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Bean polygalacturonase inhibitor protein-1 (PGIP-1) inhibits polygalacturonases from *Stenocarpella maydis*

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Stenocarpella maydis, a fungal pathogen of maize, produced polygalacturonases (PGs) when grown on pectin or maize cell walls. An extract from bean (*Phaseolus vulgaris* L.) which contained an active inhibitor of *Aspergillus niger* PG, also inhibited *S. maydis* PG in a reducing sugar assay. Bean polygalacturonase inhibitor protein (PGIP) was purified from the extract by affinity chromatography, and this inhibited the *S. maydis* PG in reducing sugar assays and an overlay gel activity assay. Inhibition was abolished by boiling of the PGIP. Since purified PGIP could still be a mixture of PGIPs with similar physical properties but different inhibitory activities on different fungal PGs, the bean *pgip-1* gene was cloned and expressed in transgenic tomato. PGIP extracts from the transgenic tomato inhibited both *A. niger* and *S. maydis* PG, whereas extracts from a control untransformed tomato did not. This indicated that bean PGIP-1 is able to inhibit the *S. maydis* PGs.
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Keywords: *Stenocarpella maydis*; *Phaseolus vulgaris*; polygalacturonase; inhibitor; PGIP.

INTRODUCTION

Stenocarpella maydis (Berk.) Sutton [=*Diplodia maydis*] causes both ear and stalk rot on maize (*Zea mays* L.). It is a necrotrophic fungus that has been reported on an epidemic scale in certain seasons in South Africa [45] and is a localised problem in the U.S.A. [23]. Control of the disease through breeding for resistance and cultural practises has received attention for many years [23, 28, 45]. However, very little is known about the biochemical mechanisms of pathogenicity in *S. maydis*.

S. maydis colonises maize stalk, leaf and shank tissues by direct penetration of epidermal cells, which involves enzymatic degradation [5]. Ear infection is commonly the result of initial fungal infection of the shank, from where it moves up into the ear. Many pathogenic fungi produce polygalacturonase enzymes at early stages of infection to degrade the pectin component of plant cell walls [29]. Pectin is a complex polysaccharide, which is broken down by a suite of enzymes, including polygalacturonases with *endo*- and *exo*-modes of action, pectate lyases, pectin lyases and pectin methylesterases [16]. In necrotrophic fungi, it is the *endo*polygalacturonases that are largely responsible for the large-scale maceration of plant cells, whereby galacturonides are released to provide a source of nutrition for the invading fungus [17, 18].

Polygalacturonase-inhibiting proteins (PGIPs) have been identified in the cell walls of many plant species, including bean, soybean, tomato, pear, apple, raspberry, onion and leek [3, 19, 25–27, 31, 41, 42, 47]. Plant PGIPs interact with *endo*polygalacturonases from fungi, but do not appear to have an effect on those of bacterial or plant origin [13]. Certain breakdown products of the plant cell wall, including oligogalacturonides with a degree of polymerization (DP) of 10–15, are elicitors of plant defense responses [29]. Furthermore, PGIP has been shown to increase the accumulation of oligogalacturonides *in vitro*. This led to the current hypothesis that PGIPs contribute to the defense response by modulating the activity of fungal PGs to release elicitor active oligogalacturonides, thus delaying the rapid degradation of pectin to inactive monomers [14].

Recent work has shown that in some species PGIPs are expressed predominantly in fruit tissues, such as tomato, apple, and raspberry [32, 41, 46], whereas in others such as bean it is expressed in vegetative and other tissues [22, 38]. Furthermore, each species appears to have multiple PGIPs which have different specificities against different PGs [21]. For example, bean has at least five PGIPs and detailed characterization of two members, PGIP-1 and

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PGIP-2, has shown that both are effective against *Aspergillus niger* PG, whereas only PGIP-2 is effective against *Fusarium moniliforme* PG [34].

PGIPs have been identified only in dicots and the non-graminaceous monocots (*Allium* sp.), which have pectin-rich cell walls. However, it appears that PGIPs from various plants show inhibitory activity against PGs from fungi that the plant was not exposed to during evolution. For example, the bean PGIP-2 interacts strongly with a PG from a pathogen of maize, *F. moniliforme* [34]. The aim of this study was to investigate PG production of another maize pathogen, namely *S. maydis*, and determine if its activity was inhibited by bean PGIP.

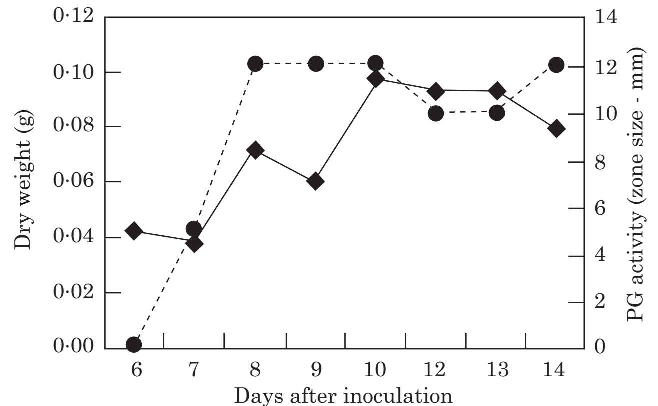

FIG. 1. Growth of *Stenocarpella maydis* cultured on Fries medium with pectin as the sole carbon source measured as mycelial dry weight (◆). Polygalacturonase (PG) activity (●) was assessed using the agarose diffusion assay.

MATERIALS AND METHODS

Fungal cultures

Stenocarpella maydis (Berk.) Sutton [=*Diplodia maydis*] was isolated from an infected maize cob collected in the Potchefstroom region of South Africa. The culture was stored as isolate PPRI No. 6353 in the culture collection of the ARC-Plant Protection Research Institute, Pretoria, South Africa. *Aspergillus niger* was isolated from onion seed collected in the Caledon area of South Africa (stored as PPRI No. 5017). Seed of French bean *Phaseolus vulgaris* L. (cv. Wintergreen) was obtained from the ARC-Roodeplaat vegetable genebank collection.

Growth of fungi for preparation of PG extracts

S. maydis was grown in Fries medium [7] in which the carbon source (sucrose/glucose) was replaced by either 0·5 % (w/v) pectin or 0·5 % (w/v) cell wall extract prepared from maize leaves by the method of English *et al.* [24]. The culture was grown at 23 °C in the dark with shaking at 125 r.p.m. for 14 days. One flask was harvested each day from day 3 to 14 by suction filtration through a Whatman No. 113 disc. The mycelia collected on the filter disc were dried and the dry weight was determined (Fig. 1). The filtrates, which contained the extracellular polygalacturonase (PG) enzymes, were passed through 0·22 μm syringe filter units (Millipore, U.S.A.), and stored at 4 °C before screening for PG activity using the agarose diffusion assay [43] (Fig. 1). Filtrates which showed the highest PG activity were subjected to a 0–80 % ammonium sulphate treatment to collect the PG proteins, which were subsequently suspended in one tenth the original volume with 40 mM sodium acetate buffer, pH 5. This step removed the pectin present in the supernatant, which would have interfered with the subsequent reducing sugar assays. The PG extracts were checked for recovery of PG activity using the agarose diffusion assay.

The *A. niger* PG extracts were prepared in the same way, except that the fungus was grown in a citrate : phosphate buffer (pH 6.0) containing the following final concentrations: 1 % pectin (w/v); 500 μg ml^{-1} ampicillin; 2 mM $MgSO_4 \cdot 7H_2O$; 0·4 μM $MnSO_4 \cdot 7H_2O$; 25 mM KNO_3; 3·4 μM $ZnSO_4 \cdot 7H_2O$; 0·6 μM $CuSO_4$ and 3·5 μM $FeSO_4$. *A. niger* growth increased in a linear manner up to day 10, whereas extracellular PG activity plateaued very early during growth at day 3 (data not shown). PG activity produced on day 7 was collected by ammonium sulphate precipitation. Only 28 % of this PG activity was inhibited by the bean PGIP extract (data not shown). An explanation for this is that there may be a mixture of PGs in the *A. niger* supernatant, some of which are not inhibited by bean PGIP. Consequently, pure *A. niger* PG was used as a control in further experiments.

Reducing sugar assay for PG activity

Release of reducing sugars was measured by the PAHBAH procedure [48] adapted from Salvi *et al.* [38]. Polygalacturonase enzyme activity was expressed as μmoles reducing ends released per min at 30 °C with 0·25 % polygalacturonic acid as substrate. Regression analysis showed that there was linear increase in release of reducing sugars by the *S. maydis* PG from 0 to 80 min, and that the fitted line accounted for 94·9 % of the variance in the data (Fig. 2). The activity plateaued after 80 min incubation. This data enabled selection of the 60 min time point, which was in the linear range of activity, for PGIP inhibition studies. *A. niger* PG (Sigma No. P3429: 0·29 mg protein ml^{-1} and 2520 units mg^{-1} protein) was used at dilutions of 1 : 750 and 1 : 1500 when the assay was performed over 30 or 60 min time periods, respectively (Fig. 2). When bovine serum albumin (BSA, 20 mg ml^{-1} in 50 mM Tris, pH 7·5, Boehringer Mannheim) was

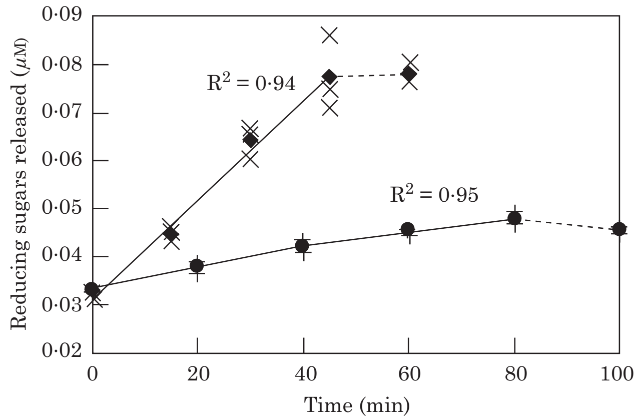

FIG. 2. Determination of time points at which *S. maydis* PG (6 μg) or pure *Aspergillus niger* PG (9 ng; Sigma) exhibit a linear increase in activity in the reducing sugar assay. *S. maydis* PG activity is represented by the mean values (●) of three replicate reactions (+), which were subjected to regression analysis to obtain the fitted thick line ($R^2 = 0{\cdot}95$). *A. niger* PG activity is represented by the mean values (◆) of three replicate reactions (×), which were subjected to regression analysis to obtain the fitted thin line ($R^2 = 0{\cdot}94$).

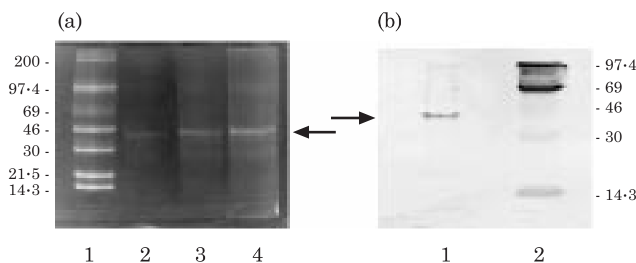

FIG. 3. SDS–PAGE gel (a) and Western blot analysis (b) of purified bean PGIP. (a) SDS–PAGE gel was stained with Coomassie blue. Lane 1, Rainbow protein markers (Amersham, U.K.), molecular weights in kDa are indicated. Lanes 2–4 contain 1, 2, and 4 μg of bean PGIP, respectively. (b) Western blot of purified PGIP (lane 1) probed with antiserum raised to a PGIP peptide. Lane 2 contains the same molecular weight markers as those in (a), lane 1. The arrows indicate the position of the PGIP protein.

required in excess, it was added at a final concentration of 100 μg ml^{-1}. Phenylmethylsulfonyl fluoride (PMSF) was added at a final concentration of 0·2 mM. PGIP inhibition studies were carried out by first mixing the PG with an equal volume of the purified bean PGIP or PGIP extracts from bean or transgenic tomato, and incubation for 20 min at 25°C before the assay. Then the PG : PGIP reactions were mixed with the PGA substrate and incubation was continued at 30°C for the appropriate time period. When necessary, PGIP extracts were boiled for 30 min prior to use. Protein concentrations were determined using the method of Bradford [*8*]. Statistical analysis was carried out using the software Genstat 5 (1993 release, Genstat 5 committee of the Statistics Department, IACR-Rothamsted, U.K., Clarendon Press: Oxford).

Preparation of PGIP extracts from bean and tomato

PGIP was isolated from bean hypocotyls as described [*38*], except that 2 days prior to harvesting the bean shoots were sprayed once a day with 50 mM salicylic acid, which has been shown to induce PGIP synthesis [*6*]. PGIP extracts were made from leaves of the transgenic and untransformed control tomato as described [21].

Purification of the PGIP from bean hypocotyls

PGIP was purified from bean hypocotyls using an adaptation of the method of Cervone *et al.* [*12*]. The PGIP extract, induced with 50 mM sodium salicylate, was concentrated by 0–90 % ammonium sulphate precipitation, dialysed against 20 mM sodium acetate buffer pH 5·0 (buffer A) and purified by affinity chromatography. The *A. niger* PG affinity matrix was prepared by coupling CNBr-activated Sepharose 4B to the ligand, 6 mg homogeneous *A. niger* PG (Sigma), according to the procedure recommended by the manufacturer (Pharmacia). PGIP extracts were applied to the column (1·6 × 10 cm) in buffer A at a flow rate of 20 ml h^{-1} and unbound proteins were eluted with buffer A. Bound PGIP was desorbed with phosphate buffered saline (PBS, 10 mM sodium phosphate buffer containing 0·13 M NaCl, pH 7·3). The PGIP fractions were pooled and concentrated against powdered sucrose using dialysis tubing with a cut-off of $M_r = 12$ kDa. Minor contaminants were separated from PGIP by centrifugal ultrafiltration through a 30 kDa membrane. The average yield was 1 mg PGIP purified from 100 g hypocotyls. The protein was electrophoretically homogeneous with a M_r of 40 kDa [Fig. 3(a)].

Polygalacturonase overlay gels

The overlay gels were performed as described [*33*]. Isoelectric focusing of the *S. maydis* PGs was carried out in a 5 % polyacrylamide gel with ampholytes in the pH 3–10 range. The overlay gels consisted of 0·8 % agarose on gelbond support film and the substrate was 0·1 % polygalacturonate in 50 mM sodium acetate pH 5·0 as buffer. When necessary, the purified bean PGIP was incorporated in the overlay gel at a final concentration of 0·425 mg ml^{-1}.

Western blotting method

Proteins were separated by electrophoresis on a 12 % SDS–PAGE gel using the Mighty small SE250 apparatus

(Hoefer Scientific Instruments) and then electroblotted at 30 V overnight onto a Hybond C membrane (Amersham) using a Hoefer TE series Transphor apparatus. The PGIP antiserum was the same as that used by Bergmann *et al*. [6], and was raised in rabbits against a peptide corresponding to amino acids 10–21 of mature bean PGIP-1. It was used at a dilution of 1/700. The second antibody used was a goat anti-rabbit IgG-alkaline phosphatase conjugate diluted 1/1000 (Sigma). Detection was carried out colourimetrically. The substrates for alkaline phosphatase were NBT (nitroblue tetrazolium salt) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, toludinium salt) (Boehringer Mannheim).

Isolation of bean pgip-1 *gene*

Bean plants (*Phaseolus vulgaris* L. cv. Wintergreen) were grown from seed in a Phytotron with a 15 h:9 h cycle of light (33°C):dark (18°C). The plants were left in the dark for 3 days prior to DNA isolation to reduce the polysaccharide content. Genomic DNA was isolated by the method of Dellaporta *et al*. [20] and yields obtained were 55 μg DNA g^{-1} leaves (dry weight). PCR amplification of the bean *pgip-1* gene was carried out using 30 ng bean genomic DNA in 10 μl aliquots in 0·2 ml thin-walled tubes, and the primers $PGIP_L$ (5′GC-**TCTAGA**-ATGACTCAATTCAATATCCCAG-3′), which contains an ***Xba*I** site and $PGIP_R$ (5′-GCAC-**GAGCTC**-TTAAGTGCAGGAAGGAAG-3′), which contains a ***Sac*I** site. PCR was conducted in a MJ Research Minicycler with the following final concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 9·0 at 25°C), 0·1% Triton X-100, 1·5 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP, dTTP, 0·5 μM of each primer and 0·5 U *Taq* DNA polymerase (Promega, Madison, U.S.A.). PCR cycle conditions were as follows: initial denaturation of 94°C for 90 s followed by 34 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 45 s, followed by a final extension at 72°C for 3 min. The 1047 bp *pgip-1* PCR product was digested with *Xba*I and *Sac*I, eluted from a 0·9% SeaPlaque agarose gel using β-agarase (FMC Bioproducts, U.S.A.), and cloned into the plasmid pBI221 (Clontech, CA, U.S.A.) digested with *Xba*I and *Sac*I to produce pLD1. Competent cells of the *Escherichia coli* strain DH5α were prepared as described [15].

DNA sequencing of the bean pgip-1 *gene*

DNA sequencing of the insert in pLD1 was carried out manually by the dideoxy chain termination method using a Sequenase kit (USB Corp., U.S.A.) and [α-^{35}S]dATP (1200 ci $mmol^{-1}$). Plasmid templates for sequencing were prepared by ultracentrifugation in a CsCl gradient. Sequence analyses were done using the computer software *Genepro Version 6.10* (Riverside Scientific Enterprises, WA, U.S.A.). Sequence templates were overlapping subclones of pLD1 obtained by exonuclease III shortening [30] or subcloning. The sequence was identical to that of the genomic *pgip-1* clone from the bean cultivar Saxa [44] (GenBank accession No. X64769).

Cloning of the bean pgip-1 *gene into the plant transformation vector*

Plasmid pLD1 was digested with *Xba*I, and the ends were filled-in with Klenow-enzyme. The plasmid was digested further with *Sac*I to release a fragment of approx. 1 kb containing *pgip-1*. This was inserted into the vector pRTL2 [37] which had been digested with *Nco*I (filled-in with Klenow-enzyme) and *Sac*I, to produce pRTL2-pgip. The cloning junction point at the 5′end of the *pgip-1* gene was checked by sequencing. This placed *pgip-1* under control of the enhanced CaMV 35S promoter and Tobacco Etch Virus (TEV) leader element, which is a translational enhancer, and the 35S terminator [37]. The cassette containing the enhanced 35S promoter-TEV leader-*pgip1*-35S terminator was then subcloned as a *Hind*III fragment into the *Hind*III site situated between the T-DNA borders of the binary vector pGA482 [2] to produce pGA482-pgip. This step required a partial *Hind*III digest of pRTL2-pgip, since there is a *Hind*III site within the bean *pgip-1*.

Tomato transformation

Plasmid pGA482-pgip was transferred to *Agrobacterium tumefaciens* LBA4404 by triparental mating [4], and this was used for Agrobacterium-mediated transformation of cotyledons of tomato (*Lycopersicon esculentum* L. Mill) cultivar UC82B [35]. Transgenic plants were selected on kanamycin and hardened off in the greenhouse, together with control untransformed plants. Leaves of greenhouse grown plants served as a source of PGIP extracts for the assays.

RESULTS

Stenocarpella maydis *produces polygalacturonase activity when grown on different carbon sources*

Stenocarpella maydis produced polygalacturonase (PG) activity when grown in liquid culture on pectin as a sole carbon source. Extracellular PG activity reached a plateau after 8 days growth, which was 2 days before mycelial growth was maximum (Fig. 1). Enzyme activity was assessed using an agarose diffusion assay [43]. Growth on an alternative pectin source, namely a maize cell wall extract, resulted in a two-fold greater mycelial dry mass. However, PG activity was only

observed at day 8, 2 days after mycelial growth had plateaued (data not shown).

The PG activity produced at day 10 was separated from the pectin in the growth medium by ammonium sulphate precipitation. Recovery of enzyme activity was tested using the agarose diffusion assay (data not shown), and then quantified using a reducing sugar assay in preparation for PGIP inhibition studies (Fig. 2).

Inhibition of the S. maydis *PG by the bean PGIP extract*

An extract containing polygalacturonase inhibiting protein (PGIP) was made from *Phaseolus vulgaris* L. (French bean cv. Wintergreen) hypocotyls. This extract was tested using a control PG, a pure preparation from *A. niger* (Fig. 2). Using the reducing sugar assay, the extract was shown to contain an active PG inhibitor, which inhibited 97 % of the *A. niger* PG activity in a statistically significant manner (data not shown). Furthermore, inhibition was heat denaturable and not affected by excess BSA or the protease inhibitor PMSF (data not shown). This bean PGIP extract inhibited 66 % of the *S. maydis* PG activity present in the fungal culture supernatant when tested in the reducing sugar assay (Table 1). The bean PGIP extract also inhibited the *S. maydis* PG in the presence of excess BSA (83 % inhibition; Table 1).

Inhibition of the S. maydis *PG by the purified bean PGIP*

The bean PGIP was purified from bean hypocotyls to yield a protein of approx. 40 kDa on SDS–PAGE [Fig. 3(a)]. This was shown to be PGIP by Western blot with antiserum directed against a surface-exposed peptide of bean PGIP-1 [Fig. 3(b)] [*6*]. This preparation contained active PGIP since it inhibited the purified *A. niger* PG in the reducing sugar assay, and the inhibition increased with increasing concentrations of PGIP (Fig. 4).

Consistent with data obtained with the PGIP extract, the purified bean PGIP inhibited 66 % of the *S. maydis* PG activity when it was grown in pectin as a sole carbon source (Fig. 5), and 92 % of the *S. maydis* PG activity when it was grown on a maize cell wall extract (Fig. 5). Inhibition of both sources of PG activity was removed by boiling of the purified PGIP (Fig. 5).

Inhibition of S. maydis *PG isozymes in overlay activity gels*

S. maydis PG extract derived from the culture grown on pectin was subjected to isoelectric focusing (IEF). IEF was conducted in a polyacrylamide gel with the pH range from 3 to 10. PG isozymes were detected by staining for activity in an overlay placed on top of the IEF gel after electrophoresis. The overlay contained the substrate polygalacturonic acid, which would be degraded by PGs that diffused into the overlay. Activity was visualized as bands of clearing in the overlay after staining with

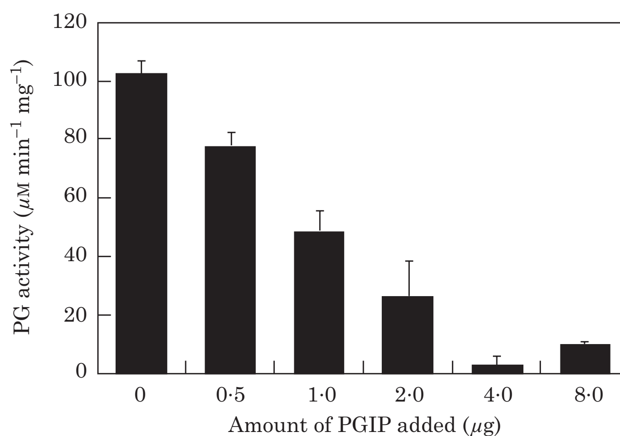

FIG. 4. Inhibition of the *A. niger* PG increases with the addition of increasing amounts of purified bean PGIP. Bean PGIP at different dilutions was mixed with *A. niger* PG (12 ng) for 20 min at 25°C prior to addition of the substrate PGA and incubation for 30 min at 30°C. The amount of reducing sugars released was assessed using the PAHBAH method. Values are the means of three separate reactions and standard deviations are indicated.

TABLE 1. *Inhibition of* Stenocarpella maydis *polygalacturonases by bean PGIP extract*

S. maydis PG	Addition‡	PGIP extract†	PG activity* (nmoles min^{-1} mg^{-1})	Inhibition (%)
+	–	–	25 ± 2	–
+	–	+	11 ± 3	66
+	BSA	–	18 ± 4	–
+	BSA	+	3 ± 2	83

* PG activity was determined by the reducing sugar assay and is shown as the means of five separate reactions and is representative for two separate experiments. Standard deviations are shown. The mean PG activities (without BSA) were significantly different from one another (one-way ANOVA, residual degrees of freedom = 8, $P < 0{\cdot}001$). The mean PG activities (with BSA) were significantly different from one another (one-way ANOVA, residual degrees of freedom = 8, $P < 0{\cdot}001$).

† Bean PGIP extract (5 μg) was mixed with *S. maydis* PG (9 μg) for 20 min at 25°C prior to addition of the substrate PGA, and incubation at 30°C for a further 60 min.

‡ BSA was added at a final concentration of 100 μg ml^{-1}.

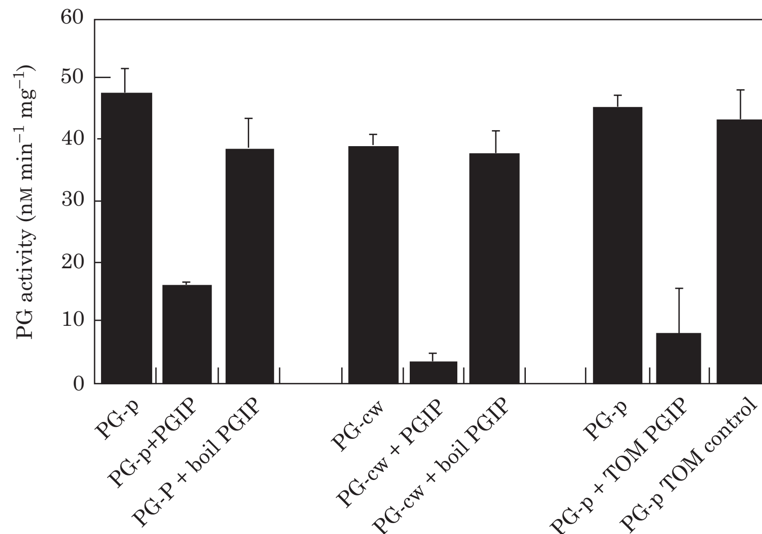

FIG. 5. Inhibition of *S. maydis* PG by purified bean PGIP, and PGIP extract from tomato transformed with the bean *pgip-1* gene. *S. maydis* PGs were produced from growth on either pectin (PG-p) or cell walls (PG-cw). PG-p (9 μg) or PG-cw (11 μg) were mixed with purified bean PGIP (9 μg) where indicated. In a separate experiment, Pg-p (5 μg) was mixed with a PGIP extract from leaves of a tomato transformed with the bean *pgip-1* gene (TOM PGIP; 0·5 μg), or an extract from leaves of an untransformed control tomato (TOM control; 0·5 μg). For each reaction, the PG was mixed with the PGIP for 20 min at 25°C prior to addition of the substrate PGA and incubation for 60 min at 30°C. The amount of reducing sugars released was assessed using the PAHBAH method. Values are the means of three separate reactions and standard deviations are indicated.

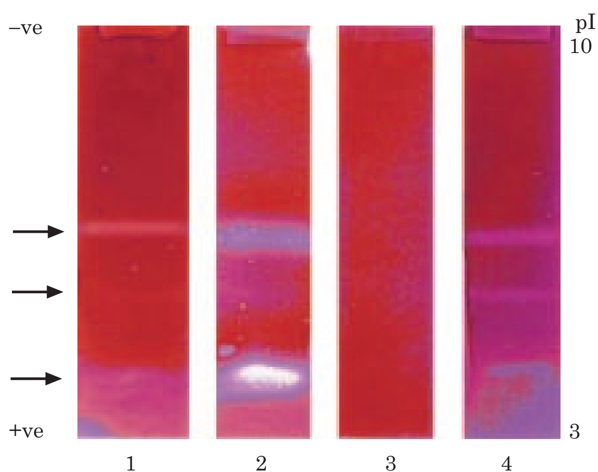

FIG. 6. Effect of bean PGIP on *S. maydis* PG isozymes in an overlay gel assay. PG isozymes were separated by isoelectric focusing and activity was determined by placing on top an overlay gel containing 0·25 % PGA substrate for 2 h (lane 1) or 17 h (lane 2). PG activity bands were detected by staining with ruthenium red. Lanes 3 and 4 contain bean PGIP or boiled bean PGIP incorporated into the overlay gel. The arrows indicate the three activity bands of PG activity with approximate pIs of 4, 5·5 and 7.

ruthenium red, which reacts with unhydrolysed substrate. Two major bands of PG activity were detected corresponding to isozymes with approximate pIs of 4 and 7 (Fig. 6, lane 1). These two activity bands were more pronounced when the overlay was exposed to the IEF gel for longer (17 h), and an additional activity band became apparent (pI of 5·5) (Fig. 6, lane 2). The purified bean PGIP was incorporated into the overlay together with the substrate, and this completely inhibited PG activity of all three *S. maydis* PG isozymes (Fig. 6, lane 3). The effective PG : PGIP ratio in these experiments was approximately 1 : 100, assuming that there are equal amounts of each of the three isozymes in the extract. Furthermore, this inhibition was shown to be due to active PGIP, since PG activity was recovered when boiled PGIP was incorporated into the overlay (Fig. 6, lane 4).

Isolation of the bean pgip-1 *gene and insertion into a plant transformation and expression vector*

The biochemical evidence indicated that bean PGIP was effective against *S. maydis*. However, it had recently been reported that extracts or purified PGIP samples from bean may contain mixtures of PGIP proteins [*21*, *36*]. These may be difficult to separate biochemically, but have different specificities against fungal PGs. On account of this, a bean *pgip* gene was cloned with the aim of expressing it independently in transgenic plants to determine its specificity against the *S. maydis* PGs.

The *pgip-1* gene was PCR amplified from genomic DNA of bean, and sub-cloned under control of a constitutive promoter into a binary vector for plant transformation. This was used to produce transgenic tomato by Agrobacterium-mediated transformation and one event, named PGIP3, rooted on selection medium and

Table 2. *Tomato transformed with the bean* pgip-1 *gene expresses an inhibitor of* Aspergillus niger *polygalacturonase*

A. niger PG	PGIP extract†	PG activity* (μmoles min^{-1} mg^{-1})	Inhibition (%)
+	–	85 ± 13	–
+	Bean	4 ± 3	95
+	Transgenic tomato	24 ± 6	72
+	Control tomato	135 ± 4	0
+	Transgenic tomato (boiled)	102 ± 6	0
+	Control tomato (boiled)	167 ± 2	0

* PG activity was determined by the reducing sugar assay and is shown as the means of three separate reactions and is representative for two separate experiments. Standard deviations are shown. The mean PG activities for all treatments are significantly different from each other [one-way ANOVA, least significant difference (1 %) = 16, residual degrees of freedom = 12, $P < 0{\cdot}001$].

† *A. niger* PG (6 ng) was mixed with either bean PGIP extract (3 μg), or an extract from transgenic tomato PGIP-3 transformed with the bean *pgip1* gene (2 μg), or an extract from an untransformed control tomato (2 μg). Where indicated, the extracts had been boiled for 30 min and cooled prior to mixing with the PG. 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.

was hardened off in the glasshouse. This plant was phenotypically normal, flowered and produced T1 seed.

Bean pgip-1 *expressed in transgenic tomato inhibits* A. niger *and* S. maydis *polygalacturonases*

PGIP extracts were made from leaves of transgenic tomato PGIP3, containing the bean *pgip-1* gene, and from an untransformed control tomato plant. Reducing sugar assays showed that extracts from the transgenic tomato plant contained an active PGIP, which inhibited the PG activity of *A. niger* by 72 % (Table 2), whereas an extract containing the same amount of total protein from leaves of an untransformed control plant did not inhibit the *A. niger* PG (Table 2). The PGIP activity in the transgenic tomato extract was abolished by boiling (Table 2).

The bean PGIP-1 extract from the transgenic tomato PGIP3 also inhibited 80 % of the *S. maydis* PG activity, whereas the control tomato extract had no effect on the PG activity (Fig. 5). Based on total proteins within the extracts, the PG was calculated to be in two-fold excess to the PGIP in the tomato extract, although the ratio of PG:PGIP in this experiment was difficult to quantify since it is a PG extract containing at least three isozymes.

DISCUSSION

Polygalacturonase activity was readily obtained from *S. maydis* cultures grown in liquid medium with pectin as a sole carbon source, or on maize cell walls, which is a better reflection of the *in vivo* environment. This indicates that this class of enzymes may play a role in the biochemical armoury of *S. maydis* during infection of maize. *F. moniliforme*, another necrotrophic pathogen of maize, produces four forms of an *endo*polygalacturonase when grown on pectin [9]. These are all derived from the same gene, but subjected to differential glycosylation by post-translational modification [11]. In another fungal pathogen of maize, *Cochliobolus carbonum*, knockout mutagenesis of an *endo*polygalacturonase gene (*png1*) did not have an effect on pathogenicity [40]. This was initially interpreted as evidence that *endo*polygalacturonase was not required. However, a subsequent study indicated that there was functional redundancy of these enzymes, thereby enabling the fungus to reprogramme its physiology to grow on pectin and retain pathogenicity [39]. Future studies of the expression of these fungal enzymes *in planta* would provide some answers as to the role of polygalacturonases in pathogenicity.

Bean PGIP was initially considered to have broad-spectrum activity against polygalacturonases from a range of fungi, including *Colletotrichum lindemuthanium*, *Fusarium oxysporum*, *F. moniliforme*, and two species of Aspergillus [1, 13]. However, recent data showed that preparations of "purified" bean PGIP could contain mixtures of PGIP proteins with very similar physical properties, but different inhibitory activities against different PGs [21]. For example, two species of PGIP, designated PGIP-I and PGIP-II, were isolated from bean pods after extensive purification steps [36].

In this study, a variety of biochemical assays were used to determine if PGIP derived from bean was effective as an inhibitor of *S. maydis* PGs. First, as had been carried out with earlier studies of bean PGIP [19], a hypocotyl extract was prepared. Careful experiments were carried out to show that this contained an active, heat-denaturable inhibitor of the PG from *A. niger*, which was used as a control.

A purified preparation of PGIP was prepared from this extract by affinity chromatography with the *A. niger* PG

as ligand. This PGIP could be more accurately called "bulk PGIP" since it could contain a mixture of PGIPs. Over 65% of the PG activity produced by *S. maydis* grown on pectin was inhibited by this bulk PGIP, whereas a larger proportion of the PG activity from the cell wall grown fungus was inhibited (see Fig. 5). An explanation for this is that growth on the different carbon sources may induce expression of different proportions of PG isozymes or different *endo*PG:*exo*PG ratios. *Endo*PGs are likely to exhibit variation in susceptibility to PGIP, whereas *exo*PGs are likely to be unaffected [*13*]. All three PG isozymes produced when pectin was used as a carbon source were completely inhibited by the bulk PGIP in the overlay assay. However, this may be due to the fact that the PGIP was calculated to be at 100-fold excess in this experiment, whereas the PG:PGIP ratio was 1:1 in the reducing sugar assays.

Another important factor is that the bulk PGIP from the affinity column may contain a subset of the bean PGIP species expressed in bean hypocotyls, namely those that bind efficiently to the PG from *A. niger* used as the ligand. Other PGIPs present in the hypocotyl extract may not have been recovered in the bulk PGIP sample. The two most characterised bean PGIPs, PGIP-1 and PGIP-2, are known to be present in hypocotyl extracts and bind to the *A. niger* PG affinity column [*10*, *44*].

Mindful that the purified bulk PGIP could contain a mixture of PGIPs which are difficult to separate by protein fractionation techniques, a recombinant DNA approach was taken to obtain a source of a specific bean PGIP. This entailed cloning of the bean *pgip-1* gene and transformation of a different plant, namely tomato, which did not have a high level of endogenous PGIP in leaf material. Transgenic tomato leaf extracts did indeed express bean PGIP-1, which inhibited *A. niger* and *S. maydis* PGs (Table 2 and Fig. 5).

During the course of this work, an independently isolated bean *pgip-1* gene was transformed into a different cultivar of tomato [*21*]. PGIP-1 extracts were made and shown to inhibit *A. niger* PG, thus verifying the results obtained in this work. Subsequently, the group of Cervone have developed a more elegant and rapid recombinant method for assessing different PGIPs by transient expression in *Nicotiana benthamiana* using a PVX vector [*34*].

Unfortunately, tomato is not a host for the maize pathogen *S. maydis* so it was not feasible to do pathogenicity assays. However, maize transformation with the bean *pgip-1* gene would address whether the evidence from the *in vitro* and tomato expression data can be reflected by increased tolerance to *S. maydis in vivo*.

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