CHAPTER 6

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

6.1 Introduction

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

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

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*pgip1* gene has been isolated previously at ARC-Roodeplaat and transformed into LA Burley tobacco (Arendse and Berger, unpublished).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

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

Reducing sugar assay to determine PG activity

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

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

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

6.2 Materials and Methods

6.2.1 *V. dahliae* PG isolation

6.2.1.1 Fungal isolate and growth media

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

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

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

6.2.1.3 Growth of *V. dahliae* for PG production

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

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

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

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

6.2.2 Preparation of PGIP extracts from plant material

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

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

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

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

6.2.3 Assay for PGIP activity

6.2.3.1 Agarose diffusion assay (ADA)

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

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

6.2.3.2 Reducing sugar assay

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

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

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

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

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

6.2.3.2.2 Linear range of *V. dahliae* PG activity

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

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

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

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

6.2.4 Protein concentration determination

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

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

6.3 Results

6.3.1 *V. dahliae* PG isolation

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

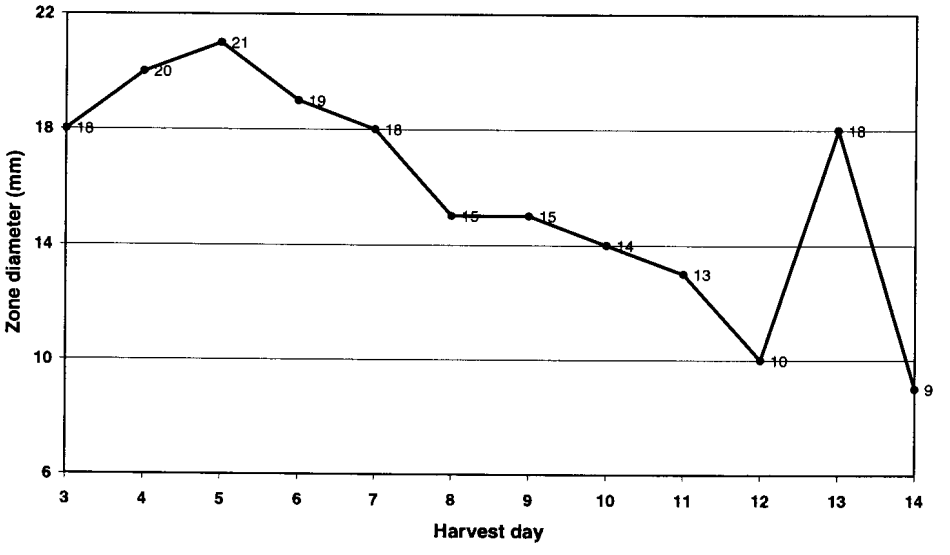

Figure 6.1 **Agarose diffusion assay (ADA) of *V. dahliae* culture supernatants.** Culture supernatants from different harvest days were assayed using the agarose diffusion assay and the zone diameters measured in mm.

6.3.2 ADA to determine the efficiency of AS precipitation

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

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

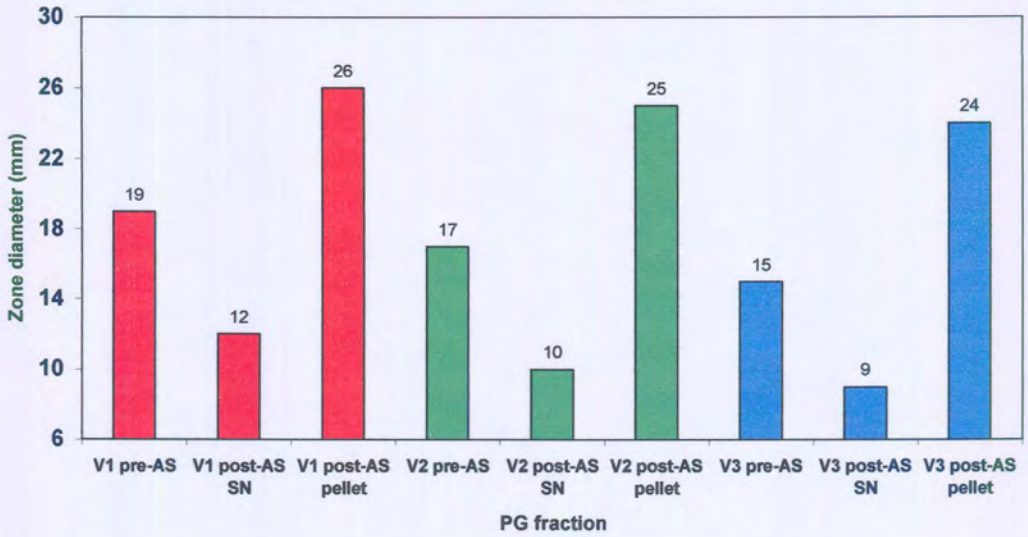

Figure 6.2 **ADA of pools of *V. dahliae* PGs before and after ammonium sulphate precipitation.** The explanations of the codes used in the figure are as follows: *V1 to V3*: pool 1 to 3; *Pre-AS*: Sterilised culture filtrate before ammonium sulphate precipitation; *Post-AS SN*: Supernatant decanted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

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

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

6.3.3 ADA to test PGIP inhibition of precipitated PG

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

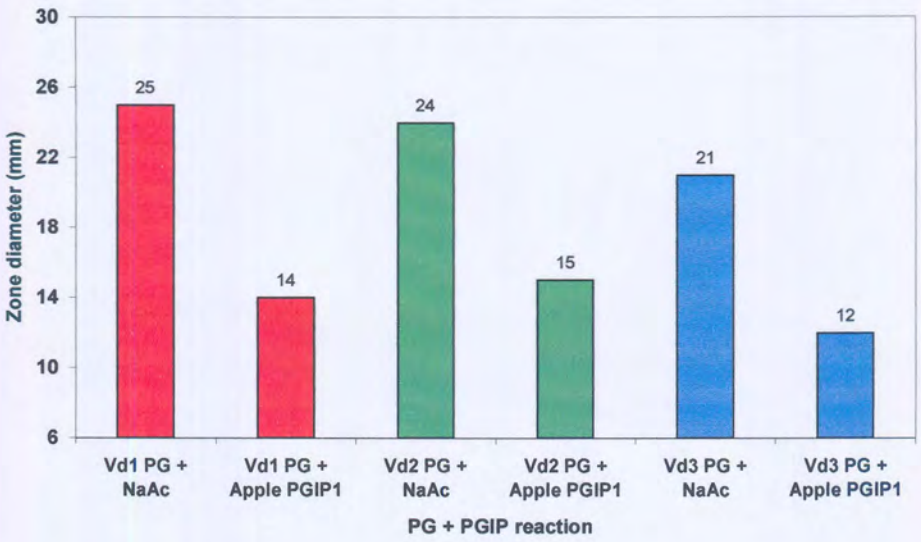

Figure 6.4 **ADA of apple PGIP1 inhibition of *V. dahliae* PGs isolated from different pools.** Vd1 to Vd3 PG: PG precipitated from pool 1 to 3. NaAc: buffer used for 100% activity of PG.

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

6.3.4 Assay for PGIP activity

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

6.3.4.1 Agarose diffusion assay (ADA)

6.3.4.1.1 Quick PGIP extraction (without dialysis) and ADA

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

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

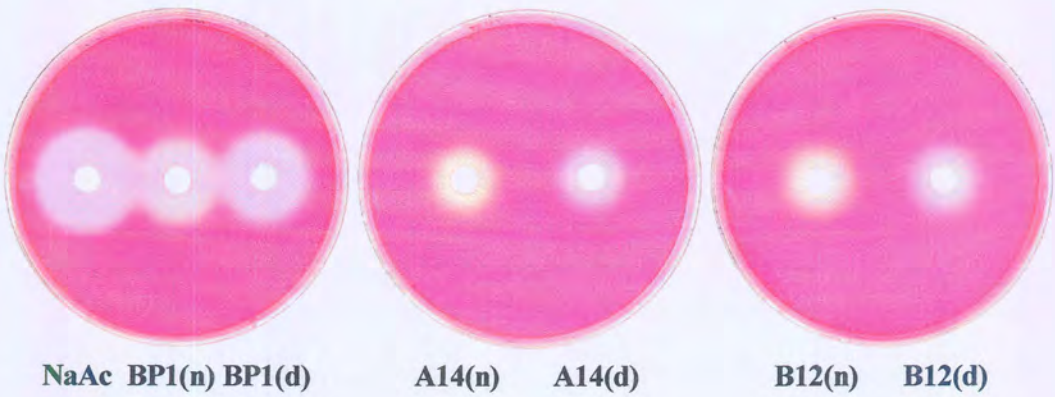

Figure 6.5 **ADA with *V. dahliae* PGs to compare the inhibiting activity of dialysed PGIP extracts with extracts that have not been dialysed.** n: non-dialysed; d: dialysed. The *V. dahliae* PGs were incubated with NaAc buffer alone, and with extracts prepared from untransformed BP1 potato and transgenic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

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

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

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

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

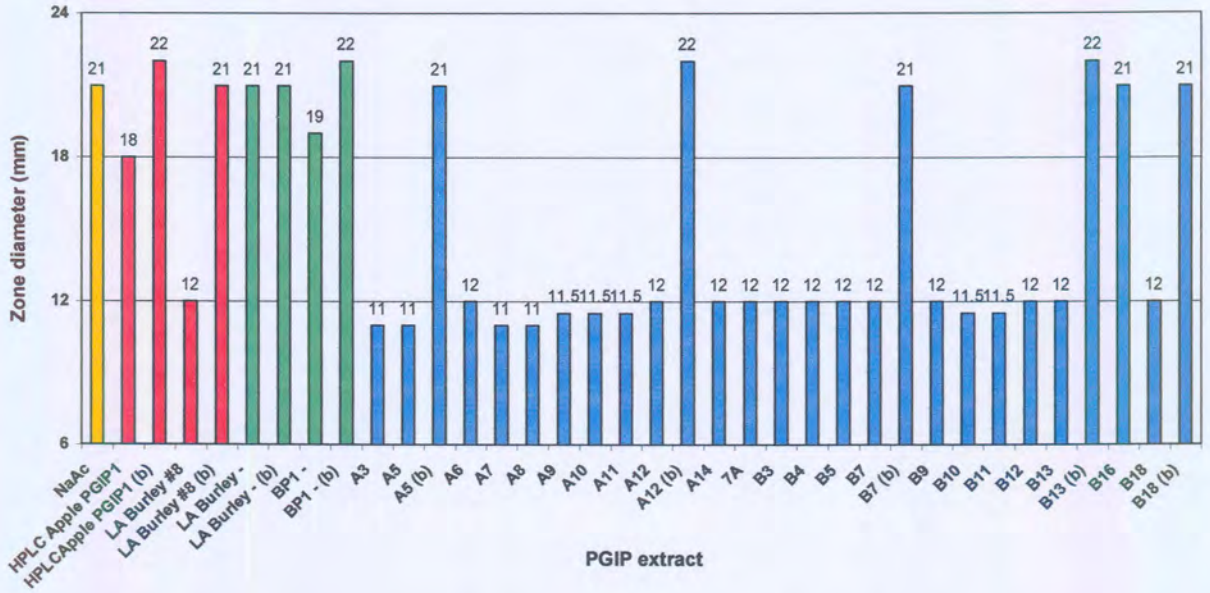

Figure 6.6 **ADA of *V. dahliae* PG activity with PGIP extracts from *in vitro* leaf material.** (b): PGIP extract boiled.

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

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

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

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

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

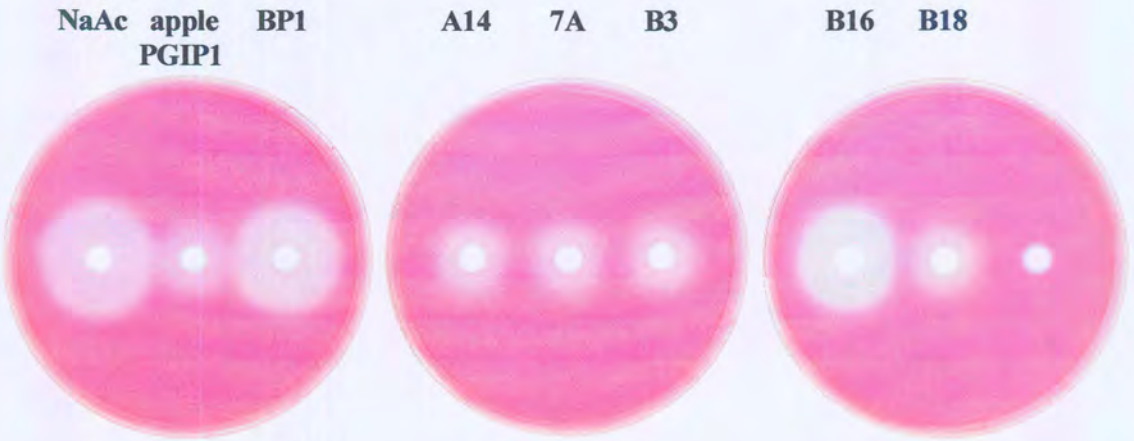

Figure 6.7 **ADA with *V. dahliae* PGs and PGIP extracts prepared from *in vitro* transgenic potato root material.** *V. dahliae* PG in the presence of NaAc buffer, HPLC purified apple PGIP1 or PGIP extract from the indicated potato line.

6.3.4.2 Reducing sugar assay

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

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

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

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

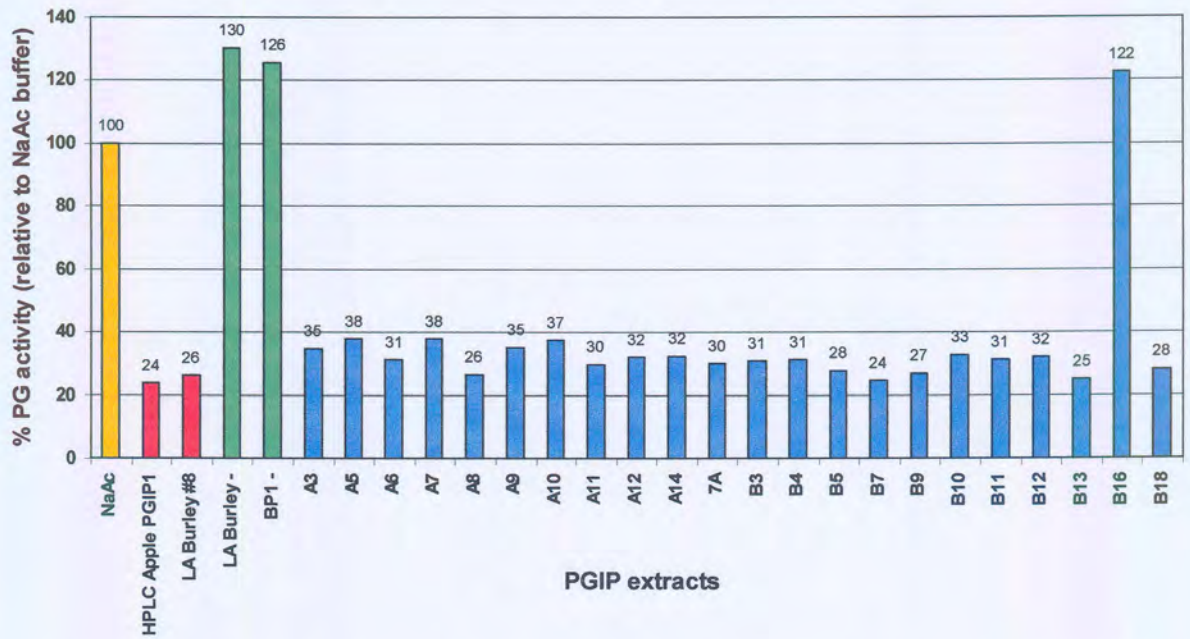

Figure 6.8 **Quick PAHBAH assay of *V. dahliae* PG activity with PGIP extracts from *in vitro* transgenic material.**

6.3.4.2.2 Linear range of *V. dahliae* PG activity

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

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

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

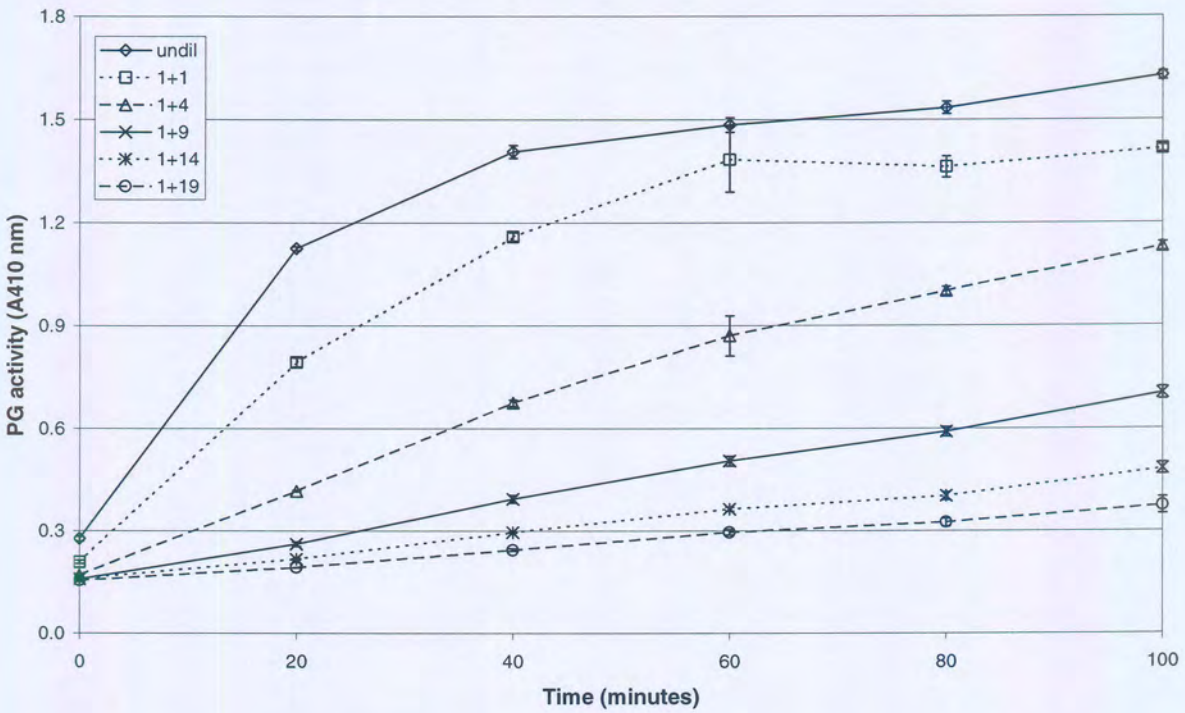

Figure 6.9 **Determination of time points at which different dilutions of *V. dahliae* PG exhibit a linear increase in activity in the reducing sugar assay.** *V. dahliae* PG activity is represented by the mean values of three replicate reactions, and the standard deviations are plotted as vertical bars.

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

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

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

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

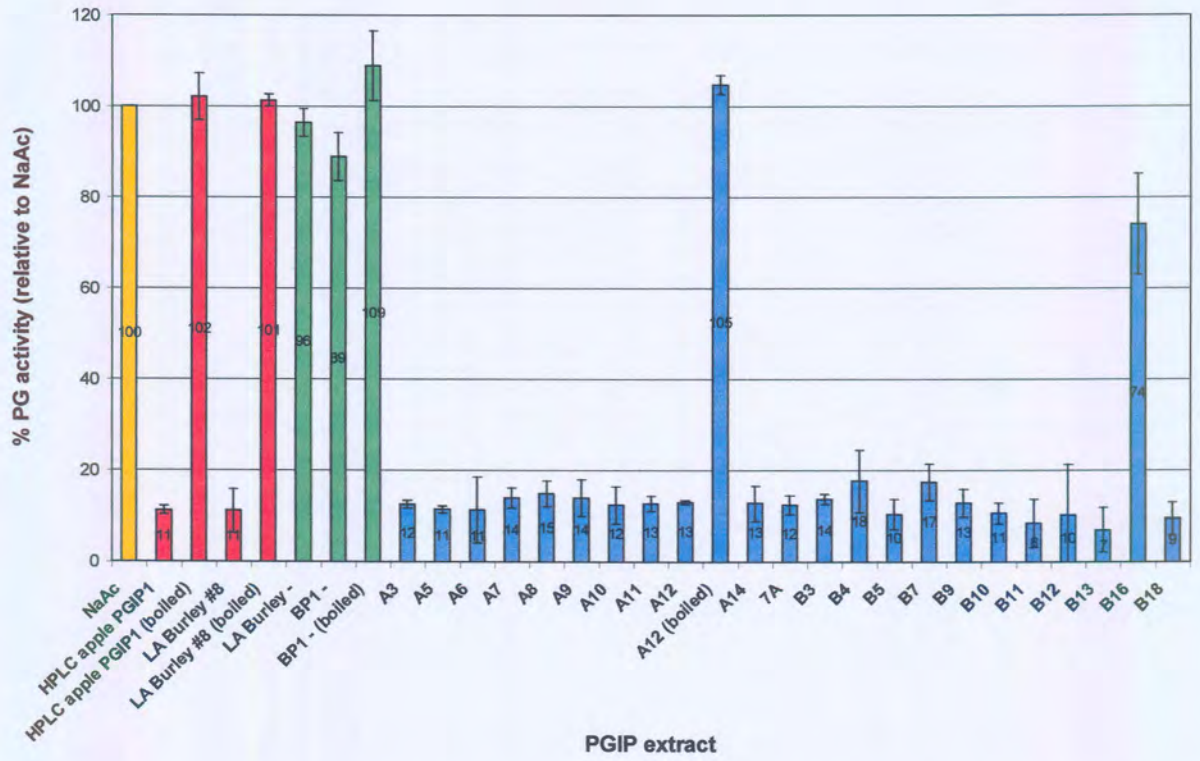

Figure 6.10 **Reducing sugar assay of *V. dahliae* PG activity with PGIP extracts from *in vitro* material.** *V. dahliae* PG activities are represented by the mean values of three replicate reactions, and the standard deviations are plotted as vertical bars.

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

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

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

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

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

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

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

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

[4] Purified PGIP1 = HPLC purified apple PGIP1.

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

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

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

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

6.3.5 Protein concentration determination of PG and dialysed PGIP extracts

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

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

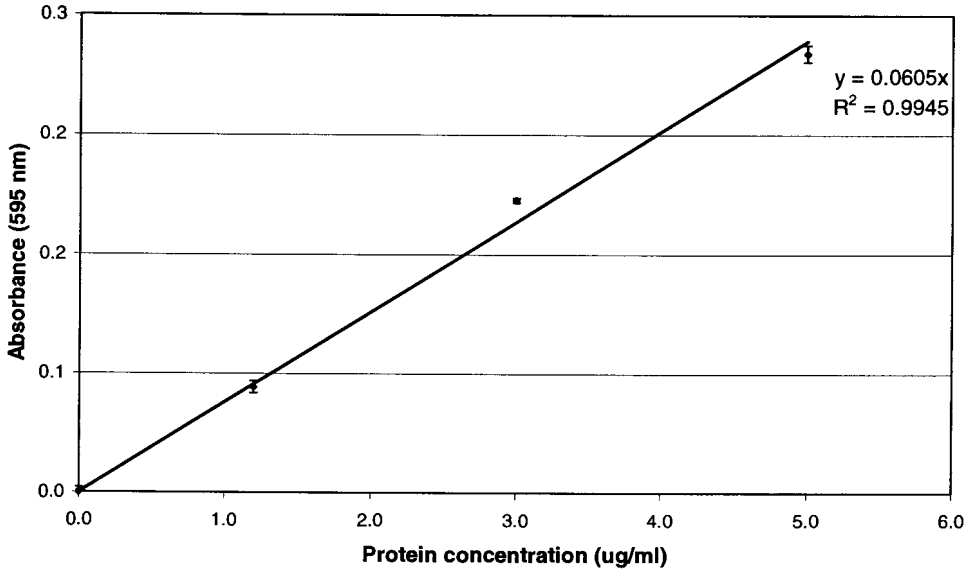

Figure 6.11 **Standard curve for the Bio-Rad protein assay using BSA as protein standard.** The average absorbance at 595 nm of each triplicate sample are plotted against protein concentration (µg/ml). The standard deviations are presented by vertical bars. A regression line is fitted to the points.

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

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

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

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

[2] BP1- = untransformed BP1 potato.

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

[4] Untransformed tobacco = LA Burley -.

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

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

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

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

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

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

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

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

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

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

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

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

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

6.4 Discussion

6.4.1 *V. dahliae* PG isolation and AS precipitation

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

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

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

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

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

6.4.2 Quick PGIP extraction and ADA

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

6.4.3 Assay for PGIP activity

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

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

6.4.3.1 Agarose diffusion assay (ADA)

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

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

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

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

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

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

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

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

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

6.4.3.2 Reducing sugar assay

6.4.3.2.1 Quick PAHBAH assay

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

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

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

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

6.4.3.2.2 Linear range of *V. dahliae* PG activity

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

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

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

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

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

6.4.4 Protein concentration determination of PG and dialysed PGIP extracts

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

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

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

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

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

6.4.5 Combined results of the inhibition assays

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

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

6.4.6 Conclusion

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

CHAPTER 7

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

7.1 Introduction

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

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

Examples of transgenic plants with enhanced resistance to fungal pathogens

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

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

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

7.2 Materials and Methods

7.2.1 Planting of tubers in the glasshouse

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

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

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

7.2.2 Preparation of *V. dahliae* microsclerotia inoculum

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

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

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

7.2.3 Visual assessment of *V. dahliae* symptoms

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

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

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

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

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

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

7.2.5 Calculation of the disease index

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

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

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

7.3 Results

7.3.1 Planting of tubers in the glasshouse

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

7.3.2 Visual assessment of *V. dahliae* symptoms

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

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

7.3.3 Plating out of stem sections onto PDA

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

7.3.4 Calculation of the disease index

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

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

7.3.5 Statistical analysis of the disease index data

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

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

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

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

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

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

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

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

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

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

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

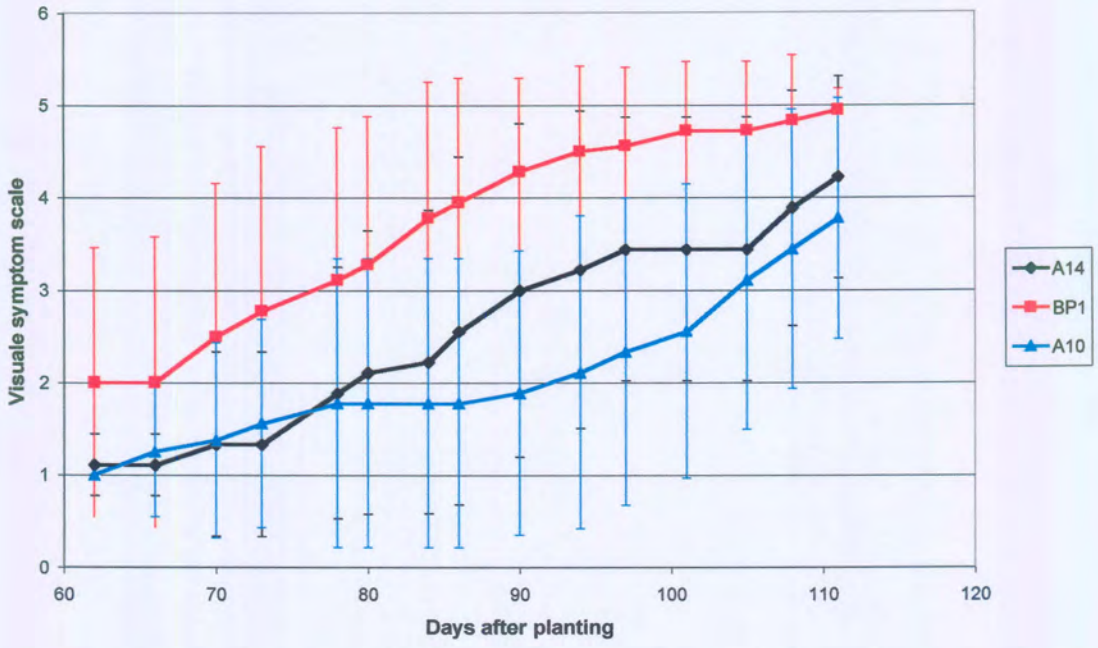

Figure 7.2 **Progression of visual disease symptoms over time for three potato lines.** The average visual disease symptom class for the nine replicates of each line is plotted against the number of days after planting of the tubers when the visual assessments were made.

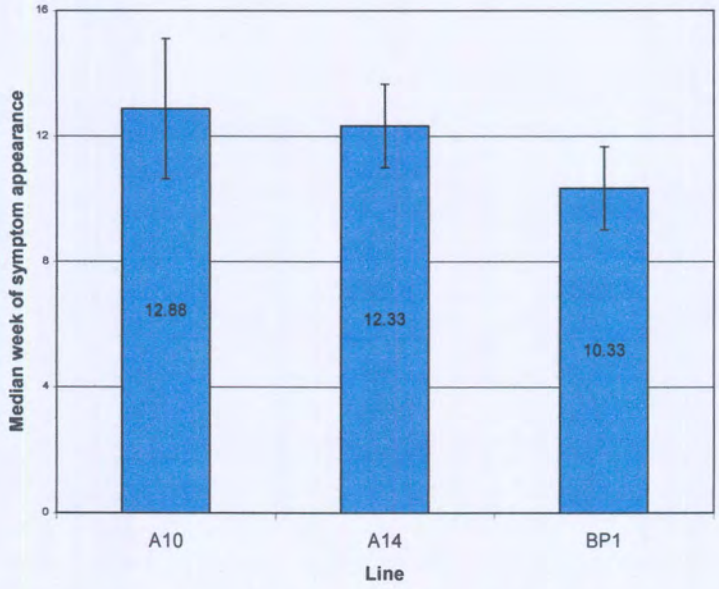

Figure 7.3 **Median time (in weeks) after planting of symptom development of three potato lines grown in inoculated soil.** The mean medians for nine replicates are presented, and their standard deviations are indicated by vertical bars.

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

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

7.3.6 Median week of symptom appearance

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

7.4 Discussion

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

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

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

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

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

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

Possible reasons why PGIP was not effective in the glasshouse trial

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

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

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

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

CHAPTER 8

Concluding Discussion

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

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

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

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