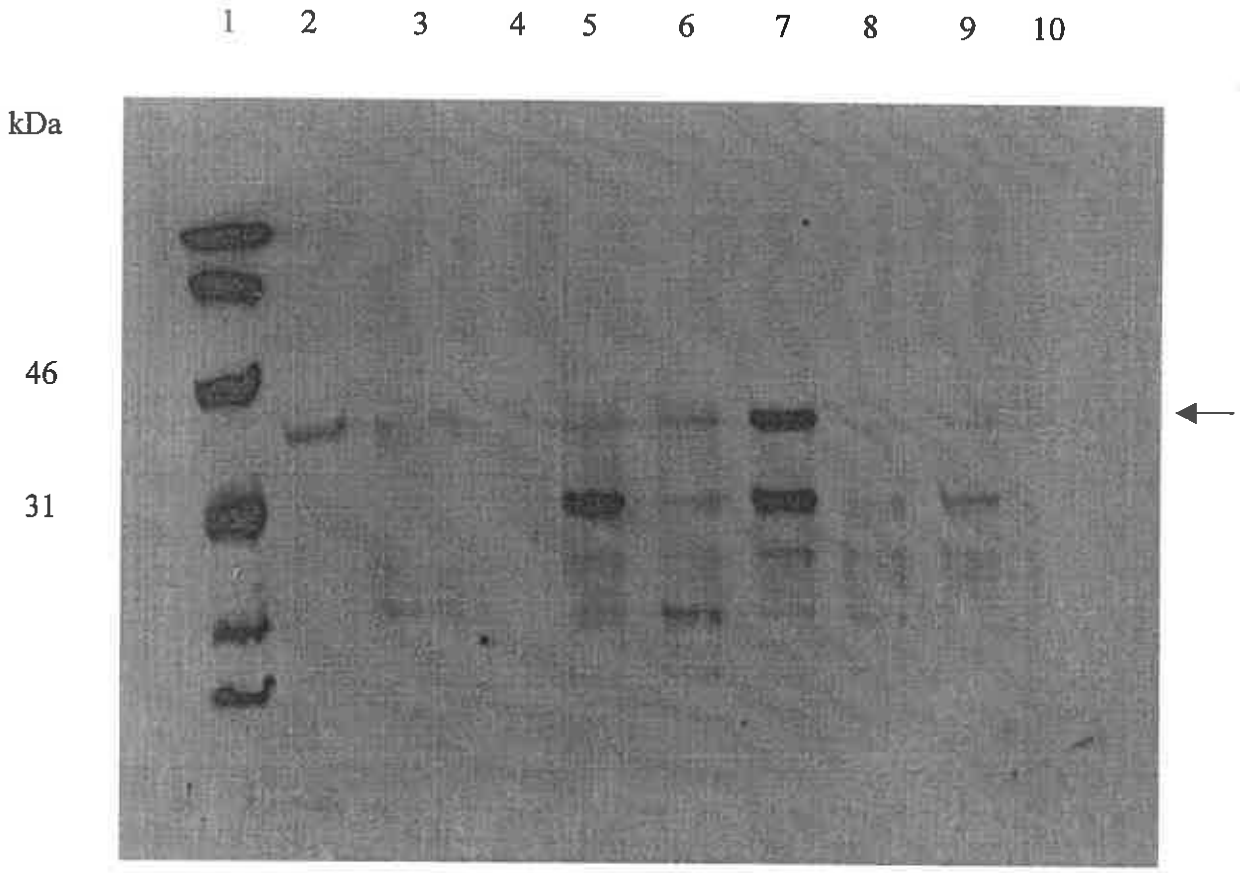

Figure 1: Western blot of bean *pgip1* transgenic maize extracts. Lane 1: ECL molecular weight marker; Lane 2: 300ng purified bean PGIP1 (positive control); Lane 3: 2µg PVX:PGIP2 extract (prepared in Rome)(positive control); Lane 5: 2µg PGIP extract from untransformed maize leaves (extract #1); Lane 6: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 212/8; Lane 7: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 158/6; Lane 8: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 216/8; Lane 9: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 292/5; Lane 10: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 103/6. Arrow indicates the presence of the PGIP protein band.

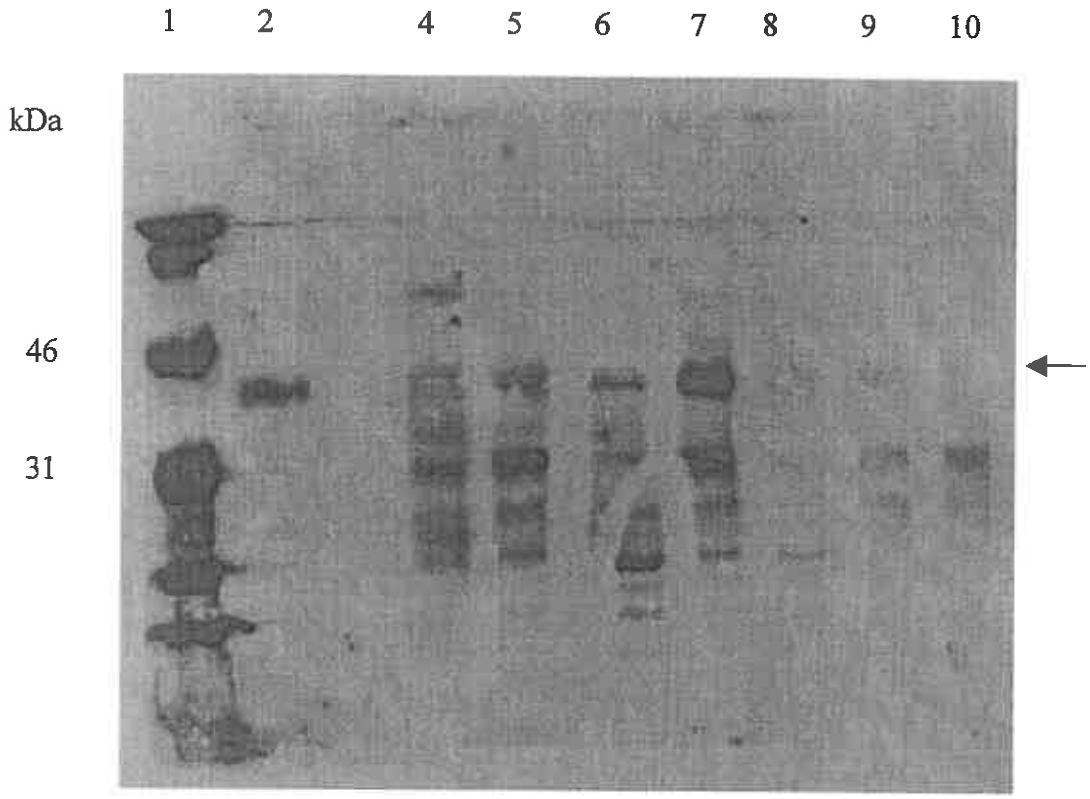

Figure 2: Western blot of bean *pgip1* transgenic maize extracts. Lane 1: ECL molecular weight marker; Lane 2: 300ng purified bean PGIP1 (positive control); Lane 4: 2µg PGIP extract from untransformed maize leaves (extract #1); Lane 5: 2µg PGIP extract from untransformed maize leaves (extract #2); Lane 6: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 212/8; Lane 7: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 158/6; Lane 8: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 216/8; Lane 9: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 292/5; Lane 10: 2µg PGIP extract from bean *pgip1* transformed maize leaves of line 103/6. Arrow indicates the presence of the PGIP protein band.

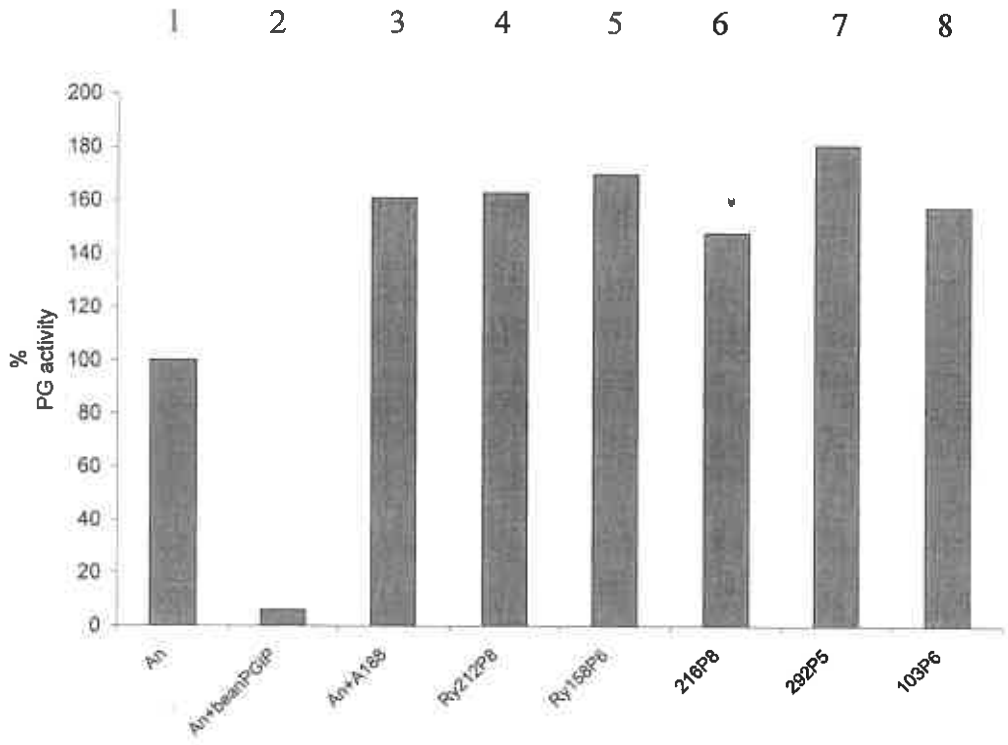

Figure 3: Inhibition study of the *Aspergillus niger* PGs (An) by bean hypocotyl PGIP (bean PGIP), PGIP extracted from bean *pgip1* transgenic (212/8, 158/6, 216/8, 292/5 and 103/6) maize leaves and PGIP extracts from untransformed maize leaves (A188) using the QuickPAHBAH assay. The PGIP was extracted from the maize leaves using a modified high salt extraction protocol (Desiderio *et al.*, 1997). Control experiments were performed by incubation the fungal PG extracts with 20mM NaAC, pH 4.7

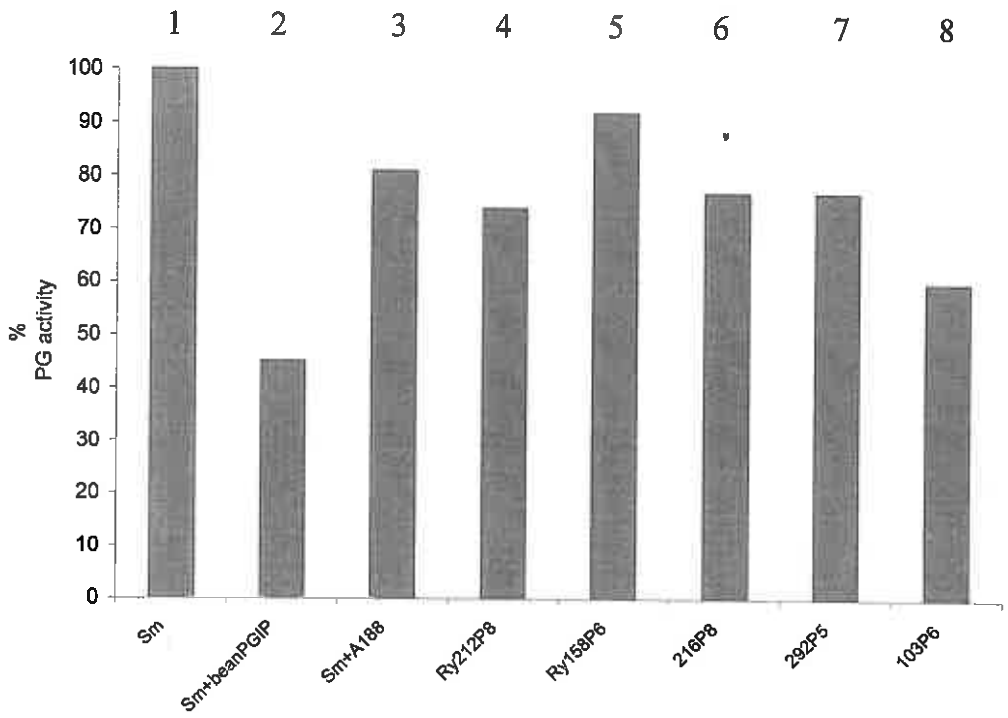

Figure 4: Inhibition study of the *Stenocarpella maydis* PGs (Sm) by bean hypocotyl PGIP (bean PGIP), PGIP extracted from bean *pgip1* transgenic (212/8, 158/6, 216/8, 292/5 and 103/6) maize leaves and PGIP extracts from untransformed maize leaves (A188) using the QuickPAHBAH assay. The PGIP was extracted from the maize leaves using a modified high salt extraction protocol (Desiderio *et al.*, 1997). Control experiments were performed by incubation the fungal PG extracts with 20mM NaAC, pH 4.7

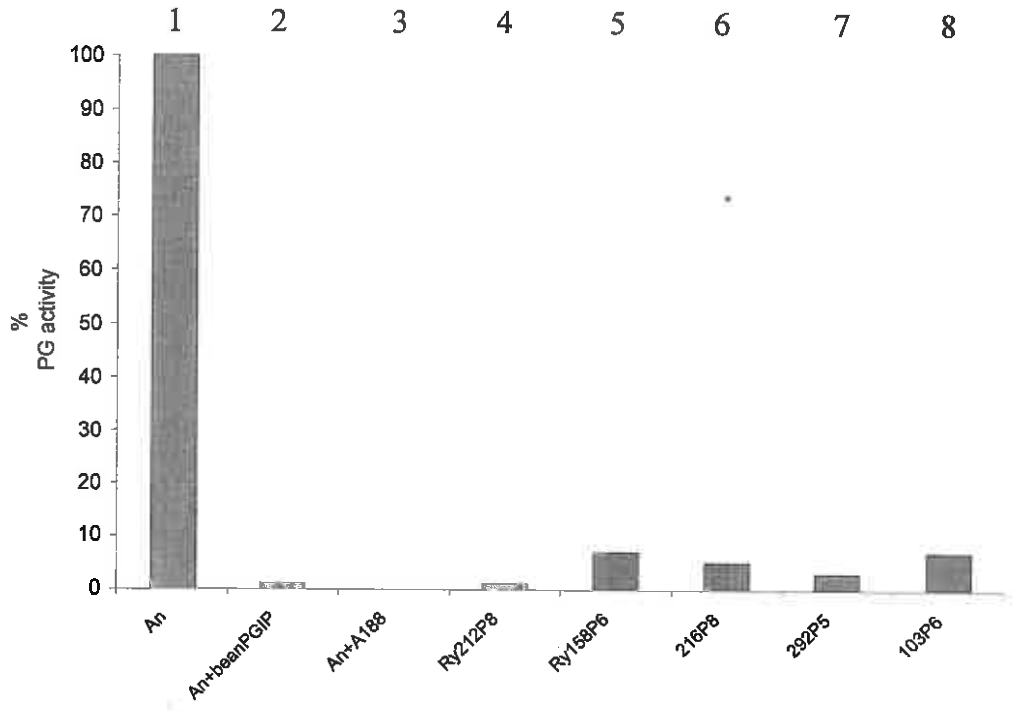

Figure 5: Inhibition study of the *Aspergillus niger* PGs (An) by bean hypocotyl PGIP (bean PGIP), PGIP extracted from bean *pgip1* transgenic (212/8, 158/6, 216/8, 292/5 and 103/6) maize leaves spiked with bean hypocotyl PGIP, and by PGIP extracts from untransformed maize leaves (A188) spiked with bean hypocotyl PGIP, using the QuickPAHBAH assay. The PGIP was extracted from the maize leaves using a modified high salt extraction protocol (Desiderio *et al.*, 1997). Control experiments were performed by incubation the fungal PG extracts with 20mM NaAC, pH 4.7

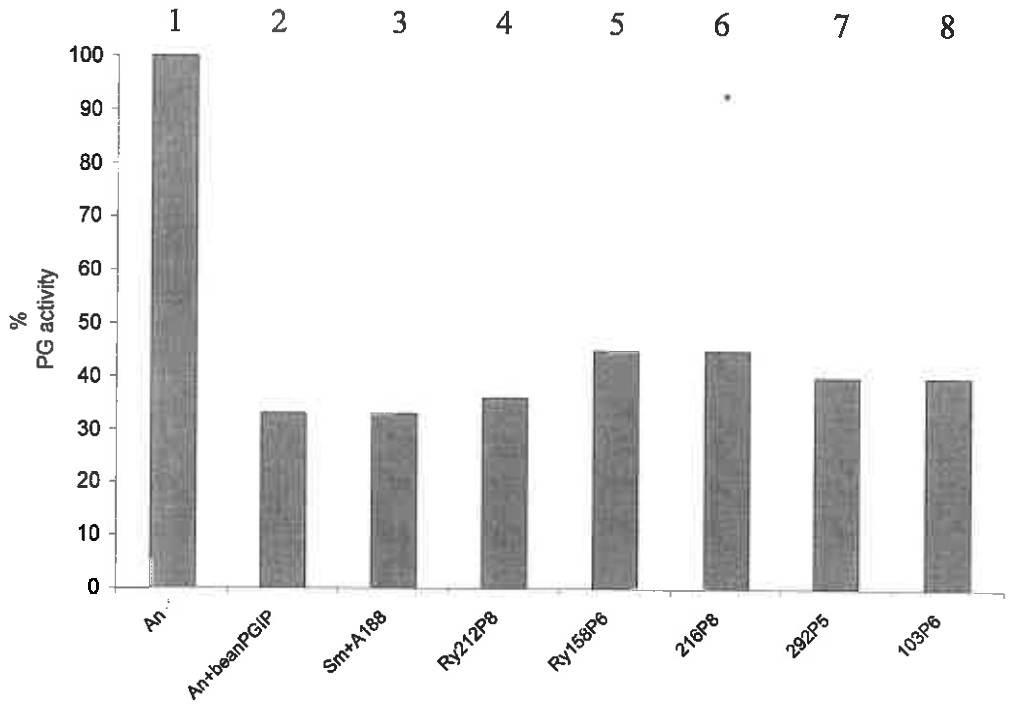

Figure 6: Inhibition study of the *Stenocarpella maydis* PGs (An) by bean hypocotyl PGIP (bean PGIP), PGIP extracted from bean *pgip1* transgenic (212/8, 158/6, 216/8, 292/5 and 103/6) maize leaves spiked with bean hypocotyl PGIP, and by PGIP extracts from untransformed maize leaves (A188) spiked with bean hypocotyl PGIP, using the QuickPAHBAH assay. The PGIP was extracted from the maize leaves using a modified high salt extraction protocol (Desiderio *et al.*, 1997). Control experiments were performed by incubation the fungal PG extracts with 20mM NaAC, pH 4.7

MATERIALS AND METHODS (Tasks 1, 2, 3 &4)

Transformation of maize

Callus induction, transformation procedures and regeneration of transgenic Hi-II maize is as described in O'Kennedy et al., (1998) and milestone reports for Phases 1-3.

Analysis of transgenic plants

DNA extraction. Genomic DNA was extracted from maize transformants using the extraction procedure of Dellaporta *et al.* (1983).

PCR analysis. Pgip (5'-GCTCTAGAATGACTCAATTCAATATCCCAG-3' and 5'-GCACGAG TCTTAAGTGCAGGAAGGAAG-3'), and *bar* (BAR_L: 5'-CATCGAGACAAGCACGGTCAACT C-3' and BAR_R: 5'-CTCTTGAAGCCCTGTGCCTCCAG-3') specific primers were used to amplify 1 Kb and 0.28 Kb fragments, respectively, from genomic DNA preparations of putative transgenic maize plantlets. PCR samples were denatured at 94°C for 2 minutes, and subjected to 35 cycles of a denaturing step at 94°C for 45 seconds, an annealing step at 64°C for 30 seconds and an elongation step at 72°C for 45 seconds.

Southern blot analysis. Five microgrammes of maize genomic DNA either digested or undigested with restriction enzymes were separated on an agarose gel and analysed by Southern blotting as described by O'Kennedy *et al.* (1998). The *pgip* and *bar* genes were excised from pUBI-PGIP by restriction digestion, the respective internal gene fragments gel purified, and labeled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche Molecular Biochemicals).

RNA isolation. Leaf material was cut from T5 generation maize plants from a field trial at ARC Grain Crops Institute. The leaf material was placed on ice and transported to CSIR Bio/Chemtek where the samples were frozen in liquid nitrogen and frozen at -70°C until use.

Total RNA was isolated from maize leaves using the SV Total RNA isolation system (Promega, cat. no: Z3100). Leaf tissue (30mg) was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was added to 175 µl SV RNA lysis buffer in an eppendorf tube and mixed well. SV DNA dilution buffer (350µl) was added to the lysate which was then mixed gently by inversion, incubated at 70°C for three minutes and centrifuged at 14 000 x *g* for 10 minutes. The supernatant was collected and 200µl of 95% ethanol was added to it. The spin column assembly consisted of a spin basket and collection

tube. The supernatant and ethanol mix was to transferred to the spin basket, and the spin column assembly was centrifuged at 14 000 x *g* for one minute. The liquid in the collection tube was discarded. SV RNA wash solution (600µl) was added to the spin basket and the spin column assembly was centrifuged at 14 000 x *g* for one minute. A DNase solution was freshly prepared (40µl of yellow core buffer, 5µl of 0.9 M $MnCl_2$ and 5µl of DNase 1). The DNase solution (50µl) was added directly to the membrane inside the spin basket. The spin basket was then incubated at room temperature for 15 minutes before addition of 200µl of SV DNase stop solution. The spin column assembly was centrifuged at 14 000 x *g* for one minute. SV RNA wash solution (600µl) was added to the spin basket before centrifugation at 14 000 x *g* for one minute. The collection tube was emptied and 250µl SV RNA wash solution was added to the spin basket before centrifugation at 14 000 x *g* for 2 minutes. Nuclease free water (100µl) was added to the spin basket membrane and RNA was eluted into an elution tube by centrifugation at 14 000 x *g* for one minute. An aliquot of RNA was used for concentration estimation and gel electrophoresis. The RNA was then stored at -70°C.

The yield of total RNA obtained was determined spectrophotometrically at 260 nm, where one absorbance unit (A260) equals 40 ug of single stranded RNA/ml. The integrity of the purified RNA was determined on a 1.2% denaturing agarose gel containing formaldehyde. Two microlitres of RNA denaturing buffer (412 ul formamide, 94 ul formaldehyde, 50 ul 10 X MOPS and 25 ul glycerol) were added to five micrograms of RNA, the sample was denatured at 94°C for 4 minutes, and chilled on ice before being loaded onto the agarose gel. The gel was run for 3 hours at 70 V. After electrophoresis, the gel was stained in a 5 ug/ml ethidium bromide solution, and the RNA was visualised over a UV light source.

DNAse treatment of RNA samples. Five micrograms of total RNA was incubated with five units RNase free DNAse I and 1X DNase I reaction buffer in a total volume of 30µl for 15 minutes at room temperature. Thereafter, the reaction was inactivated by the addition of EDTA to a final concentration of 2.5 mM and heating for 10 min at 65°C.

One-Step RT-PCR. One-step RT-PCR was performed using the Titan One-Step RT-PCR System (Roche Biochemicals). For each sample to be amplified, two 25µl master mixes of reagents were prepared in separate nuclease free microcentrifuge tubes. The first master mix contained 200µM each dNTP, 0.4 µM each pgip primers (5'-CTCTAGAATGACTCAATTCAATATCCCAG-3' and 5'-GCACGAGCTCTTAAGTGCAGGAAGGAAG-3'), 1µg RNA, 5 mM DTT and 5 units RNase

Inhibitor (Rnasin, Promega). The second master mix contained 1.5 mM $MgCl_2$ and 1µl Titan enzyme mix. The two master mixes were combined in a 0.2 ml PCR tube (on ice). For first strand cDNA synthesis, the sample was incubated in a Perkin Elmer GeneAmp 2400 thermal cycler for 30 min at 50°C. Thereafter, samples were denatured at 94°C for 2 minutes, and then subjected to 35 cycles of amplification. Each cycle consisted of a denaturing step of 94°C for 30 seconds, an annealing step of 64°C for 30 seconds and an elongation step at 72°C for 45 seconds. A final elongation step of 72°C for 7 minutes was included. PCR products were separated in 1.2% agarose gels and visualized by staining with ethidium bromide.

Two-step RT-PCR. Each reverse transcription reaction was set up in a nuclease free microfuge tube on ice. The reaction contained the following components; 1 X C. therm RT buffer, 5 mM DTT, 0.8 mM each dNTP, 5% DMSO, 1 µM of the 5' pgip primer, 6 units C.therm polymerase (Roche Biochemicals), 1 µg of total RNA and sterile water to 20 µl. Reverse transcription took place at 60°C for 30 minutes, where after reactions were incubated at 94°C for 2 minutes to inactive the C.therm polymerase.

The PCR reaction contained 0.5 uM of each *pgip* primer, 0.1 mM dNTPs, 1.5 mM $MgCl_2$ and 5 µl of the reverse transcription reaction. BioTaq (Whitehead Scientific) Taq DNA polymerase and buffer were used in all reactions. Probes were labelled with digoxygenin (DIG) DNA by including DIG-dUTP (Roche Biochemicals) in the PCR reaction mix.

Reaction mixtures were subjected to an initial denaturation step of 94°C for two minutes. This was followed by 35 cycles of amplification using a denaturation step of 94°C for 20 seconds, an annealing step of 60°C for 20 seconds, and an elongation step of 72°C for 45 seconds. A final elongation step of three minutes at 72°C was included. DNA amplification cycles were performed with a GeneAmp 2400 thermocycler (Perkin Elmer). After amplification, samples were electrophoresed through a 1% agarose gel.

Extractions from transgenic and nontransgenic maize leaf material for PGIP assays and Western blots. PGIP extractions were performed on leaf material of the transformed maize lines 212/8, 158/6, 216/8, 292/5 and 103/6 as well as on leaf material of the untransformed maize line A188. The leaf material had been harvested from T_5 progeny of transgenic event BB87C/2 planted in the maize field trial at the ARC Grain Crops Institute, Potchefstroom. Duplicate samples were collected from each plant: one was provided to CSIR-Biochemtek for

PCR, Southern and RT-PCR analysis and the other was given to ARC-Roodeplaat for the Western blot analysis and PGIP extractions.

Desideio *et al.* (1997) had previously developed a high salt extraction method for isolation of cell wall-associated PGIPs from bean, tomato and tobacco. This method was adapted to obtain maize extracts enriched for PGIP. PGIP protein extractions were performed by grinding approximately 2g of leaf material to a fine powder using a mortar and pestle in liquid nitrogen. Four millilitres of extraction buffer (1M NaCl in 20mM NaAc, pH 4.7) were added to the leaf material, and gently agitated for 1 hour at 4°C. Extracts were centrifuged at 10 000rpm for 20 minutes at 4°C, the supernatant passed through mira-cloth, and dialysed O/N at 4°C against 20mM NaAc, pH 4.7. The samples were then centrifuged at 10 000rpm for 20 minutes at 4°C and the supernatants transferred to clean Eppendorf tubes. Samples were aliquoted and stored at -70°C

Protein concentration determination of the PGIP samples. The protein concentrations of the various maize PGIP extracts were determined using the Bio-Rad protein assay kit using the following modified protocol:

BSA is used at a stock concentration of 1mg/ml

1 to 5:	standards
6 to 8:	212/8 extract
9 to 11:	158/6 extract
12 to 14:	A188 extract
15 to 17:	216/8 extract
18 to 20:	292/5 extract
21 to 23:	103/6 extract

Tube no.	BSA (µl)	Sample (µl)	H_2O (µl)
1	0	x	800
2	1	x	799
3	5	x	795
4	10	x	790
5	20	x	780
6	x	20	780
7	x	30	770
8	x	40	760
9	x	20	780
10	x	30	770
11	x	40	760
12	x	20	780
13	x	30	770
14	x	40	760
15	x	20	780
16	x	30	770
17	x	40	760
18	x	20	780
19	x	30	770
20	x	40	760
21	x	20	780
22	x	30	770
23	x	40	760

PGIP extracts were added to H_2O, after which 200µl Bio-Rad reagent was added to all tubes. These were vortexed and the timer was started after vortexing of the first tube. The reaction was allowed to take place for 10 minutes and the A_{595} values obtained after blanking the spectrophotometer with H_2O. Protein concentrations were calculated using the A_{595} values.

Silver staining of SDS-PAGE gels of the maize PGIP extracts. Two micrograms of maize PGIP extract were loaded in each lane. Proteins were separated by SDS-PAGE on a 10% gel. Solutions needed for silver staining are listed in Appendix A. All steps are performed at room temperature. After electrophoresis the gel is placed in 50ml fixing solution for 30 minutes with gentle agitation, after which the gel is washed 2x10 minutes with 50ml

washing solution. The gel is placed in pre-treatment solution for 1 minute, followed by 3x30 second washes in 50ml ultrapure H_2O. The gel is subsequently placed in silver staining solution for 10 minutes, followed by 2x20 second washes with 50ml ultrapure H_2O. The protein bands are visualised by placing the gel in 50ml developing solution until the desired resolution is obtained, the reaction being terminated by placing the gel in 50ml stop solution.

Western Blot of the maize PGIP extracts. Two micrograms of maize PGIP extract were loaded in each lane. Proteins were separated by SDS-PAGE on a 10% gel. Two gels were run, one for visualisation of the proteins using silver staining, and the second for Western blotting, followed by silver staining in order to ascertain the efficiency of transfer. Transfer was performed at 30 volts O/N at 4°C in transfer buffer. The solutions needed for Western blotting are listed in Appendix B. After blotting the membrane was placed in blocking buffer and gently agitated for 1 hour and 30 minutes at room temperature. The membrane was washed 1 x 10 minutes and 2 x 5 minutes in washing buffer after blocking. The membrane was incubated with the first antibody (raised in rabbits against PGIP purified from bean PODS – kindly supplied by Professor F. Cervone, University of Rome, Italy) at room temperature in a hybridisation bottle O/N in a hybridisation oven. After the first antibody incubation step, the blot was washed with washing buffer for 1 x 10 minutes and 2 x 5 minutes. The membrane was then incubated with the second antibody (anti rabbit– supplied with the Amersham ECL Western blotting kit) at room temperature for 2 hours. After the second antibody incubation step, the membrane was washed with washing buffer for 1 x 10 minutes and 2 x 5 minutes.

For detection the Amersham ECL detection kit was used as suggested by the manufacturers. For visualisation of bands, the membrane was exposed to autorad film for 1 minute before developing.

Inhibition of A. niger and Stenocarpella maydis PGs by the bean pgip1 transgenic maize PGIP extracts using the Quick PAHBAH assay protocol. In these experiments a bean hypocotyl PGIP extract was used as a positive control (Berger et al., 2000), and 5 PGIP extracts from bean pgip1 transformed maize lines were used. A PGIP extract from a non-transformed maize line, A188, was used as a negative control. The *A. niger* PG was used at a 1:750 dilution and the *S. maydis* PGs at a 1:10 dilution with 20mM NaAc (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}) and a single Eppendorf tube for blanking of the spectrophotometer. First, 750µl substrate (0.025% PGA in 50mM NaAc, pH 4.7) was added to each of the Eppendorf tubes. The PG (30µl) was mixed with either 20mM NaAc buffer (30µl) or PGIP

extract (30µl) and incubated for 20 minutes at 25°C before the assay. Then, 25µl of this PGIP:PG mix was added to 1 set of the series of Eppendorf tubes prepared at 15" intervals (T_{30}). These were left to incubate for 30 minutes at 30°C. During the incubation period, 750µl PAHBAH was added to the second set of Eppendorf tubes prepared. The PAHBAH reagent was made fresh each time by mixing the PAHBAH with 4 volumes of 0.5M NaOH and 1 volume of 0.5N HCl to give a final PAHBAH concentration of 1%. To obtain the T_0 values, 25µl of the PGIP:PG mix was added to the second set of tubes. After the 30 minutes incubation period, 750µl PAHBAH was added to the T_{30} Eppendorf tubes at 15" intervals, after which all Eppendorf tubes were boiled for 10 minutes, cooled and the A_{410} values obtained spectrophotometrically.

Inhibition of A. niger and Stenocarpella maydis PGs by the bean pgip1 transgenic maize PGIP extracts, spiked with bean hypocotyl PGIP, using the Quick PAHBAH assay protocol.
In these experiments a bean PGIP extract from bean hypocotyls (Berger *et al.*, 2000) was used to spike the 5 PGIP extracts from bean *pgip1* transformed maize lines and a PGIP extract from a non-transformed maize line, A188, in order to determine whether a compound that could block the PGIP:PG interaction was possibly present in the maize leaf extracts. The *A. niger* PG was used at a 1:750 dilution and the *S. maydis* PGs at a 1:10 dilution with 20mM NaAc (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}) and a single Eppendorf tube for blanking of the spectrophotometer. First, 750µl substrate (0.025% PGA in 50mM NaAc, pH 4.7) was added to each of the Eppendorf tubes. The PG (30µl) was mixed with either 20mM NaAc buffer (30µl) or maize PGIP extract (30µl) together with bean hypocotyl PGIP (30µl) and incubated for 20 minutes at 25°C before the assay. Then, 40µl of this PGIP:PG mix was added to 1 set of the series of Eppendorf tubes prepared at 15" intervals (T_{30}). These were left to incubate for 30 minutes at 30°C. During the incubation period, 750µl PAHBAH was added to the second set of Eppendorf tubes prepared. The PAHBAH reagent was made fresh each time by mixing the PAHBAH with 4 volumes of 0.5M NaOH and 1 volume of 0.5N HCl to give a final PAHBAH concentration of 1%. To obtain the T_0 values, 40µl of the PGIP:PG mix was added to the second set of tubes. After the 30 minutes incubation period, 750µl PAHBAH was added to the T_{30} Eppendorf tubes at 15" intervals, after which all Eppendorf tubes were boiled for 10 minutes, cooled and the A_{410} values obtained spectrophotometrically.

APPENDIX A

Solutions for silver staining

Fixing solution

- 50% methanol
- 12% acetic acid
- 0.185% formaldehyde

Washing solution

50% ethanol

Pre-treatment

1ml sodium thiosulphate (200mg/10ml stock) in 100ml ultrapure H_2O

Silver staining solution

- 0.2% $AgNO_3$
- 0.028% formaldehyde

Developing solution

- 6% sodium carbonate
- 0.0185% formaldehyde
- 20µl sodium thiosulphate (200mg/10ml stock)

Stop solution

- 50% methanol
- 12% acetic acid

Important note: All solutions are made using ultrapure H_2O only

APPENDIX B

Solutions for Western blotting

Transfer buffer

- 70ml 10 x TG Bio-Rad (250mM Tris, 1.92M glycine, pH 8.3)
- 200ml methanol
- 730ml distilled water

Washing buffer

- 1 x PBS (100ml 10 x stock)
- 0.2% Tween 2 (0.4ml)
- to 1L with Ultra pure water

Blocking buffer

- 100ml washing buffer
- 2.5g Amersham membrane blocking reagent

First antibody

- 10ml washing buffer
- 0.5% BSA
- first antibody

Second antibody

- 10ml washing buffer
- 0.5% BSA
- second antibody

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