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ORIGINAL PAPER

The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases

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Abstract Polygalacturonase-inhibiting proteins (PGIPs) selectively inhibit polygalacturonases (PGs) secreted by invading plant pathogenic fungi. PGIPs display differential inhibition towards PGs from different fungi, also towards different isoforms of PGs originating from a specific pathogen. Recently, a PGIP-encoding gene from *Vitis vinifera* (*Vvpgip1*) was isolated and characterised. PGIP purified from grapevine was shown to inhibit crude polygalacturonase extracts from *Botrytis cinerea*, but this inhibitory activity has not yet been linked conclusively to the activity of the *Vvpgip1* gene product. Here we use a transgenic over-expression approach to show that the PGIP encoded by the *Vvpgip1* gene is active against PGs of *B. cinerea* and that over-expression of this gene in transgenic tobacco confers a reduced susceptibility to infection by this pathogen. A calculated reduction in disease susceptibility of 47–69% was observed for a homogeneous group of transgenic lines that was statistically clearly separated from untransformed control plants following infection with *Botrytis* over a 15-day-period. VvPGIP1 was subsequently purified from transgenic tobacco and used to study the specific inhibition profile of individual PGs from *Botrytis* and *Aspergillus*. The heterologously expressed and purified VvPGIP1 selectively inhibited PGs from both *A. niger* and *B. cinerea*, including BcPG1, a PG from *B. cinerea* that has previously been shown to be essential for virulence and symptom development. Altogether our data confirm the antifungal nature of the VvPGIP1, and the in vitro inhibition data suggest at least in part, that the VvPGIP1 contributed to the observed reduction in disease symptoms by inhibiting the macerating action of

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certain *Botrytis* PGs *in planta*. The ability to correlate inhibition profiles to individual PGs provides a more comprehensive analysis of PGIPs as antifungal genes with biotechnological potential, and adds to our understanding of the importance of PGIP:PG interactions during disease and symptom development in plants.

Keywords *Vitis vinifera* · Polygalacturonase-inhibiting protein · Polygalacturonases · *Botrytis cinerea* · *Aspergillus niger*

Introduction

Polygalacturonase-inhibiting proteins (PGIPs) have been isolated from many mono- and dicotyledonous plants and in recent years, a large amount of data has emerged regarding the regulation, structure and function of PGIPs (for recent reviews, see De Lorenzo et al. 2001; De Lorenzo and Ferrari 2002; Gomathi and Gnanamanickam 2004). PGIPs are cell wall proteins with a role(s) in plant defence, most notably their interaction with and inhibition of fungal polygalacturonases (PGs). PGs are among the first enzymes secreted by a number of fungal and bacterial pathogens when breaching plant cell walls (Herlache et al. 1997; De Lorenzo et al. 2001; Idnurm and Howlett 2001). Fungal pathogens such as *Botrytis cinerea*, *Aspergillus flavus* and *Altenaria citri* are all dependent on PG genes to maintain full virulence on their respective hosts (Shieh et al. 1997; ten Have et al. 1998; Kars et al. 2005). PGs and their action on plant cell walls have been comprehensively studied and the contribution of this interaction to the molecular dialogue between host and pathogen is well established (Esquerré-Tugayé et al. 1999). PGs cleave the α-1,4 linkages of non-esterified galacturonic acid residues within the homogalacturonan domain (Esquerré-Tugayé et al. 1999), but the specific activity, pH optimum and substrate preference of PGs can vary considerably (De Lorenzo and Ferrari 2002; Kars et al. 2005).

PGIPs may specifically inhibit PGs by either interacting with residues within the active cleft of the enzyme, thereby inhibiting binding of the PG to its substrate while simultaneously blocking the active site (De Lorenzo and Ferrari 2002; Federici et al. 2001), or by binding to the opposite site of the PG molecule, preventing the conformational changes necessary for ligand binding (King et al. 2002). These observations are consistent with the hypothesis that the action of PGIPs during fungal attack is 2-fold; the physical interaction between PGIPs and PGs slows down the infection rate of the fungus and facilitates the prolonged existence of mid-sized oligogalacturonides (degree of polymerisation 11–20) which in turn can elicit a general defence response from the plant (De Lorenzo et al. 1994; Hahlbrock et al. 1995; Reymond et al. 1995).

The importance of PGIPs in defence against fungal pathogens has been further demonstrated by the over-expression of various PGIP-encoding genes in native as well as heterologous hosts. These include the over-expression of pear *pgip* in tomato (Powell et al. 2000), the over-expression of bean *pgip* in tobacco (De Lorenzo and Ferrari 2002) and the over-expression of *Arabidopsis pgip* genes in *Arabidopsis* (Ferrari et al. 2003). In all cases, a reduction in disease symptoms was reported when transgenic plants were infected with *B. cinerea*. This reduction of susceptibility towards *B. cinerea* can presumably be attributed to the inhibition of a PG(s) that is an important pathogenicity factor(s) for the fungus. None of the previous studies explored the nature of the observed resistance further and no data exist regarding the inhibition of individual PGs from these transgenic experiments. It was recently shown that *B. cinerea* has at least six PGs that are differentially regulated during the infection process and contribute differentially to virulence and symptom development (Wubben et al. 1999; ten Have et al. 2001; Kars et al. 2005). The study of PGIPs and individual PGs from fungal pathogens not only provides valuable insight into the nature and dynamics of these interactions, but helps also to identify promising candidate genes for biotechnological approaches to improve disease resistance.

A grapevine PGIP encoding gene, *Vvpgip1*, has previously been isolated in our laboratory and PGIP purification from grapevine berries yielded a protein with strong inhibition activity against a crude extract of PGs from *B. cinerea*

(De Ascensao 2001). Here we over-express the *Vvpgip1* gene in tobacco, confirm PGIP activity in the heterologous host and show that *B. cinerea* symptom development is reduced in transgenic lines. VvPGIP1 was subsequently purified from transgenic tobacco and used to evaluate its interaction with and inhibition of individual PGs from *Aspergillus niger* and *B. cinerea*.

Materials and methods

Plant growth and light conditions

In vitro tobacco (*Nicotiana tabacum* L. Havana petit SR1) plantlets were cultured on MS medium (Murashige and Skoog 1962) and incubated at 26°C with a 16 h light 8 h dark photoperiod regime. Tobacco leaf discs transformed with the grapevine polygalacturonase-inhibiting protein encoding gene (*Vvpgip1*) were allowed to form shoots on MS media supplemented with 0.5 μg/ml 6-benzyl-aminopurine (BAP) and 100 μg/ml kanamycin. Rooting was induced on MS media supplemented with 0.1 μg/ml naphthalenacetic acid (NAA) and 100 μg/ml kanamycin. To establish greenhouse plants, seeds were germinated in peat moss (Jiffy International AS, Kristiansand, Norway) and plants were maintained in a greenhouse under natural light at 26°C and 65% humidity.

Vector constructs and plant transformation

Oligonucleotide primers 5′-CTGCAGATGGAGACTTCAAAACTTTT-3′ (*Pst*I site underlined) and 5′-GGATCCACTTGCAGCTCTGGAGTGGAG-3′ (*Bam*HI site underlined) were used to amplify the 1002 bp ORF of the *Vvpgip1* gene (Genbank accession: AF499451). The fragment was cloned into the pGEM-T-Easy vector (Promega Corporation, Madison, USA) and confirmed by sequencing. The *Vvpgip1* insert was subcloned into the *Pst*I and *Bam*HI sites of pBluescriptSK(+) (Stratagene, La Jolla, USA) for mobilisation into a plant expression vector. The fragment was subsequently excised from pBluescriptSK(+) with *Eco*RV and *Sac*I and cloned into the *Sma*I and *Sac*I sites of the binary vector pBI121 (Jefferson et al. 1987), replacing the *β*-glucuronidase gene to yield pBI(Vvpgip1) and placing the gene under control of the 35S CaMV promoter and nopaline synthase (NOS) terminator. The construct was mobilised into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation. Tobacco was transformed with pBI(Vvpgip1) using the leaf disc method of Gallios and Marinho (1995) and plantlets were regenerated under kanamycin selection (100 μg/ml). Several putative primary transgenic lines were recovered and acclimatised for growth in a greenhouse (T0 lines). The T0 lines were allowed to self-pollinate and the offspring (T1 lines) were germinated and grown as described.

PCR and Southern blot analyses of transgenic lines

Transgene integration in primary transformants was confirmed by PCR with the *Vvpgip1* gene specific primers (results not shown). All PCR analyses were conducted in a Whatman Biometra Trio-thermoblock automated temperature cycler (Göttingen, Germany). Typical PCRs consisted of 1× PCR buffer, 0.25 μM primer, 0.2 μM dNTPs, 100 ng genomic DNA as template and 0.5 U of *Taq* DNA polymerase in a 50 μl reaction volume. PCR cycle conditions typically included an initial denaturation step at 95°C for 3 min and 30 amplification cycles consisting of denaturation at 95°C for 30 s, annealing at 58°C for 40 s and elongation at 72°C for 40 s. Reactions also typically included a final elongation step of 5 min at 72°C.

Genomic DNA was extracted from 0.1 g of tobacco leaves obtained from greenhouse acclimatised T0 and T1 generation transformants according to the method of McGarvey and Kaper (1991). For Southern blot analysis, genomic DNA from the transgenic tobacco lines (10 μg) was digested with *Eco*RV and separated on a 0.8% (w/v) agarose gel. *Eco*RV cuts on the 3′ end (just 5′ of the *Vvpgip1* gene) of the 35S CaMV promoter, but not in the *Vvpgip1* gene, providing an estimate of transgene insertion events. Gels were transferred to positively charged Hybond-N nylon membranes according to Sambrook et al. (1989). Pre-hybridisation (2 h) and hybridisation (16–20 h) reactions were carried out at 42°C in DIG

Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany). The *Vvpgip1* gene was used as a probe and digoxigenin-labelled according to the DIG System User's Guide (Roche Diagnostics GmbH, Mannheim, Germany). After hybridisation, membranes were washed sequentially at room temperature (two washes with double strength SSC [1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0] and 0.1% SDS [w/v] for 5 min each) and 68°C (two washes with half strength SSC and 0.1% SDS [w/v] for 15 min each). Chemiluminescent detection of nucleic acids was done using CSPD as substrate (Roche Diagnostics GmbH, Mannheim, Germany).

Northern blot analysis of *Vvpgip1* transcript levels

Total RNA was extracted from the leaves of greenhouse acclimatised T0 and T1 generation transformants using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's specifications. Total RNA (10 μg per lane) was size fractionated by electrophoresis on a 1.2% (w/v) formaldehyde agarose gel and blotted to Hybond-N nylon membranes using standard techniques as described by Sambrook et al. (1989). The membranes were pre-hybridised at 50°C for 4 h in DIG Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany). The membranes were then hybridised in the same solution with the addition of the digoxigenin-labelled *Vvpgip1* probe. After hybridisation, membranes were washed twice in double strength SSC and 0.1% SDS (w/v) at room temperature for 15 min and twice in half strength SSC and 0.1% SDS (w/v) at 68°C for 15 min. Chemiluminescent detection proceeded as previously described for Southern blot detection.

Detection of VvPGIP1 activity in transgenic tobacco

Crude protein extracts of independent transgenic tobacco lines (T0 generation) and untransformed control tobacco plants were analysed for the presence of PGIP activity by an agarose diffusion assay (Taylor and Secor 1988). This assay uses polygalacturonic acid (PGA) as substrate for polygalacturonases (PGs) and inhibition of PG activity by active PGIP results in a size-reduction of clearing zones. Finely crushed leaf tissue (0.4 g) was homogenised in extraction buffer consisting of 0.1 M sodium acetate buffer (pH 6.0), 10 mM β-mercaptoethanol and 1% (w/v) PVP-40. The homogenate was centrifuged at 10,000*g* for 15 min, followed by two more extractions in extraction buffer without PVP-40, each followed by centrifugation. The remaining insoluble tissue was resuspended in 2 volumes of 50 mM sodium acetate buffer (pH 5.2) also containing 1 M NaCl and stirred for 1 h at 4°C. The insoluble debris was removed by centrifugation at 10,000*g* for 20 min. The proteins precipitating at 80% (w/v) saturated ammonium sulphate were collected, resuspended in 20 mM sodium acetate (pH 5.2) and dialysed extensively at 4°C against 20 mM sodium acetate (pH 5.2). The protein concentration was determined according to Bradford (1976), using a Bio-Rad (Hercules, USA) protein assay kit and bovine serum albumin (BSA) as standard. The dialysed samples were assayed for PG inhibition by the agarose diffusion assay using crude PGs from *Botrytis cinerea* (see next section). All agarose diffusion assays were done in triplicate and allowed to proceed for 16 h at 30°C. A boiled sample of crude extract was also loaded on the plates to confirm the proteinaceous origin of the reduction in clearing zones. Zones were visualised by staining of the plates with 0.05% Ruthenium red. Equal amounts of total protein were used in the assay. The diameter of the clearing zones as a result of PG hydrolysis on the pectic substrate was compared with those formed when plant cell extracts containing inhibitor was added, and expressed as a percentage reduction.

Fungal endopolygalacturonases

Crude PG preparations from *B. cinerea* were prepared by culturing the fungal spores in citrate phosphate buffer (pH 6.0), supplemented with 1% (w/v) citrus pectin, 2 mM $MgSO_4 \cdot 7H_2O$, 0.6 μM $MnSO_4 \cdot H_2O$, 25 mM KNO_3, 30 μM $ZnSO_4 \cdot 7H_2O$, 0.9 μM $CuSO_4$ and 65 μM $FeSO_4$ for 10 days at 22°C. The cultures were filtered through Whatman No. 1 paper and precipitated

overnight with 80% $(NH_4)_2SO_4$ at 4°C. Proteins were recovered by centrifugation at 10,000*g* for 20 min at 4°C and resuspended in 40 mM sodium acetate (pH 5.0). Purified PGs from *Aspergillus niger* (AnPGII, AnPGA and AnPGB) and *B. cinerea* (BcPG1, BcPG3, BcPG4 and BcPG6), were obtained as previously reported (Pařenicová et al. 1998; Kars et al. 2005).

Plant infections and disease assessment

Pathogenic cultures of *B. cinerea* were isolated from a South African vineyard and were maintained on sterile halved apricots (Naturlite, Tiger Food Brands Limited, South Africa), in a dark growth chamber at 23°C until sporulation occurred. Spore inocula were harvested using sterile distilled water following incubation on apricot halves for 12 days. Mycelial debris was removed from the spores by filtration. Spore viability was evaluated by plating an aliquot of the spore suspension on 0.8% (w/v) water agar (Difco, Detroit, MI) and calculating the percentage of spores that had germinated following incubation for 24 h at 23°C.

Susceptibility to *B. cinerea* of the T0 population was evaluated using a detached leaf assay according to Carstens et al. (2003). Leaves of 7- to 8-week-old greenhouse acclimatised plants (including an untransformed control) were detached with a scalpel blade and placed in a sealed tissue culture container, with the petiole embedded in 0.8% (w/v) water agar. Three leaves per plant line were inoculated on the adaxial side, without wounding the surface, with two aliquots of 5×10^3 spores per spot. The containers were sealed to maintain 100% humidity and placed in a growth room at 23°C. Disease symptoms were scored 3 days after inoculation by measuring the diameter of the lesions, this data was used to calculate the percentage decrease in disease susceptibility in the transgenic lines compared to the untransformed control.

The detached leaf assay was followed by a whole plant infection on 7- to 8-week-old T1 progeny of six of the independent transgenic lines over a period of 15 days. Lines 1, 5, 24, 37, 45 and 47 were randomly selected for this assay and used together with untransformed control plants. For the whole plant infections, 5 μl of a spore suspension (1×10^3 in a 50% grape juice medium) was spotted on the adaxial surface of leaves without detaching or wounding the leaves. Three leaves per plant line were inoculated with four spots per leaf. Mature, fully expanded leaves from positions 3, 4 and 5 were selected for inoculation and care was taken to ensure that leaf position of inoculated leaves were consistent between plant lines. Plants were placed in a humidity chamber at 100% relative humidity and incubated for a period of 15 days following inoculation at 23°C. The success of the infections was monitored and disease susceptibility was determined by measuring the diameters of the lesions daily from day three to seven and again at day 15. The decrease in disease susceptibility was calculated by comparing the average lesion diameter at day 15 of all lines to that of the untransformed control. Two independent whole plant infections were carried out on the mentioned lines. Significant differences and homogeneous groups were calculated by performing a one-way Analysis of Variance (ANOVA) using the STATISTICA 6 (StatSoft Inc., Tulsa, OK, USA) software package at 95% confidence intervals.

Isolation and purification of VvPGIP1 from transgenic tobacco plants

Purified PGIP was isolated from transgenic tobacco plants (line 37) using a modified approach from Favaron et al. (1994). Healthy leaves from 6- to 8-week-old T1-generation tobacco plants (450 g) were homogenised in a blender with 1 l of acetone. The pulp was filtered through a nylon cloth, squeezed, dried and blended with 300 ml of acetone (repeated three times), and allowed to dry out completely (overnight at room temperature). The dried pulp was soaked in 1 l of extraction buffer (20 mM sodium acetate, pH 5.5, 1 M NaCl) and stirred for 24 h at room temperature. The material was centrifuged at 9000*g* for 30 min. The supernatant was recovered, filtered through Whatmann GF/A glass filter paper and dialysed against 20 mM sodium acetate (pH 5.5) over a period of 3 days in standard cellulose dialysis tubing (12,000–14,000 molecular weight

cut-off) with at least six buffer changes. The dialysed sample was centrifuged for 30 min at 10,000*g* and the supernatant recovered.

The protein content was separated by cation exchange chromatography using a 5 ml Econo-Pac high S cartridge (Biorad, Hercules, USA) at a flow rate of 1 ml/min. The column was equilibrated with buffer A (20 mM sodium acetate, pH 5.0) and the bound fraction was pulse-eluted with buffer B (20 mM sodium acetate, 300 mM NaCl, pH 5.0). The eluted fraction was diluted 1:2 in double strength ConcavalinA (ConA) buffer (200 mM sodium acetate, pH 6.0, 2 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$) (Favaron et al. 1994) and diluted again 1:2 in ConA buffer. The sample was loaded onto a ConA Sepharose 4B column (Amersham Biosciences, NJ, USA) the flow-through collected and eluted with ConA elution buffer (250 mM α-methyl-D-manno-pyranoside in ConA buffer) (Favaron et al. 1994). The flow-through was loaded on the column again and eluted as described. This procedure was repeated one more time to ensure maximum recovery of VvPGIP1.

The eluted sample was concentrated to 20 ml and diluted 1:10 in 20 mM sodium acetate (pH 5.0). This procedure was repeated three times to remove all traces of the elution buffer. Before proceeding to chromatography, the final concentrate was diluted 1:10 in 20 mM sodium acetate (pH 5.0). The diluted sample was loaded onto a Hi-Trap S cation exchange column (Amersham Biosciences, NJ, USA) at a flow rate of 1 ml/min. The column was again equilibrated with buffer A and eluted with buffer B, using a gradient of 1% buffer B per minute. Protein content in the fractions were quantified according to Bradford (1976) using a Bio-Rad protein assay kit and BSA as a standard. Eighty 0.5 ml fractions were analysed for PGIP activity against PGB from *Aspergillus niger* (AnPGB).

Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)

Fractions eluted from the Hi-Trap S cation exchange column that contained PGIP activity were analysed on a Hewlett Packard G2025A MALDI-TOF mass spectrometer (Palo Alto, CA, USA). The matrix used was a saturated sinapinic acid (3,5 dimethoxy-4-hydroxy-*trans*-cinnamic acid) solution in 50% acetonitrile. Equal volumes of sample and matrix (approximately 1 μl) were loaded onto the probe and were dried under vacuum. The laser energy ranged from 10 to 14 μJ.

Quantitative endopolygalacturonase inhibition assays with purified VvPGIP1

Endopolygalacturonase inhibition by the purified grapevine PGIP was followed by measuring the hydrolytic activity of the PGs on a pectic substrate in the absence and presence of the inhibitor. Approximately 100 ng purified VvPGIP1 was used to test inhibition against BcPGs 1, 3, 4 and 6 as well as AnPGs II, A, and B at pH 3.75, 4.0, 4.2, 4.5, 4.75, 5.0, 5.5 and 6.0. Approximately 50 ng PG was used in each assay. Reactions were incubated for 1 h at room temperature with the respective PGs in 0.025% (w/v) polygalacturonic acid (Sigma, St Louis, USA) as substrate and buffered in 50 mM sodium acetate. Following incubation, the inhibition was quantified by measuring the decrease in the release of reducing sugars spectrophotometrically at 410 nm, using the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York et al. 1985). Controls lacking PGs were included to compensate for possible reducing sugars and native PGs present in the chromatographic fractions. To compensate for the possible influence of the chromatography buffer gradient on the enzyme activity, equivalent amounts of salt were added to all assays. All assays were done in triplicate.

Results

Construction of a plant expression cassette and subsequent tobacco transformations

The complete coding region of the grapevine polygalacturonase inhibiting protein encoding

gene (*Vvpgip1*) was inserted between the CaMV 35S promoter and nopaline synthase terminator in the plant expression vector pBI121. pBI(Vvpgip1) was mobilised into *Agrobacterium tumefaciens* and subsequently used for tobacco transformation. Independent transgenic tobacco lines were identified with PCR and Southern blot analyses. PCR analysis performed on 46 independent primary transgenic tobacco lines yielded a fragment corresponding to the size (1002 bp) of the *Vvpgip1* gene in all of the lines tested (results not shown). Untransformed lines were used as negative controls and did not result in any amplification. Southern blot analysis (Fig. 1) confirmed integration of the *Vvpgip1* ORF in most lines tested. The integration number of the transgenes varied per transformed line (T0 generation) and was estimated from the Southern results to range from one to approximately eight or more insertion events. Expression of the *Vvpgip1* transgene in the transformed tobacco lines was analysed by Northern blot analysis using the *Vvpgip1* gene as probe. Hybridisation analysis of total RNA from leaf tissue demonstrated that *Vvpgip1* transcripts of the correct size were expressed in 19 transgenic lines. No transcripts could be detected in untransformed plants (Fig. 2). The transgenic population was phenotypically analysed and compared to untransformed controls and did not show any abnormalities with regards to growth, size or reproduction.

Crude protein extracts from transgenic tobacco over-expressing *Vvpgip1* can inhibit crude polygalacturonase (PG) preparations from *Botrytis cinerea*

To assess the activity of VvPGIP1 in transgenic tobacco plants over-expressing the *Vvpgip1* gene, crude protein extracts prepared from greenhouse acclimatised T0 transgenic tobacco leaves (all 19 Northern positive lines) were used to assess inhibition of crude PG preparations from *B. cinerea* in an agarose diffusion assay described by Taylor and Secor (1988). The results confirmed PGIP activity in the crude extracts from all the transgenic lines, showing clear reductions in the clearing zones when the plant extracts were incubated with the PGs on the pectic substrate. The levels of PG inhibition ranged from 25 to 80% (Table 1).

Transgenic tobacco plants over-expressing *Vvpgip1* are less susceptible to *B. cinerea* infection

Initial experiments to determine the effect of VvPGIP1 over-expression in transgenic tobacco on disease susceptibility was conducted using greenhouse acclimatised T0 Northern positive transgenic lines in a detached leaf assay. Successful infections occurred on all leaves and proceeded aggressively in the untransformed controls. Lesion sizes were significantly reduced

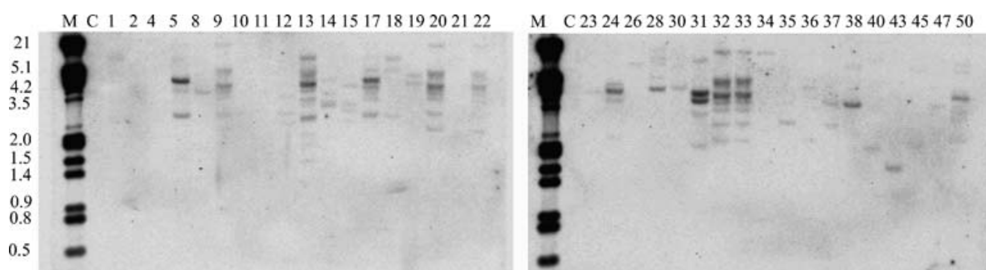


Fig. 1 Southern blot analyses of T0 generation tobacco plants transformed with the grapevine PGIP1 encoding gene. Genomic DNA from tobacco plants were digested with *Eco*RV and hybridised with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of the VvPGIP1 encoding gene. Digoxigenin-labelled lambda DNA was used to visualise the molecular marker (lambda DNA digested with *Eco*RI and *Hin*dIII). The numbers identify each independent transgenic plant tested. Untransformed tobacco genomic DNA digested with *Eco*RV is shown in lane C. The marker lane (M) contains *Eco*RI and *Hin*dIII digested lambda DNA. Sizes of the standard DNA fragments are indicated in kb

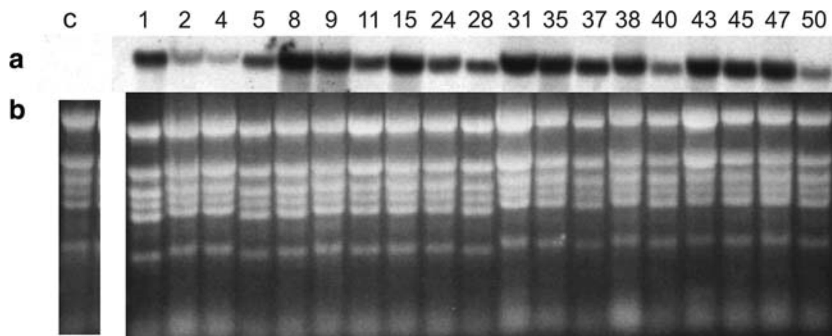


Fig. 2 Northern blot analysis of the expression of the grapevine polygalacturonase-inhibiting protein encoding gene (*Vvpgip1*) in T0 generation transgenic tobacco lines. Total RNA was extracted from leaf tissue and probed with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of the grapevine *pgip1* gene as shown in **a**. Ethidium bromide staining of the formaldehyde agarose gel is shown in **b**. Numbers identify each transgenic plant line that showed mRNA *pgip1* expression. Total RNA extracted from an untransformed tobacco plant is shown in lane C

Table 1 Analysis of transgenic tobacco over-expressing a grapevine PGIP: Inhibition of polygalacturonases (PGs) from *Botrytis cinerea* by protein extracts[a] from the transgenic plants and assessment of lesion development on detached leaves of the transformed plants when infected by *B. cinerea*

PG control and pant lines[b]	PGIP activity assay		Detached leaf assay	
	Clearing zone (mm) in agarose plate assay	PG inhibition[c] (%)	Lesions (mm) on detached leaves infected with *B. cinerea*	Decrease in lesion size of detached leaves infected with *B. cinerea* (%)
PG control	21 ± 2.0	N/A	N/A	N/A
Untransformed	20.5 ± 1.8	2.4	16.17 ± 1.5	0
Boiled extract	20.5 ± 0.5	2.4	N/A	N/A
1	7.7 ± 0.6	63.3	8.17 ± 0.6	49.5
2	15 ± 1.0	28.6	10.50 ± 1.2	35.1
4	14.8 ± 1.6	29.5	10.00 ± 1.2	38.1
5	7 ± 1.0	66.7	7.83 ± 0.9	51.5
8	8.2 ± 1.0	61.0	8.83 ± 1.2	45.4
9	6.5 ± 0.5	69.0	7.33 ± 1.1	54.6
11	8.3 ± 1.2	60.5	9.33 ± 1.0	42.3
15	7.3 ± 1.2	65.2	8.33 ± 0.9	48.5
24	11.3 ± 0.6	46.2	10.50 ± 1.2	35.1
28	10.3 ± 0.6	51.0	9.67 ± 0.9	40.2
31	4.2 ± 0.3	80.0	6.33 ± 1.5	60.8
35	5 ± 0.5	76.2	7.00 ± 1.9	56.7
37	5.7 ± 0.6	72.9	7.17 ± 1.5	55.7
38	4.7 ± 0.6	77.6	6.33 ± 1.4	60.8
40	11 ± 0.0	47.6	10.17 ± 0.9	37.1
43	9.5 ± 0.5	54.8	9.00 ± 0.9	44.3
45	8.5 ± 0.9	59.5	9.50 ± 1.0	41.2
47	8.2 ± 1.3	61.0	9.00 ± 0.8	44.3
50	15.8 ± 1.3	24.8	11.33 ± 1.1	29.9

[a]Crude extracts were prepared from leaves

[b]Numbers identify each independent transgenic line tested

[c]PG inhibition was determined by the agarose diffusion assay (Taylor and Secor, 1988), 100 ng of extract was used for each sample

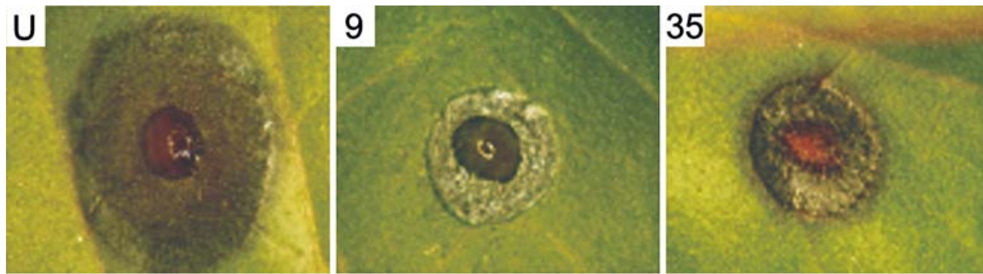


Fig. 3 *Botrytis cinerea* lesion development on detached leaves of untransformed and transgenic T0 generation *Nicotiana tabacum* L. Havana petit SR1 plants. Shown is the lesion development on tobacco plants lines 9 and 35 over-expressing the grapevine polygalacturonase inhibiting protein (VvPGIP1) relative to the untransformed control plants (U). Lesions were photographed 3 days after inoculation using similar magnification conditions

in the transgenic population at 3 days post-infection (dpi) and up to 61% reduction in lesion size was obtained (Table 1 and Fig. 3).

To assess whether transgenic tobacco whole plants expressing VvPGIP1 showed reduced susceptibility to *B. cinerea* infection, untransformed as well as six *Vvpgip1* expressing T1 progeny lines were infected with *B. cinerea* spores. The infection was followed for 15 days. The high spore-load as well as the incubation conditions favoured disease development and 75–92% of inoculum sites developed into primary lesions within three dpi. Lesion size was scored daily for the period 3–7 dpi and again after 15 dpi (see Table 2 for the lesion size at 15 dpi). Comparing lesion development over the 15 days, lesion development as well as lesion size remained comparable in all transgenic lines for up to 7 dpi, yet consistently showed reduced lesion sizes compared to the untransformed control (Fig. 4). During the analysis period, lesions on the untransformed plants remained wet and started to expand very rapidly, while lesions on transgenic plants expanded at significantly reduced rates and became dry and necrotic.

Statistically significant differences in lesion development and size between the various transgenic lines as well as between transgenic lines and untransformed control only occurred in the period between 7 and 15 dpi (Fig. 4). Statistical analysis grouped the transgenic lines with high confidence into two distinct homogeneous groups based on lesion sizes (at 15 dpi) (Table 2). Lines 5, 24, 37 and 45 were placed in one group, while lines 1 and 47 were placed with the untransformed plants in a separate group. Group two

Table 2 Analysis of lesion development on transgenic tobacco lines over-expressing a grapevine PGIP following whole plant infections with *Botrytis cinerea*

Plant line	Percentage of successful infections per plant line[a]	Average lesion diameter (mm) [a]	Percentage decrease in disease susceptibility (compared to WT) [a]	Homogenous groups[a]
Wild-type	75%	40.94 ± 3.50	0	1
VvPGIP1	75%	37.66 ± 4.24	8.0	1
VvPGIP 5	83%	12.58 ± 1.36	69.2	2
VvPGIP 24	83%	15.43 ± 0.94	62.3	2
VvPGIP 37	83%	12.60 ± 1.06	69.2	2
VvPGIP45	92%	21.84 ± 3.10	46.7	2
VvPGIP 47	92%	36.77 ± 3.24	10.2	1

The decrease in disease susceptibility was calculated by comparing the average lesion diameter at day 15 of all lines to that of the untransformed control

[a]Two independent whole plant infections were carried out on the mentioned lines. Significant differences and homogeneous groups were calculated by performing a one-way Analysis of Variance (ANOVA) using the STATISTICA 6 (StatSoft Inc, Tulsa, OK, USA) software package at 95% confidence intervals

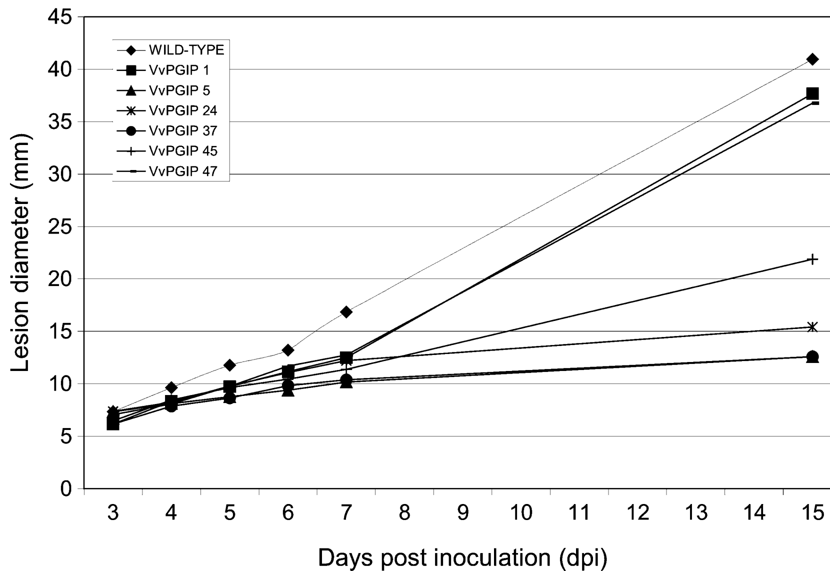


Fig. 4 Disease assessment of *Botrytis cinerea* infections on untransformed and VvPGIP1 transgenic tobacco lines at the whole-plant level. *B. cinerea* spores were inoculated on the adaxial surface of the leaves and the infection progression followed for a period of 15 days. Lesions were measured daily from day 3 to 7 and again at day 15. The mean lesion diameter of developing lesions at the indicated days post-inoculation of untransformed wild-type and transgenic lines are shown. One-way ANOVA indicated significant differences between mean lesion diameters of plant lines at 15 dpi. Lesion diameters and respective standard error at 15 dpi of all lines are indicated in Table 2

plants showed reductions in disease susceptibility ranging from 47 to 69% (Table 2) and lesions on these plants did not exhibit the same rapid expansion phenotype as those on wild-type plants (Fig. 4). Plants from group one showed high susceptibility to *B. cinerea* infection.

VvPGIP1 purified from transgenic tobacco differentially inhibits endo-polygalacturonases from *Aspergillus niger* (AnPGs) and *B. cinerea* (BcPGs)

To evaluate the inhibition of individual PGs from *A. niger* and *B. cinerea* by VvPGIP1, PGIP was purified from transgenic tobacco line 37. Eighty 0.5 ml fractions were collected from the final cation exchange chromatography step and tested for PGIP inhibitory activity against AnPGB (Fig. 5). Fractions containing PGIP inhibitory activity were further analysed with MALDI-TOF MS to assess the purity and integrity of the samples. All PGIP-containing fractions showed three peaks corresponding to 37, 40, and 42 kDa. A contaminating peak of about 24 kDa was observed in the fractions with the highest PGIP activity, but inhibition assays with fractions not containing this peak showed that it did not influence PGIP activity in any way (results not shown). The MALDI-TOF profile of fraction 45 (highest PGIP inhibitory activity) is shown in Fig. 6.

The activity of seven fungal PGs; AnPGA, AnPGB, AnPGII, BcPG1, BcPG3, BcPG4 and BcPG6 were assayed over a wide pH range in the presence or absence of the grapevine inhibitor. The enzymatic activity of the PGs tested displayed variable pH optimums on a homogalacturonan substrate using reducing sugar analysis (Fig. 7). The AnPGs A and B were most active at pH 4.2 and pH 5.5, respectively (Fig. 7a and b), whereas the AnPGII displayed optimum activity around pH 4.75 (Fig. 7c). BcPG1, BcPG3 and BcPG4 required a more acidic pH that ranged between 3.75 and 4.0 (Fig. 7d–f) for optimal activity while BcPG6 was most active in a less acidic environment with an optimum pH of 5.5 (Fig. 7g).

The addition of PGIP caused a significant reduction in activity of AnPGA and AnPGB, as well as BcPG1 and BcPG6, whereas the activity of BcPG3 was virtually unchanged by VvPGIP1

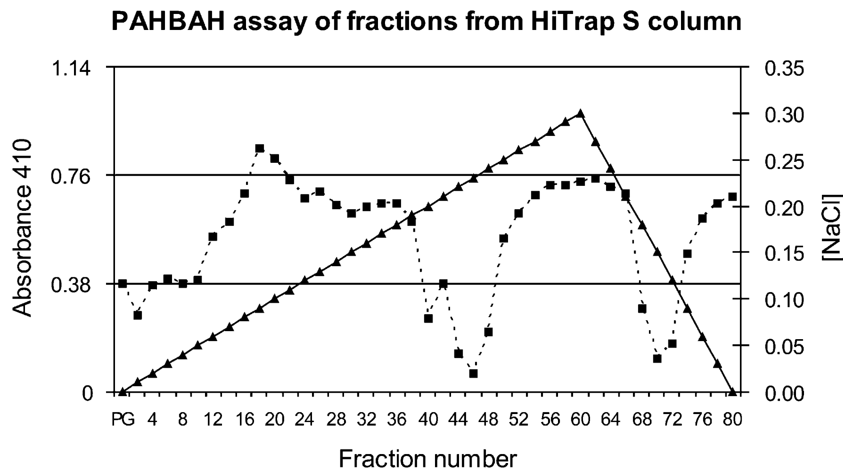


Fig. 5 Analysis of grapevine polygalacturonase-inhibiting protein (VvPGIP1) activity in 80 fractions collected from the final cation exchange chromatography step during the purification of VvPGIP1 from transgenic tobacco. Fractions were eluted with a NaCl gradient (solid line, ▲) and assayed against *Aspergillus niger* PGB (AnPGB). Inhibition was quantified by measuring the decrease in the release of reducing sugars spectrophotometrically at 410 nm, using the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York et al. 1985). The relative activity of AnPGB (as indicated by the A_{410} values) alone (PG) or in combination with the different fractions (numbers 2–80) is indicated on the graph (dotted line, ■). Every second fraction was assayed for PGIP activity

(Fig. 7). BcPG4 was only inhibited in the lower pH range (3.75–4.75), whereas AnPGII was not inhibited by VvPGIP1. The inhibition assays were repeated with fraction 69 (Fig. 4) which was less homogeneous, but did not contain the 24 kDa contaminating peak that fraction 45 contained. The inhibition data for this fraction did not differ significantly from fraction 45 (results not shown).

Discussion

In recent years, several studies have reported that polygalacturonase-inhibiting proteins (PGIPs) are effective in reducing the disease susceptibility of plants towards pathogens (De Lorenzo et al. 2001 and references therein). Apart from these transgenic over-expression studies, biochemical

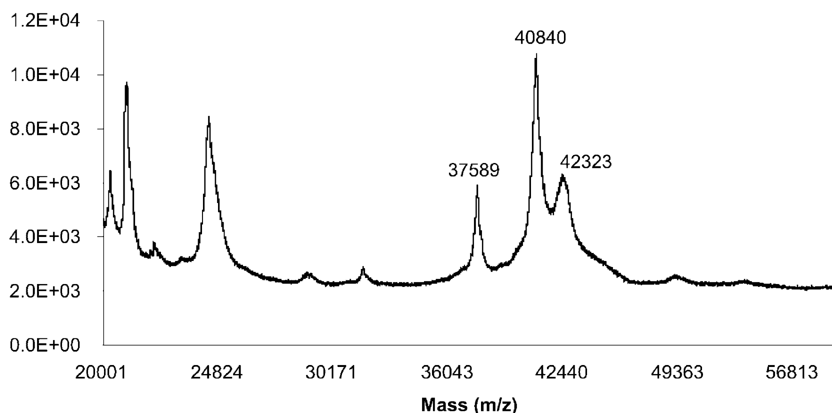
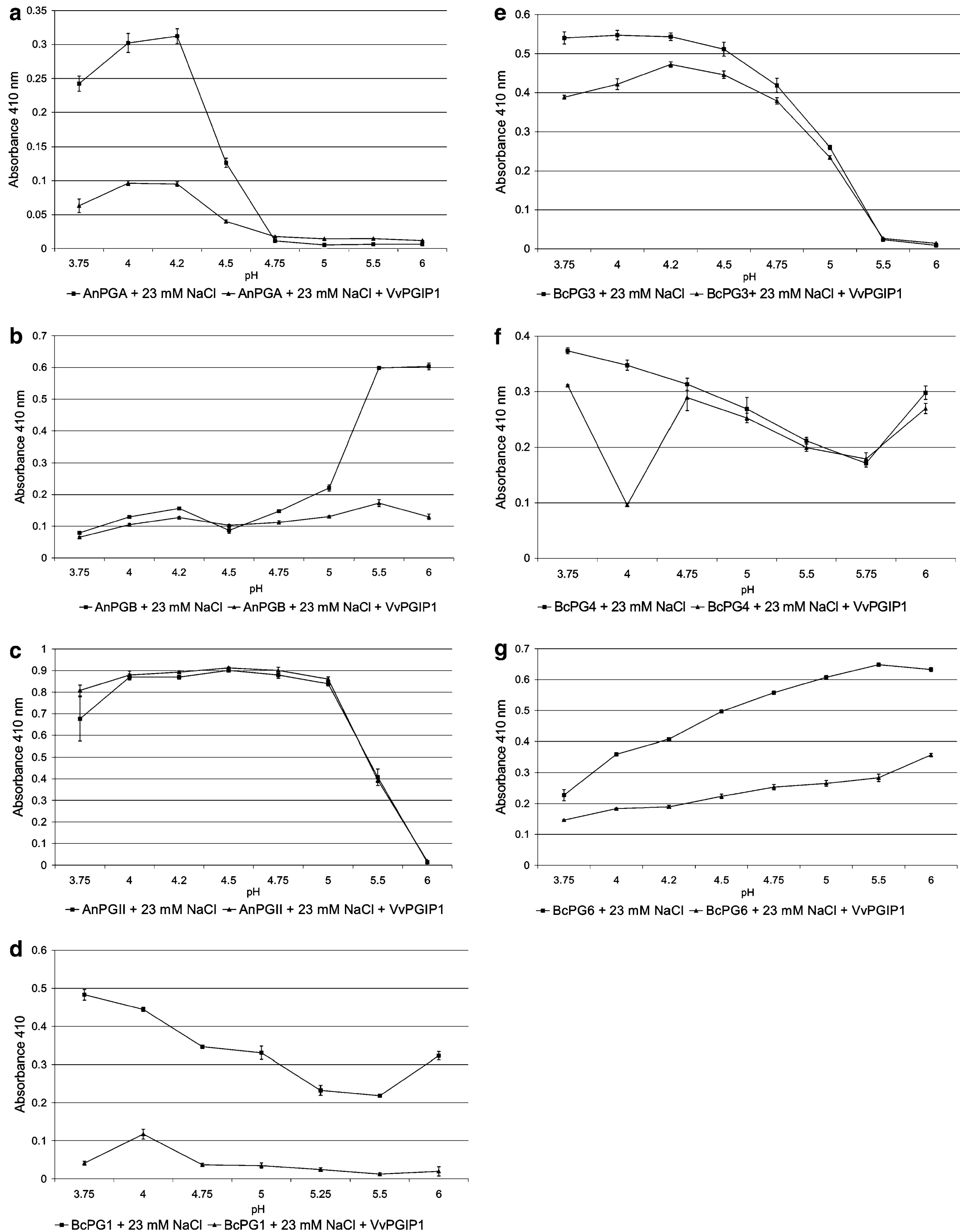


Fig. 6 Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) analysis of the fraction containing the highest PGIP activity eluted from the final cation exchange chromatography step during the purification of VvPGIP1 from leaves of transgenic tobacco. Samples were analysed on a Hewlett Packard G2025A MALDI-TOF mass spectrometer to assess the integrity and purity of PGIP containing fractions. The matrix used was a saturated sinapinic acid (3,5 dimethoxy-4-hydroxy-*trans*-cinnamic acid) solution in 50% acetonitrile. Equal volumes of sample and matrix (approximately 1 μl) were loaded onto the probe and dried under vacuum. The laser energy ranged from 10 to 14 μJ. VvPGIP1-containing fractions showed three peaks corresponding to 37, 40 and 42 kDa



analysis and protein–protein interaction data confirmed the direct inhibition of fungal polygalacturonases (PGs) by PGIPs. Moreover, *pgip* genes have been shown to be transcriptionally upregulated by pathogen infection and the presence of defence signaling molecules. Although a role for PGIPs in plant defence is well-established, more work is needed to explore their role(s) *in*

◀ **Fig. 7** The inhibition profile of VvPGIP1 purified from transgenic tobacco was determined against polygalacturonases (PGs) from *Aspergillus niger* and *Botrytis cinerea*. Inhibition was quantified by measuring the release of reducing sugars spectrophotometrically at 410 nm, using the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York et al. 1985) by fungal polygalacturonases (PGs) alone (■) or in combination with VvPGIP1 (▲). Assays were done at room temperature using the PAHBAH reagent across a wide pH range with 0.025% polygalacturonic acid as substrate. For the assays with PGs alone, NaCl was added to the same concentration as that of the fraction containing VvPGIP1 used in the combined assays. All assays were done in triplicate. (**a**–**c**) Reducing sugar (PAHBAH) assays of *Aspergillus niger* PGs A, B and II, respectively. (**d**–**g**) Reducing sugar (PAHBAH) assays of *Botrytis cinerea* PGs 1, 3, 4 and 6

planta in the progression and signaling of the defence response in plants following infection.

Powell et al. (2000) were the first to report that the heterologous expression of pear PGIP in tomato successfully conferred resistance against *B. cinerea* by targeting and inhibiting the tissue-macerating functions of the fungus. The availability of PG encoding genes, purified PGs, and data regarding the contribution of individual PGs to virulence and symptom development now facilitate studies to evaluate PGIPs more accurately and comprehensively.

A PGIP encoding gene has been isolated from grapevine and it was shown that grapevine has strong PGIP activity against a crude extract of PGs from *B. cinerea* (De Ascensao 2001). PGIPs in grapevine are strictly regulated, and expression has been found to be tissue specific, developmentally regulated and are inducible by *B. cinerea* infection amongst other factors (DA Joubert, G de Lorenzo, IS Pretorius and MA Vivier, unpublished data). In this study, the *pgip1* gene from *Vitis vinifera* cv Pinotage was over-expressed in tobacco plants, resulting in the accumulation of *Vvpgip1* transcripts in 19 individual transgenic tobacco lines. These lines were used to show using an in vitro plate assay, that the over-expression of this gene resulted in active PGIP in the plant extracts of the transgenic lines, causing inhibition of crude *B. cinerea* PGs (Table 1). A boiled plant extract sample did not show any PGIP activity, confirming that the PG inhibitions observed was not an artefact of the extraction procedure.

Having established that the grapevine PGIP gene is indeed active in the heterologous host, its ability to decrease disease susceptibility was first assessed with a detached leaf assay to screen all primary transformants accumulating VvPGIP1. At 3 dpi a clear reduction in lesion sizes was observed in the transgenic population compared to the untransformed control (Table 1 and Fig. 3). A very high spore load and high humidity conditions ensured very effective infection rates. From these detached leaf assays, reductions in disease susceptibility ranging from 30 to 61% were observed. These promising results were corroborated by whole plant infections conducted over 15 days on six of the transgenic lines, as well as on untransformed control plants. The whole plant infection assay was carried out on T1 progeny lines randomly chosen and yielded statistically significant separation of four of the six lines from the untransformed control. Successful *Botrytis* infections occurred with high percentages, with primary lesions developing at the inoculation sites within 3 dpi. The lines over-expressing VvPGIP1 showed a reduction in lesion size, spread and subsequent expansion of fungal mass over the 15-day evaluation period, with the most significant statistical differences being observed at 15 dpi. Calculated reductions in disease susceptibility, expressed relative to the untransformed control approached 70% in the least susceptible lines in this analysis. The detached leaf assay and the whole plant infections confirmed that the VvPGIP1 can influence plant defence positively, presumably through the observed inhibitory activity towards *B. cinerea* PGs. Further investigation is ongoing to clarify the susceptible phenotype of transgenic lines 1 and 47.

The *Vvpgip1* gene was isolated from *V. vinifera* cv Pinotage and as with most of the cultivars within *V. vinifera*, it has rather poor resistance to most fungal pathogens. Our results of the grapevine PGIP in transgenic tobacco indicate it to be an effective antifungal gene, but in the native genetic background it clearly does not act with the same efficiency. During grape berry maturation an accumulation of numerous developmentally regulated defence-associated proteins occur up to *véraison*, and the green berries are quite resistant to fungal pathogens. Some of the most prominent

defence-proteins in the pre-*véraison* stage include acidic chitinases, thaumatin-like proteins and lipid-transfer proteins (Salzman et al. 1998; Davies and Robinson 2000); grapevine *pgip* is also strongly expressed at *véraison* (DA Joubert, G de Lorenzo, IS Pretorius and MA Vivier, unpublished data). During the post-*véraison* stages (comprising ripening-related events such as accumulation of sugars and pigments as well as softening of cell walls) most of these genes, including *pgip*, are down regulated and the berries also become highly susceptible to fungal infections. It has been speculated that the berries remain resistant to fungal attack until the seeds are fully formed (*véraison*-stage) and that from an evolutionary perspective there is no need (for the plant) after that to protect the berries. Grapevine susceptibility to *B. cinerea* should therefore not primarily be used to gauge the efficiency of the grapevine PGIP as a possible antifungal agent.

During fungal infection, it is thought that spreading lesion development and the expansion of fungal biomass is dependent on the expression and secretion of PGs (ten Have et al. 2002; Kars et al. 2005). *Botrytis* has been shown to express at least six PGs (encoded by *Bcpg1-6*) during the infection process (ten Have et al. 2001). BcPG1 and 2 have been shown to be important pathogenicity factors (ten Have et al. 1998; Kars et al. 2005).

Tobacco transgenic lines were also used to purify VvPGIP1; this provided a mechanism to link a specific PG inhibition profile to the *Vvpgip1* encoded PGIP. PGIP purified from grape berries showed inhibition against PGs from several fungal pathogens (De Ascensao 2001), but the inhibition profile of individual grapevine PGIPs has not been determined yet. Leaf material from line 37 was used in a purification protocol that yielded several active fractions; these were confirmed to contain polypeptides of the expected sizes corresponding to previous data regarding the glycoforms of the PGIP purified from grapevine berries (De Ascensao 2001). The heterologously produced and purified VvPGIP1 were also used to evaluate the interaction and inhibition of this inhibitor towards individual PGs from *B. cinerea* and *Aspergillus niger*. These PGs have earlier been shown to be homogeneous (Kemp et al. 2004; Krooshof et al. 2004; Kars et al. 2005). Differential inhibition was observed for PGs from both *A. niger* and *B. cinerea*. VvPGIP strongly inhibited BcPG1 and 6 under all conditions tested, whereas inhibition towards BcPG3 was less pronounced. The inhibition of BcPG4 was very pH dependent, as was the inhibition for AnPGA and B. Unlike the results of Kemp et al. (2004) for a PGIP from *Phaseolus vulgaris* (PvPGIP2), PG activation could not be detected.

These inhibition assays confirmed the ability of VvPGIP1 to inhibit fungal PGs. The observed decrease in *Botrytis* symptom development in tobacco lines over-expressing VvPGIP1, could therefore be due to the inhibition of the fungal PGs, specifically the strong inhibition of BcPG1. BcPG1 has been shown to be essential for pathogen virulence and causes tissue maceration, chlorosis and necrosis when applied in purified form to tobacco plants (Kars et al. 2005). The ability of PGIP to inhibit fungal PGs in vitro suggests that the *in planta* role of PGIPs includes protection of the plant cell walls by inhibiting pathogen PG action.

In conclusion, the over-expression of a grapevine PGIP encoding gene in tobacco and the subsequent purification of the inhibitor confirmed that this gene encodes a PGIP with strong activity against *Botrytis* PGs. Moreover, the VvPGIP1 differentially inhibits individual PGs from *B. cinerea*, as well as *A. niger* and this data provide one of the first reports of detailed inhibition studies of a PGIP and the individual PGs produced by a pathogen. Transgenic tobacco plants expressing VvPGIP1 displayed reduced symptom development when inoculated with *Botrytis*, suggesting that the in vitro PG inhibition by this PGIP also occurred *in planta*, at least in part contributing to the observed reduced disease symptoms. This study provides a strong base to study the currently available and other potential grapevine PGIPs further, to identify inhibitors with specialised PG inhibition profiles and to also study and understand the *in planta* roles of PGIPs in disease responses and signalling.

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