Figure 4.4 **Inhibition of *V. dahliae* PG activity by PGIP extracts from putative apple *pgip1* transgenic *A. thaliana* leaf material.** Zone diameters obtained during an agarose diffusion assay are presented. The activity of PG in the presence of PGIP extracts from each line is measured. Blue bars 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-*appgip1*-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-*apgip1*-pCAMBIA#30 and pCAM2300-*apgip1*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 constraints, 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<sub>2</sub>O<sub>2</sub>, 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* transcripts 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-*appgip1A* and pCAMBIA2300-*appgip1B*) 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<sup>3,7</sup>]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  $-70^{\circ}\text{C}$ . 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  $\mu\text{l}$  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)  $\beta$ -mercaptoethanol], preheated to  $60^{\circ}\text{C}$ . The samples were then incubated at  $60^{\circ}\text{C}$  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  $4500\times g$  for 10 minutes at  $22^{\circ}\text{C}$ . The DNA-containing aqueous phase (400-450  $\mu\text{l}$ ) 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  $-20^{\circ}\text{C}$  for 30 minutes to overnight.

The precipitated DNA was pelleted by centrifugation at  $6500\times g$  for 15 minutes. The DNA pellet was washed with 500  $\mu\text{l}$  70% ethanol, air-dried and resuspended in 20 to 40  $\mu\text{l}$   $1\times$  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  $\mu\text{l}$  with  $1\times$  TE (pH 8.0). Two and a half microlitres RNase A (10 mg/ml) was added and incubated at  $37^{\circ}\text{C}$  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  $\mu\text{l}$  isopropanol and 10 min incubation at  $-20^{\circ}\text{C}$ . The tubes were centrifuged at  $6500\times g$  for 10 min at  $4^{\circ}\text{C}$ , and the pellet washed with 500  $\mu\text{l}$  70% ethanol. After centrifugation, all the liquid was removed and the pellet allowed to air-dry. The pellet was dissolved in  $1\times$  TE (pH 8.0) to a final concentration of 100 ng/ $\mu\text{l}$ .

### 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  $-70^{\circ}\text{C}$ . 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)  $\beta$ -mercaptoethanol] in a 250 ml centrifuge tube. Eight millilitres of 20% SDS was added and the sample mixed thoroughly. The samples were incubated at  $65^{\circ}\text{C}$  for 30 minutes while shaking. Twenty millilitres of 5 M KOAc was added and the samples incubated at  $0^{\circ}\text{C}$  for 20 min. The cell debris was pelleted by centrifugation at  $6500\times g$  for 20 minutes at  $4^{\circ}\text{C}$ . The supernatant was filtered through muslin cloth wetted with 40 ml cold isopropanol, and the sample incubated at  $-20^{\circ}\text{C}$  for 30 min. The DNA was pelleted by centrifugation at  $6500\times g$  for 15 minutes at  $4^{\circ}\text{C}$ . The supernatant was decanted and the pellet dried by inversion of the tube for 10 min. The pellet was resuspended in 3 ml  $1\times$  TE (pH 8.0), 150  $\mu\text{l}$  RNase A (10 mg/ml) was added, and the pellets allowed to dissolve overnight at  $10^{\circ}\text{C}$ . The dissolved DNA was split into three 2.2 ml Eppendorf tubes, and each tube extracted twice with 1000  $\mu\text{l}$  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  $6500\times g$  for 10 minutes at  $4^{\circ}\text{C}$ . Each tube was extracted once with 1 volume (1000  $\mu\text{l}$ ) chloroform, and the phases separated by centrifugation. One tenth of the volume (100  $\mu\text{l}$ ) 3 M NaOAc and an equal volume (1000  $\mu\text{l}$ ) cold 100% ethanol was added to each tube, mixed and incubated at  $-70^{\circ}\text{C}$  for 10 minutes. The precipitated DNA was pelleted by centrifugation at  $4500\times g$  for 10 minutes at  $4^{\circ}\text{C}$ . 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  $\mu\text{l}$   $1\times$  TE (pH 8.0) each and pooled. The tubes were centrifuged twice at  $4500\times g$  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  $\mu\text{l}$  with  $1\times$  TE (pH 8.0). It was extracted once with 1 volume phenol: chloroform (1:1), centrifuged at  $6500\times g$  for 10 min at  $15^{\circ}\text{C}$ , 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  $\mu\text{l}$   $1\times$  TE (pH 8.0) overnight at  $4^{\circ}\text{C}$  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  $\mu$ l, contained 1 $\times$  Taq reaction buffer, 200  $\mu$ M of each dNTP, 0.5  $\mu$ M of each primer, 1.5 mM MgCl<sub>2</sub>, 1U Taq DNA polymerase (Promega) and 50 - 120 ng gDNA as template. The reaction volume was made up to 10  $\mu$ l using sterile dH<sub>2</sub>O. 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<sub>2</sub>O 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  $\mu$ g/ml ethidium bromide in 0.5 $\times$  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  $\mu$ g 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  $1.2 \times 10^6$  genome copies. One picogram of DNA represents  $0.965 \times 10^6$  kb, thus 1 kb of DNA equals  $1.03 \times 10^{-6}$  pg. The size of pAppRTL2 is 4784 bp, which equals  $4.956 \times 10^{-6}$  pg. The

mass of  $1.2 \times 10^6$  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<sub>2</sub>, PCR DIG mix and Expand High fidelity enzyme mix. The reaction was made up to a total volume of 50 µl with dH<sub>2</sub>O. 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/ *HindIII* with DIG-dUTP to use as DNA molecular weight marker

λDNA digested with *HindIII* (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 *HindIII*), 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/ *HindIII* 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 *NsiI*, *NcoI*, *BamHI*, *PvuI* and *PstI*. 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 (*NsiI*, *NcoI* and *BamHI*). 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 1/20<sup>th</sup> volume 5 M NaCl and

2.5 volumes cold ethanol and incubation at  $-20^{\circ}\text{C}$  for 1 h. After centrifugation at  $6500\times g$  for 20 min, the pellet was air-dried and resuspended in  $30\ \mu\text{l}$   $1\times$  TE (pH 8.0). The fragments were separated by overnight electrophoresis at  $7^{\circ}\text{C}$  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  $0.5\ \mu\text{g}/\text{ml}$  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  $0.25\ \text{M}$  HCl for 10 min, denatured for  $2 \times 15$  min in denaturation solution [ $0.4\ \text{N}$  NaOH,  $0.6\ \text{M}$  NaCl] and neutralised in neutralisation solution [ $0.5\ \text{M}$  Tris [pH 7.5],  $1.5\ \text{M}$  NaCl] for  $2 \times 15$  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  $20\times$  SSC [ $3\ \text{M}$  NaCl,  $0.3\ \text{M}$  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 Biotech, 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  $2\times$  SSC to remove the excess  $20\times$  SSC, placed between clean sheets of filter paper and baked for 2 h at  $80^{\circ}\text{C}$  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  $42^{\circ}\text{C}$  in 20 ml of DIG Easy HYB solution (Roche Diagnostics) containing denatured salmon testes DNA at a final concentration of  $125\ \mu\text{g}/\text{ml}$ . 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  $125\ \mu\text{g}/\text{ml}$  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).

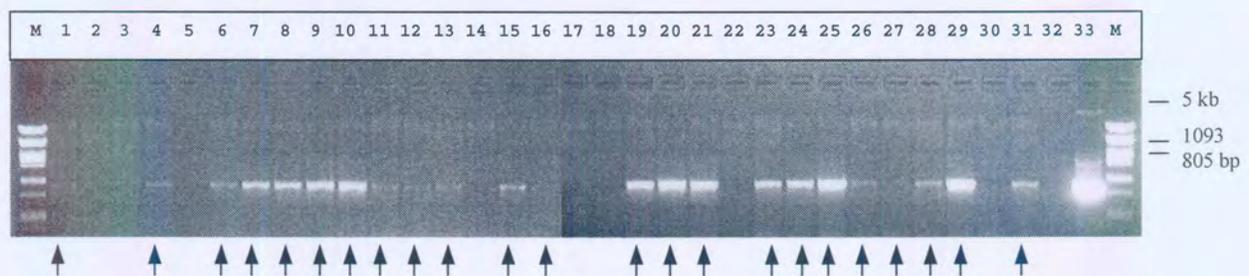


Figure 5.1 Apple *pgip1* PCR with gDNA from *in vitro* transgenic potato leaf material. M:  $\lambda$ DNA/*Pst*I marker; lane 1: LA Burley: *pgip1* #8 positive control; lane 2: untransformed LA Burley negative control; lanes 3 to 31: putative transgenic potato lines A2, A3, A4, A5, A6, A7, A8, A9, A10, 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:  $\lambda$ DNA/ *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).

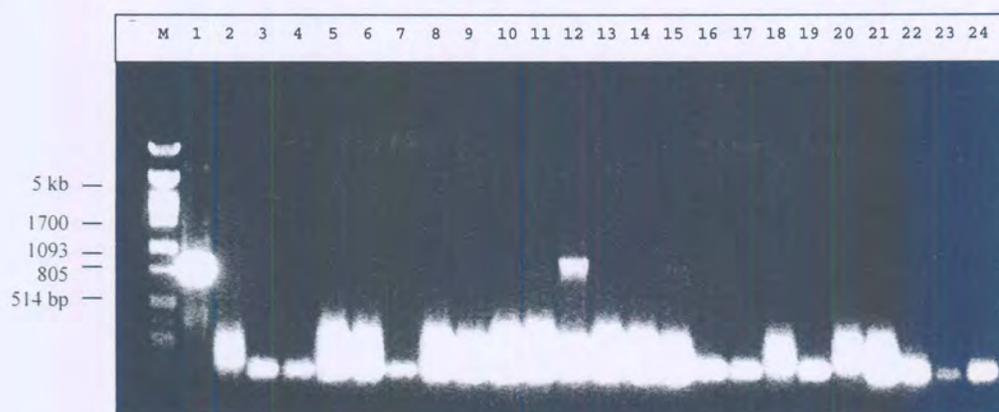


Figure 5.3 Unsuccessful PCR with gDNA from glasshouse transgenic potato leaf material. M:  $\lambda$ DNA/ *Pst*I marker; lane 1: positive control with plasmid as template; lanes 2 to 21: putative transgenic potato lines A3, A5, A6, A7, A8, A9, A10, A11, A12, A14, 7A, B3, B5, B9, B10, B11, B12, B13, B16, 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:  $\lambda$ DNA/ *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.

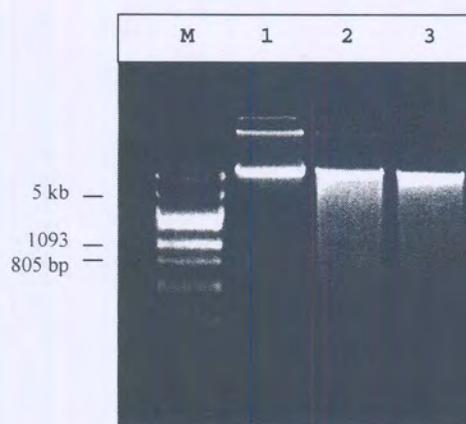


Figure 5.5 **gDNA cleaned up from RNA contamination**. M:  $\lambda$ DNA/ *Pst*I marker; lane 1: gDNA from LA Burley: *pgip1* #8 isolated using the large-scale Dellaporta *et al.* (1983) method; lanes 2 and 3: gDNA from lines B3 and B9, respectively, isolated using the small-scale CTAB method and treated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

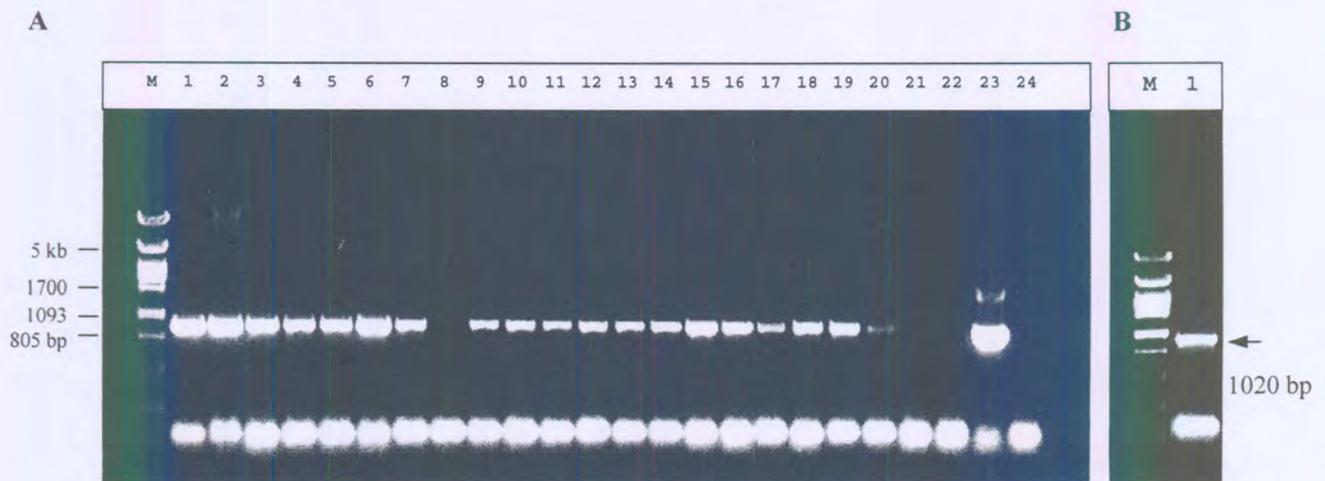


Figure 5.6 Apple *pgip1* PCR with gDNA from glasshouse transgenic potato leaf material.

- A. All glasshouse potato lines gDNA as template.** M:  $\lambda$ DNA/ *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:  $\lambda$ DNA/ *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.

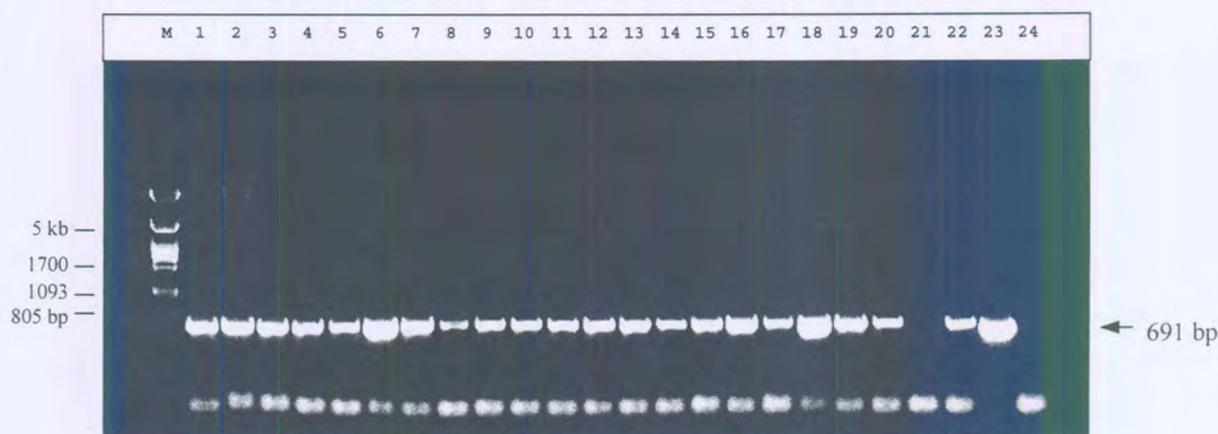


Figure 5.7 *nptII* PCR with gDNA from glasshouse transgenic potato leaf material. M:  $\lambda$ DNA/*PstI* marker; lanes 1 to 20: *nptII* PCR with gDNA from putative transgenic potato lines A3, A5, A6, A7, A8, A9, A10, A11, A12, A14, 7A, B3, B5, B9, B10, B11, B12, B13, B16, and B18, respectively; lane 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 *PstI* 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 *PstI* releases a fragment 1893 bp in length containing the CaMV e35S promoter, TEV leader and apple *pgip1* gene. Figure 5.8 shows *PstI* 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 *PstI* digestion was complete. This *PstI* digested pAppRTL2 plasmid DNA was used to spike untransformed gDNA during Southern blot of 10  $\mu$ g

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.

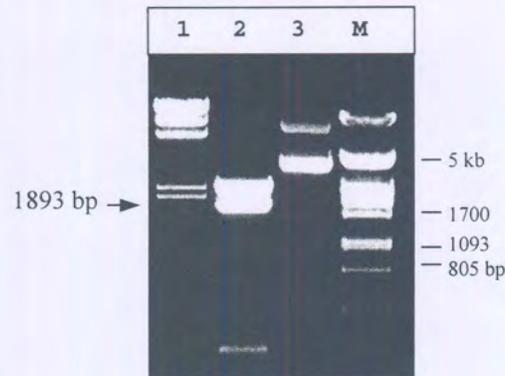


Figure 5.8 ***PstI* restriction digestion of pAppRTL2.** Lane 1: 250 ng λDNA/ *HindIII* marker; lane 2: 350 ng *PstI* digested pAppRTL2; lane 3: 250 ng undigested pAppRTL2; M: λDNA/ *PstI* marker.

### 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.

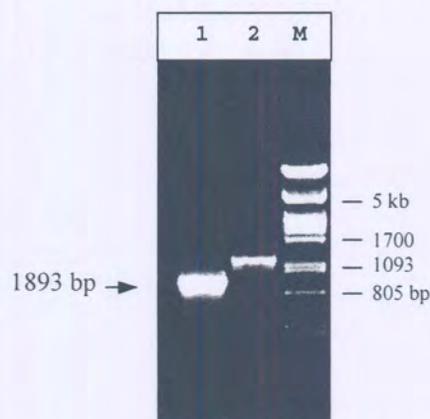


Figure 5.9 **DIG-labelled apple *pgip1* PCR product.** Lane 1: unlabelled apple *pgip1* PCR product; lane 2: DIG-labelled apple *pgip1* PCR product; M: λDNA/ *PstI* marker.

### 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  $\mu\text{g}$  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 *PvuI* and *PstI*, 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). *NcoI* and *BamHI* digested better, but digestion was still not complete (Figure 5.10, lanes 2 and 3, respectively). *NsiI* digestion was the best of all the enzymes (Figure 5.10, lanes 1 and 6), with only a little high molecular weight gDNA remaining.

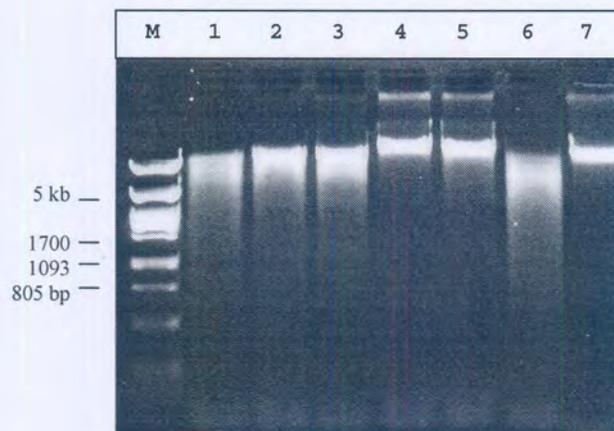


Figure 5.10 **Restriction digestion of 400 ng potato gDNA.** M:  $\lambda$ DNA/ *PstI* marker; lanes 1 to 5: 400 ng potato gDNA restriction digested with 1.2U of *NsiI*, *NcoI*, *BamHI*, *PvuI* and *PstI*, respectively; lane 6: 400 ng potato gDNA restriction digested with 3U of *NsiI*; lane 7: undigested potato gDNA.

Upon repeating the restriction digestion with 600 ng gDNA, the same results were obtained (Figure 5.11). Digestion with *NcoI* and *BamHI* was again not complete (Figure 5.11; lanes 3 to 5), with *NsiI*

again giving the best smear of frag<sup>ments</sup> (Figure 5.11, lane 2). Even increasing the *Bam*HI quantity to 5U did not improve digestion (Figure 5.11, lane 5).

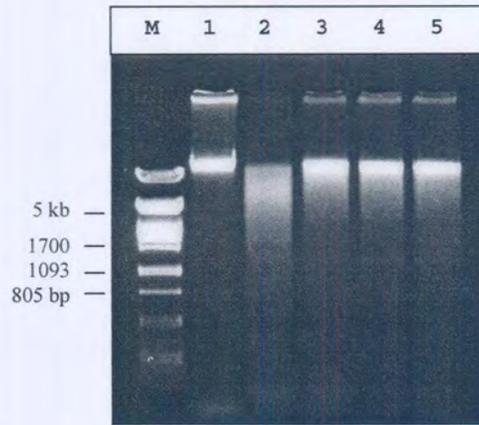


Figure 5.11 **Restriction digestion of 600 ng potato gDNA.** M:  $\lambda$ DNA/ *Pst*I marker; lane 1: undigested potato gDNA; lanes 2 to 4: 600 ng potato gDNA restriction digested with 1.8U of *Nsi*I, *Nco*I and *Bam*HI, respectively; lane 5: 600 ng potato gDNA restriction digested with 5U of *Bam*HI.

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  $\mu$ l 1 $\times$  TE (pH 8.0).

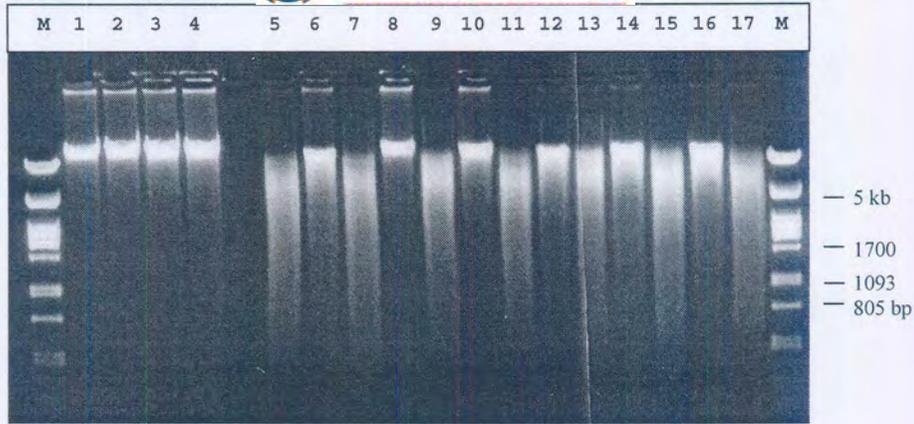


Figure 5.12 **Agarose gel to check if large-scale digestion of potato gDNA is complete.** Five hundred nanogram samples of potato gDNA digested with *NsiI*, *NcoI* or *BamHI*. M:  $\lambda$ DNA/ *PstI* marker; lanes 1 to 4: undigested gDNA from lines A9, B10, B13 and BP1, respectively; lanes 5, 7 and 9: *NsiI* digestion of lines A5, A6 and A9 gDNA, respectively; lanes 6, 8 and 10: *BamHI* digestion of lines A5, A6 and A9 gDNA, respectively; lanes 11, 13 and 15: *NsiI* digestion of lines B10, B11 and B13 gDNA, respectively; lanes 12, 14 and 16: *NcoI* digestion of lines B10, B11 and B13 gDNA, respectively; lane 17: *NsiI* 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  $\lambda$ DNA/ *HindIII* (Figure 5.13, lane 1), *NsiI* digested untransformed BP1 gDNA (lane 20) and *NsiI* 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/ <i>Hind</i> III (60 ng)	
2		
3	Untransformed BP1	<i>Nsi</i> I digested + 1 copy apple <i>pgip1</i>
4	"	" + 10 copy apple <i>pgip1</i>
5	"	" + 20 copy apple <i>pgip1</i>
6		
7	A5	<i>Nsi</i> I
8	"	<i>Bam</i> HI
9	A6	<i>Nsi</i> I
10	"	<i>Bam</i> HI
11	A9	<i>Nsi</i> I
12	"	<i>Bam</i> HI
13	B10	<i>Nsi</i> I
14	"	<i>Nco</i> I
15	B11	<i>Nsi</i> I
16	"	<i>Nco</i> I
17	B13	<i>Nsi</i> I
18	"	<i>Nco</i> I
19		
20	Untransformed BP1	<i>Nsi</i> 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.

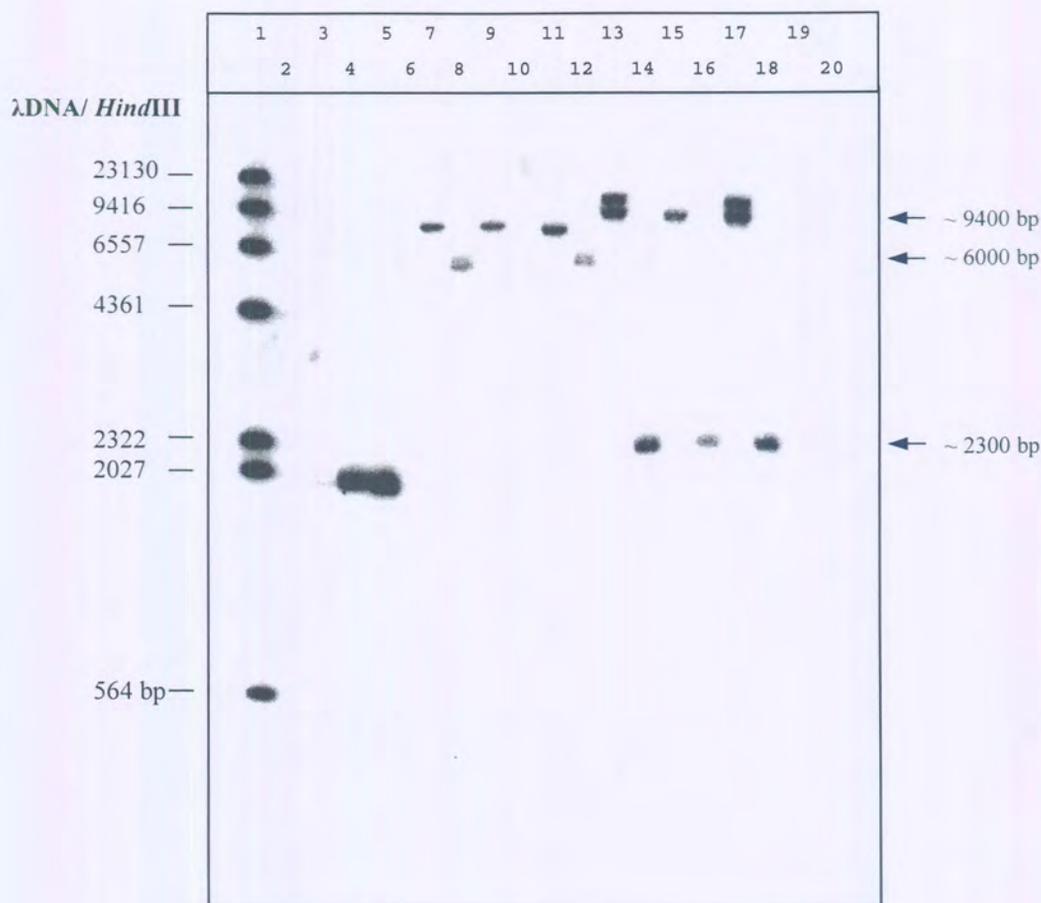


Figure 5.14 Southern blot of six apple *pgip1* transgenic potato lines. Contents of lanes are indicated in Table 5.1.

Using the non-radioactive DIG hybridisation and detection method, very low background signal was obtained. Klenow end-labelling of  $\lambda$ DNA/ *Hind*III with DIG was successful, since 60 ng of labelled  $\lambda$ DNA/ *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  $\mu$ g *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 *NsiI* and *BamHI* digested lanes (lanes 11 and 12, respectively). *BamHI* digestion of the potato A-lines are expected to give a *pgip1*-hybridising fragment of 1943 bp (see Figure 5.15 and Discussion).

*NsiI* 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). *NcoI* digestion of all three B-lines yielded fragments of approximately 2300 bp (lanes 14, 16 and 18, respectively). The intensity of the *NcoI*-fragment of line B11 (lane 16) was approximately half of that of the *NcoI*-fragments of lines B10 and B13 (lanes 14 and 18). *NcoI* 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 *NsiI*-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-*appgip1A* and pCAMBIA2300-*appgip1B* plasmids

The transgenic potato lines were generated by transforming potato cv. BP1 with the pCAMBIA2300-*appgip1A* and pCAMBIA2300-*appgip1B* constructs (**Appendix C**). These constructs were digested with *BamHI* and *NcoI*, 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-*appgip1A* with *BamHI* released a fragment of 1943 bp, that contains the apple *pgip1* gene (Figure 5.15; lane 2). Digestion with *NcoI* released a fragment of 2010 bp that does not contain the gene (lane 3). *BamHI* digestion of pCAMBIA2300-*appgip1B* released a 265 bp fragment (lane 5), while *NcoI* released a fragment of 2356 bp that is expected to hybridise with the *pgip1* probe (lane 6).

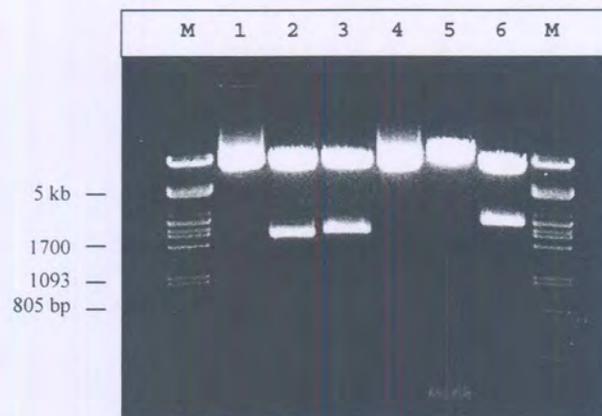


Figure 5.15 **Restriction digestion of pCAMBIA2300-*appgip1A* and pCAMBIA2300-*appgip1B* plasmids.** M:  $\lambda$ DNA/ *Pst*I marker; lane 1: undigested pCAMBIA2300-*appgip1A* plasmid; lanes 2 and 3: *Bam*HI and *Nco*I digestion of pCAMBIA2300-*appgip1A* plasmid, respectively; lane 4: undigested pCAMBIA2300-*appgip1B* plasmid; lanes 5 and 6: *Bam*HI and *Nco*I digestion of pCAMBIA2300-*appgip1B* plasmid, respectively.

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

The pCAMBIA2300-*appgip1A* and pCAMBIA2300-*appgip1B* 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-*appgip1B* 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-*appgip1A* plasmid, the potato A-lines and pCAMBIA2300-*appgip1B* plasmid (Figure 5.16, lanes 10 to 14). The negative dH<sub>2</sub>O controls were clear. The expected amplification products are described in the Discussion.

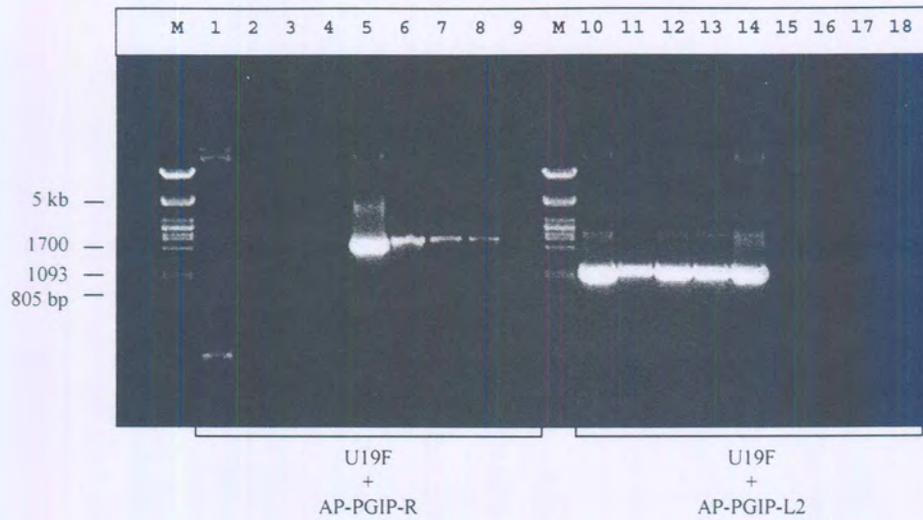


Figure 5.16 **PCR of six potato lines using U19F and AP-PGIP primers.** gDNA of the six transgenic potato lines and the pCAMBIA2300-*appgip1A* and pCAMBIA2300-*appgip1B* plasmids were used as templates for the PCR reactions. M:  $\lambda$ DNA/ *Pst*I marker; lanes 1 to 9: U19F and AP-PGIP-R primer combination; lanes 10 to 18: U19F and AP-PGIP-L2 primer combination. The templates used for PCR were the following: lanes 1 and 10: pCAMBIA2300-*appgip1A* plasmid; lanes 2 and 11: A5 gDNA; lanes 3 and 12: A6 gDNA; lanes 4 and 13: A9 gDNA; lanes 5 and 14: pCAMBIA2300-*appgip1B* plasmid; lanes 6 and 15: B10 gDNA; lanes 7 and 16: B11 gDNA; lanes 8 and 17: B13 gDNA; lanes 9 and 18: dH<sub>2</sub>O 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-*appgip1A* construct, while the B lines are transformed with the pCAMBIA-*appgip1B* construct. To determine the number of insertion events of the transgene into the potato genome, the restriction enzyme *NsiI* was chosen to digest gDNA for

Southern blot. It doesn't have a recognition site between the T-borders of pCAMBIA-app*gip1A* and pCAMBIA-app*gip1B*. 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 *NsiI* 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. *PvuI* and *PstI* 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. *BamHI* was chosen to excise the apple *pgip1* gene from the A lines and *NcoI* from the B lines. Digestions with these enzymes were never as complete as with *NsiI* (Figures 5.10 and 5.11).

A possible reason for the poor digestion of gDNA with *BamHI* is the enzyme's sensitivity to methylation. The recognition sequence of *BamHI* is GGATCC, and methylation at the first cytosine will lead to inhibition of cleavage. The sequence contains a recognition sequence (underlined) of the m-EcoDamI 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. *NcoI* (CCATGG) and *NsiI* (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 *NcoI* and *BamHI* 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 *BamHI* (Figure 5.13, especially lane 10). Even though extra units of this restriction enzyme were added and the reactions incubated for longer times, *BamHI* 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).

*NsiI* 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 *NsiI* were interesting (Figure 5.14). All three potato lines transformed with the pCAMBIA2300-*appgip1A* 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 *BamHI* 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 *NsiI* digestion, it is expected that line A6 should also show a fragment of 6000 bp. gDNA from line A6 was poorly digested with *BamHI* (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 *BamHI* digested samples, might be partial digestion by the enzyme, or that the *BamHI* 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 *BamHI* recognition site was, however, present in the pCAMBIA2300-*appgip1A* construct that was used for the transformation of the A-lines. *BamHI* 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-*appgip1A*, but are in fact clones of a transgenic B-line transformed with the pCAMBIA2300-*appgip1B* construct. A mix-up during the subculturing of transgenic *in vitro* potato plants may have lead to the mixing of transgenic lines. *BamHI* will then cut only on one side of the transgene construct (see map of pCAMBIA-2300-*appgip1B* 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-*appgip1B* construct was investigated with PCR. The results indicated that the A-lines are definitely transformed with the pCAMBIA2300-*appgip1A* construct and the B-lines with the pCAMBIA2300-*appgip1B* 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 *NsiI* and *NcoI* 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-appgip1A construct and the transgenic potato A-lines. U19F in combination with AP-PGIP-R should only amplify a 1958 bp fragment from pCAMBIA2300-appgip1B and the B-lines.

The results obtained in Figure 5.16 corresponds very well with the expected, except that the pCAMBIA2300-appgip1B 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-appgip1B plasmid preparation was contaminated with the pCAMBIA2300-appgip1A 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-appgip1B 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-appgip1A 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.