THE GENETIC ENGINEERING OF SOUTH AFRICAN MAIZE CULTIVARS FOR RESISTANCE TO *STENOCARPELLA (DIPLODIA) MAYDIS*

CSIR

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PHASE 4 : Molecular screening of field trial material

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Executive Summary

Perspective on PGIP-mediated fungal resistance in maize

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In this project, the most promising transgenic maize event was BB87C/2. This was shown to be fertile up to at least the T5 generation, and showed stable inheritance of both the *pgip1* and *bar* genes. This was shown by presence of the same Southern blot pattern in T_1 and later generations, although there were multiple insertion sites. It was not determined if these were as tandem copies or if they were scattered throughout the genome.

BB87C/2 also showed stable inheritance of the herbicide tolerance phenotype in leaves, indicating that at least one copy of the *bar* gene was expressed from the *ubi1* promoter as mRNA and translated into an active protein. The same promoter was also driving the bean *pgip1* gene, and therefore it was expected that the *pgip1* gene should have been expressed at a high level. Some evidence for *pgip1* mRNA expression was obtained in RT-PCR experiments, although it appeared to be unstable. However, extensive attempts throughout the project to detect PGIP1 protein or PGIP1 activity were unsuccessful. This may either be due to the lack of sensitive and specific detection methods, or it may be that the protein was not made in an active form.

In two sets of field trials at ARC-Grain Crops Institute, progeny of BB87C/2 were challenged with the fungus *Stenocarpella maydis*, however no statistically significant increase in fungal resistance was observed. Due to the fact that it could not be determined if bean PGIP1 was present, it is not possible to conclude at this stage whether the lack of resistance to the fungus was due to absence of PGIP1 or that PGIP1 is not an effective strategy. Since biochemical assays had shown that bean PGIP1 does inhibit the PG activity of *Stenocarpella maydis* (Berger et al., 2000), it still remains possible that transgenic PGIP may confer fungal resistance. A conclusion on this matter would only be possible if a transgenic maize event could be produced in which PGIP was shown to be expressed at a high level and in an active form.

Introduction

Stenocarpella (formerly *Diplodia*) *maydis*, or cob rot, is the most serious fungal pathogen of maize in South Africa since it affects both yield and cob quality. Like many other fungal pathogens, *S. maydis* produces polygalacturonase enzymes, which assist in penetration of plant cells by degradation of the cell wall pectin component, while simultaneously providing the invading fungus with a nutrient source. Polygalacturonase-inhibiting protein (PGIP) is a cell wall-associated protein which was successfully cloned and sequenced from a variety of plants including bean plants (Berger *et al.*, 1996). The *pgip* gene was shown, using *in vitro* studies, to be an effective antifungal protein for the control of *Stenocarpella maydis* (Berger *et al.*, 1996) (Progress report 1). The *pgip* gene was therefore cloned into the vector pAHC25 (Progress report 2, May 1996) (Fig. 1). The construct pUBI-PGIP contains the *pgip* gene that encodes for the polygalacturonase-inhibiting protein enzyme, and for selection, the *bar* gene, that encodes the enzyme phosphinothricin acetyl transferase (PAT). PAT inactivates the herbicide phosphinothricin (Basta[7] and Ignite[7]). Both these genes are under the control of the maize *Ubi1* promoter which was shown to be effective in cereals (Taylor *et al.*, 1993).

Prior to the recent development of cereal transformation, the production of improved cereal lines relied on traditional plant breeding techniques. Today, gene transfer is used to supplement conventional plant breeding, thereby contributing to improved disease resistance or to modified growth quality. This technology offers the ability to alter a trait specifically, while retaining the superior qualities of a particular cultivar. The production of stably transformed, fertile plants is crucial for the introduction of new traits into crop species. Monocotyledonous crops, and cereals in particular, appear in the past to be very recalcitrant to *Agrobacterium* infection. High efficiency *Agrobacterium*-mediated transformation of maize (*Zea mays* L.) has been reported only recently (Ishida *et al.*, 1996; Negrotto *et al.*, 2000). However, these authors reported on the efficient transformation of the laboratory strain of maize, A188, and of some crosses between A188 and other inbreds. Although a low copy number of the gene is inserted via *Agrobacterium* transformation, a limitation of the system is the strict interaction between the genotype of the plant species and the *Agrobacterium* strain (Ishida *et al.*, 1996).

The use of microprojectile bombardment-mediated transformation of embryogenic tissue culture material or immature embryos, with the subsequent generation of fertile transgenic plants, bypasses *Agrobacterium* host-specificity. Three years after the biolistic transformation of a laboratory strain of maize (Fromm *et al.* 1990, Gordon-Kamm *et al.* 1990), Koziel and

co-workers (1993) published the first paper on field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis* using the Biorad helium driven Biolistic device (PDS-1000He). Subsequently, stable transformation of elite maize by microprojectile bombardment of immature embryos has been performed by a few laboratories using the Biorad PDS1000He gun and their own proprietary elite maize lines (Armstrong and Songstad, 1993; Hill *et al.*, 1995; Koziel *et al*, 1993; Pierce, Pioneer Hi-Bred International Inc., personal communication). Furthermore, a laboratory strain of maize, Hi-II and elite white maize was stably transformed in our laboratory by bombarding either highly regenerable Type II maize embryogenic calli or immature zygotic embryos, respectively, using the particle inflow gun (O'Kennedy *et al.*, 1998; O'Kennedy *et al.*, 2001 publication submitted).

The integrated transgenes must be inherited and expressed in a predictable and stable manner over many generations of genetically modified plants in order to be useful in plant breeding programmes. Molecular and biochemical analysis of transformed plant material is done for two reasons. First, to determine whether the material is transgenic, second, to characterise the material e.g. determine copy number and/or the complexity of the DNA insert, and finally to evaluate transgene expression. Polymerase chain reaction (PCR) technology is the most popular method for screening material for the presence or absence of transgene sequences. For analysis of copy number and integration complexity, digestion of genomic DNA with SacI was performed followed by Southern blot analysis.

Objectives of phase 4

The objective of phase 4 was the molecular analysis using PCR, Southern blot, RT-PCR and Western blot analysis of plants in field trial (June 1999-May 2000). Phase 4 is subdivided into 4 tasks which are individually described:

Task 1: PCR screening of 29 selected T_4 plants and 260 selected T_5 plants for the presence of the transgenes *bar* and *pgip*.

Description: Greenhouse (bulk up material, T_4) and field grown material (T_5) planted by Dr Koos van Rensburg (ARC, Summer Grain Centre, Potchefstroom) will be screened by PCR analysis for the presence or absence of the *bar* and *pgip* transgenes.

Task 2: Southern blot analysis of 20-30 selected T_4 and T_5 plants

Description: After completion of the PCR analysis of most of the field planted material, southern blot analysis will be done to confirm the integration pattern and the copy number of the transgenes bar and *pgip.* Leaf material will be harvested, and DNA extracted from plants containing 1) both transgenes, 2) plants containing the herbicide resistance gene, *bar* but not the *pgip* gene, 3) plants containing the *pgip* gene but not the herbicide resistance gene, *bar* and 4) plants containing neither the *pgip* nor the *bar* gene (negative control).

Task 3: Confirming the expression of the *pgip* transgene of selected T_5 transformants by RT-PCR analysis

Description: On the basis of the PCR analysis, selected transformed plants containing the *pgip* gene can be analysed by RT-PCR to determine whether the *pgip* transgene is expressed. This will greatly assist us in the final outcome of the field trial, since the gene could be present as confirmed by PCR and southern blot analysis, but the gene might not be expressed. It is essential for us to know that the *pgip* gene is not only integrated into the genome of the transgenic maize, but also expressed.

Task 4: Western blot analysis of selected T_5 transformants

Description: It is proposed in tasks 1-3 that PCR, Southern blots, and RT-PCR will be used to confirm the presence and mRNA expression of the *pgip* gene, respectively. However, no information will be obtained concerning the presence of the PGIP protein. Western blots using the Italian PGIP antibodies will be used to screen the SAME plants analyzed by RT-PCR. This will make possible a correlation between presence of gene, presence of mRNA, presence of protein and resistance to *S. maydis.*

Task 1 & 2: PCR and Southern blot analysis of selected T_4 and T_5 transformants

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OBJECTIVES

Task 1: PCR screening of 29 selected T_4 plants and 260 selected T_5 plants for the presence of the transgenes *bar* and *pgip*.

Task 2: Southern blot analysis of 20-30 selected T_4 and T_5 plants

SUMMARY

The presence of the *pgip* and *bar* transgenes in the T_4 and T_5 progeny of transformation event BB87C/2 was confirmed by PCR and Southern blot analysis. Furthermore, the integration pattern of the *pgip* transgene remained unaltered when compared to the T_1-T_3 integration pattern. Apart from the negative control plants, a total of 267 plants were screened by PCR analysis for the presence or the absence of the *pgip* and *bar* transgenes in the T_4 and T_5 progeny of transformation event BB87C/2. Furthermore, 41 plants of the T_4 and T_5 progeny were screened by Southern blot analysis to confirm the integration pattern previously obtained for the T_1-T_3 generation of transformation event BB87C/2.

RESULTS AND DISCUSSION

Apart from the negative controls, 27 T_4 plants and 240 T_5 plants were screened by PCR analysis for the absence or the presence of the *pgip* and *bar* transgenes in the T_4 and T_5 progeny of transformation event BB87C/2. Furthermore, 14 T_4 plants and 27 T_5 plants were screened by Southern blot analysis to confirm the integration pattern previously obtained for transformation event BB87C/2.

PCR analysis and Southern blot analysis of the T4 generation of transgenic plants

The Southern blot results coincide with the PCR results (Table 1, Fig. 1). If the PCR analysis indicated that the transgene was present or absent, the Southern blot analysis confirmed the result. Furthermore, apart from variation in the intensity of the banding pattern, as a result of differences in the total DNA, the banding pattern for the *pgip* transgene remained identical Fig. 1). The *pgip* gene was absent in plant numbers 7/4/7, 9/9/3, 9/9/24 and 9/12/16 (Table 1, Fig. 1). Plant number 9/9/3 was used as negative control, but spiked with 2 copies of the plasmid transgene in the results of Fig 1.

PCR analysis of the T5 progeny

The T_5 progeny of plant numbers 9/8/7 (12 plants screened) and 9/9/5 (4 plants screened) were all negative (data not shown in table 2) as expected since the T_4 generation were negative for both transgenes *pgip* and *bar* (Table 1) but they were screened as controls. The T_4 generation plants which contained both transgenes were plant numbers 7/14/5, 7/14/10, 7/14/12, 9/6/3, 9/13/12, 9/13/13, 9/13/22, 9/13/37, 17/2/6, and17/5/3 (Table 2). Although the plants contained both transgenes the T_5 progeny had 1) both transgenes or 2) only the *bar* transgene or 3) neither of the transgenes of the T_4 generation. The T_4 generation which contained only the *bar* transgene but not the *pgip* gene were T_4 plants 9/13/8, 7/14/11, 9/13/2, 9/13/20 and 9/14/16 (Table 2). The bar gene were either present or absent in the progeny of these plants (T_5 generation) (Table 2). Only one transgenic plant, number 6/9/6 contained the *pgip* gene but not the bar gene and only one of its progeny still inherited the *pgip* gene (Table 2). It was clear that the *pgip* gene was more often lost in the generation to follow than the *bar* transgene, although both are driven by the constitutive promoter ubiquitin.

Table 1: PCR and Southern blot analysis of T_4 maize plants grown in the greenhouse at the ARC, Potchefstroom for the purpose of bulking up seed for a T_5 generation field trial.

T_4 generation			
Plant number	PCR analysis pgip	bar	Southern blot analysis pgip
6/9/6	+	-	+
7/4/7	-	-	-
7/14/5	+	+	
7/14/8	+	+	+
7/16/1	+	+	+
8/17/1	-	+	
9/1/14	+	+	+
9/6/3	+	+	+
9/6/15	-	+	
9/8/7	-	-	
9/8/24	-	-	
9/8/29	-	-	
9/8/42	-	-	
9/9/3	-	-	-
9/9/5	-	-	
9/9/6	-	-	
9/9/24	-	-	-
9/12/16	-	-	-
9/13/12	+	+	
9/13/13	+	+	+
9/13/22	+	+	
9/13/37	+	+	+
9/14/4	-	-	
17/2/6	+	+	+
17/5/3	+	+	+
17/6/2	-	+	
17/16/1	+	+	+

Table 2: PCR analysis of T_5 progeny of selfpollinated transgenic T_4 plants

T_4 generation	T_5 generation			
Plant number	Row number	plant numbers	PCR analysis pgip	bar
6/9/6	68	2	+	-
		1, 3-22	-	-
7/14/5	70	1, 12	+	+
		4-9, 11,15	-	+
		2, 3, 10, 13, 14, 16	-	-
7/14/10	72	1, 4, 6, 9, 11, 12, 14, 17, 18	+	+
		2, 3, 5, 7, 8, 10, 13, 15, 16, 19, 20	-	-
7/14/11	73	9	-	+
		1-8, 10-16	-	-
7/14/12	74	6, 7, 8	+	+
		2, 3, 4	-	+
		1, 5	-	-
9/6/3	103	6	+	+
		5, 8	-	+
		1-4, 7	-	-
9/13/2	155	5	-	+
		1-4, 6-7	-	-
9/13/8	156	6	-	+
		1-5	-	-
9/13/12	157	2	+	+
		1, 3-7	-	-
9/13/13	158	6	+	+
		1, 2, 5, 8	-	+
		3, 4, 7	-	-
9/13/20	161	1, 2, 9-11	-	+
		3-8, 12-14	-	-
9/13/22	162	1, 7, 11	+	+
		3, 5, 8, 10, 12, 13	-	+
		2, 4, 6, 9	-	-
9/13/37	171	1, 2	+	+
		7	-	+
		3-6	-	-
	271	8, 9, 11	-	+
		1-7, 10, 12, 13	-	-
	340	1, 9	-	+
		2-8, 10	-	-
9/14/14	181	1-18	-	-
9/14/16	183	1	-	+
		2-18	-	-
17/2/6	212	1, 3, 8, 9	+	+
		2, 4, 6, 7, 10, 11	-	+
		5, 12-14	-	-
	292	5	+	+
		1-4, 6-9	-	+
	364	1-11	-	-
17/5/3	216	4, 8	+	+
		2, 9, 12	-	+
		1, 3, 5-7, 10, 11, 13, 14	-	-

Southern blot analysis of the T5 progeny:

Although plant 6/9/6 was positive for the *pgip* transgene in the T_4 generation (Fig. 1), and the progeny designated 68/2 was PCR positive, Southern blot analysis showed that 68/2 did not inherit the *pgip* transgene (Table 3, Fig. 2). The T_5 generation plant number was compiled by a combination of the row number and the plant number in the row (row/plant). The other 26 plants screened contained both transgenes as confirmed by PCR analysis. Furthermore, all 26 plants screened had the *pgip* gene stably inherited as confirmed by Southern blot analysis (Figs 2 & 3). The inheritance of the *bar* gene was confirmed for selected plants by stripping the Southern blot and reprobing the blot with an internal fragment of the *bar* transgene (Table 3). Once more apart from variation in the intensity of the banding pattern, as a result of differences in the total DNA, the multicopy banding pattern for the pgip transgene (Figs 2 & 3) as well as the bar transgene (data not shown) remained identical. As recorded in Table 3, the *pgip* gene was also inherited in T_5 plant numbers 171/2, 216/4, 216/8 and 292/5, as confirmed by Southern blot analysis (data not shown).

There is only one *Sac* I restriction site in the construct pUBI-PGIP. Therefore each band on the Southern blots (Figs 1, 2 and 3) should represent a different integration event in the maize genome. The pattern shows at least 5 bands, indicating that at least 5 *pgip* integration events were inserted. The band at 1,4 Kb is very intense and corresponds to at least 10 copies of *pgip* (compare lanes 3 and 14 in Fig. 3), so this band should represent 10 tandem copies of *pgip* flanked by *Sac* I sites which are 1,4 Kb apart.

Furthermore, a constant loss of the *pgip* gene was observed (Table 1). Two plants which were *pgip* negative in the T_3 generation (9/8 and 9/9) were also *pgip* negative in the T_4 generation. This was shown by PCR of four T_4 progeny plants of each (9/8/7, 9/8/24, 9/8/29, 9/8/42, 9/9/3, 9/9/5, 9/9/6, 9/9/24) and Southern blot of two T_4 progeny (9/9/3 and 9/9/24).

T_4 plants that were *pgip* positive had T_5 progeny that were also consistently *pgip* positive (Tables 1 and 3). Five T_4 plants shown to be *pgip* positive (9/6/3, 9/13/13, 9/13/37, 17/2/6, 17/5/3) had T_5 progeny which were also *pgip* positive in Southern blots.

Nine T_4 plants had T_5 progeny that were *pgip* and *bar* positive (Table 3).. The number of positive progeny tested is shown in brackets: 7/14/5 (1), 7/14/10 (9), 7/14/12 (3), 9/6/3 (1), 9/13/13 (1), 9/13/22 (2), 9/13/37 (2), 17/2/6 (5), 17/5/3 (2).

The data that was obtained cannot be used to determine what the segregation of the *pgip* transgene is in the T_5 progeny because only plants that were PCR positive were analysed further by Southern blot analysis.

Table 3: PCR and Southern blot analysis of T_5 generation plants in field trial

T_4 generation	T_5 generation					
plant number	Row number	plant number	PCR analysis pgip	PCR analysis bar	Southern blot analysis *pgip*	Southern blot analysis *bar*
6/9/6	68	2	+	-	-	-
7/14/5	70	1	+	+	+	+
7/14/10	72	1	+	+	+	nd
		4	+	+	+	nd
		6	+	+	+	nd
		9	+	+	+	nd
		11	+	+	+	nd
		12	+	+	+	nd
		14	+	+	+	nd
		17	+	+	+	nd
		18	+	+	+	nd
7/14/12	74	6	+	+	+	nd
		7	+	+	+	nd
		8	+	+	+	nd
9/6/3	103	6	+	+	+	+
9/13/13	158	6	+	+	+	+
9/13/22	162	7	+	+	+	+
		11	+	+	+	+
9/13/37	171	1	+	+	+	+
		2	+	+	+	nd
17/2/6	212	1	+	+	+	+
		3	+	+	+	+
		8	+	+	+	+
		9	+	+	+	+
	292	5	+	+	+	nd
17/5/3	216	4	+	+	+	nd
		8	+	+	+	nd

\+ = positive detection of the transgene as indicated in the relevant column
\- = negative detection of the transgene as indicated in the relevant column
nd = not determined

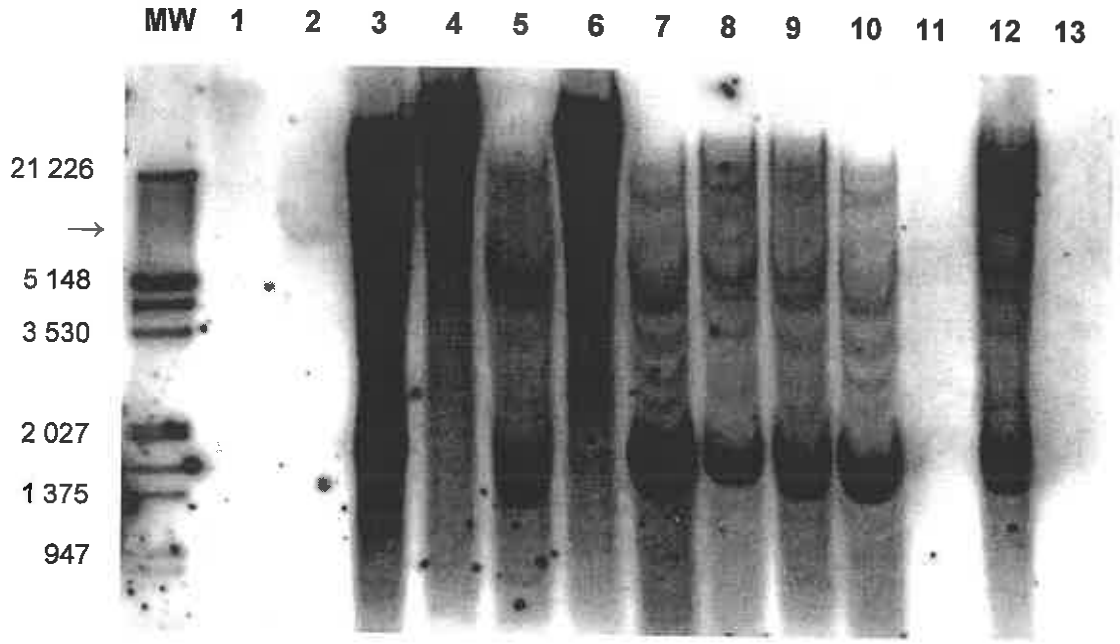

Figure 1: Southern blot analysis of T_4 progeny of maize transformation event BB87C/2. Genomic DNA was purified from plant leaf material, restricted with *Sac* I, resolved in a 0.8% agarose gel, transferred to a nylon membrane, and then probed with an internal *pgip* fragment. Approximately five microgram of genomic DNA were loaded per lane. MW, DIG labeled molecular weight marker III (Roche Molecular Biochemicals); lane 1, untransformed maize DNA, plant number 9/9/24; lane 2, represents untransformed maize plant number 9/9/3 spiked with 2 transgene copies; lane 3, plant 9/13/37; lane 4, 9/6/3; lane 5, 7/14/8; lane 6, 9/13/13; lane 7, 9/1/14; lane 8, 17/5/3; lane 9, 17/2/6; lane 10, 7/16/1; lane 11, 7/4/7; lane 12, 6/9/6; lane 13, 9/12/16. The arrow indicates the size of pUBI-PGIP restricted once with *Sac* I, which is 9.5 Kb.

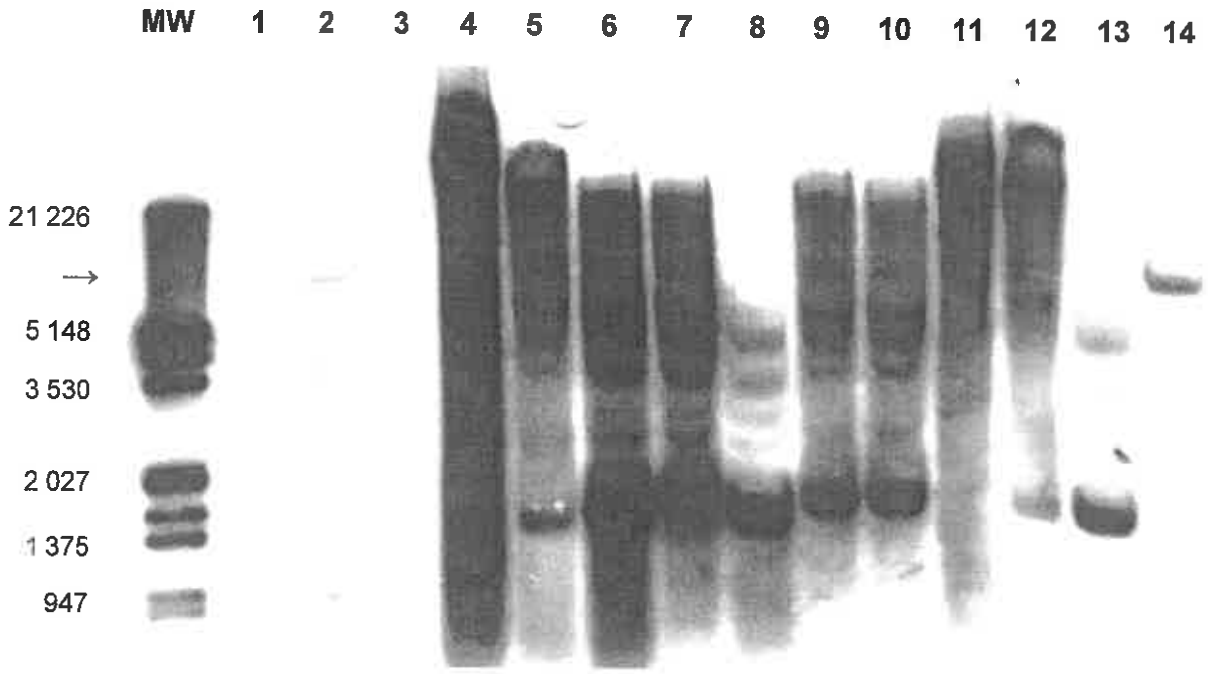

Figure 2: Southern blot analysis of T_5 progeny of maize transformation event BB87C/2. Genomic DNA was purified from plant leaf material, restricted with *Sac* I, resolved in a 0.8% agarose gel, transferred to a nylon membrane, and then probed with an internal *pgip* fragment. Approximately five microgram of genomic DNA were loaded per lane. MW, DIG labeled molecular weight marker III (Roche Molecular Biochemicals); lane 1, untransformed maize DNA; lanes 2 and 14, represent untransformed maize A188 spiked with 2 and 10 transgene copies, resepectively; lane 3, plant 68/2; lane 4, 70/1; lane 5, 158/6; lane 6, 162/7; lane 7, 162/11; lane 8, 171/1; lane 9, 212/1; lane 10, 212/3; lane 11, 212/8; lane 12, 212/9; lane 13, 103/6. The arrow indicates the size of pUBI-PGIP restricted once with *Sac* I, which is 9.5 Kb.

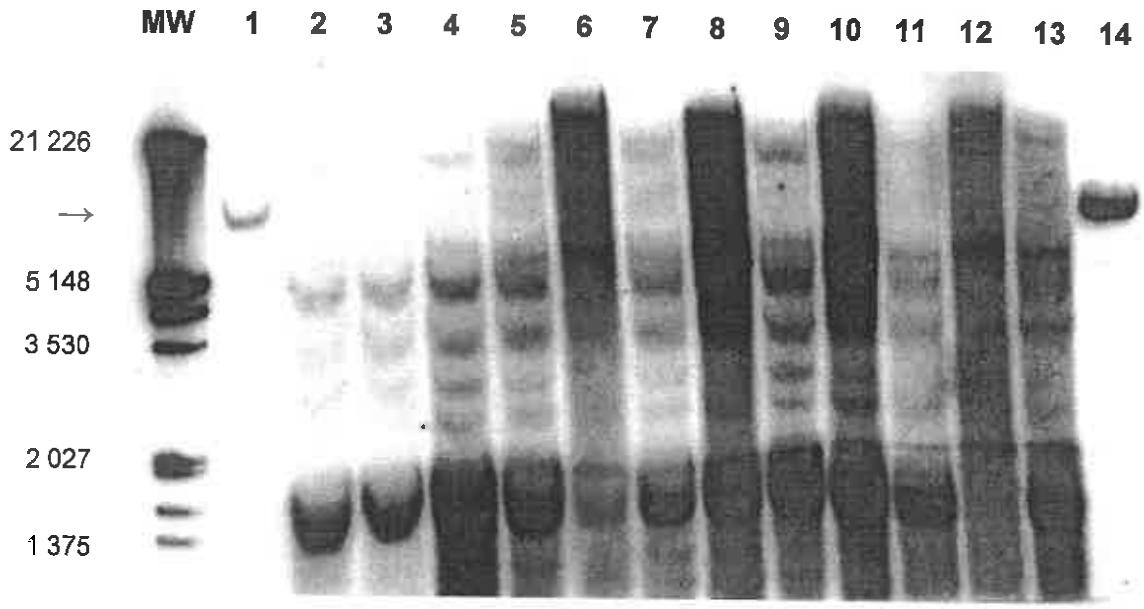

Figure 3: Southern blot analysis of T_5 progeny of maize transformation event BB87C/2. Genomic DNA was purified from plant leaf material, restricted with *Sac* I, resolved in a 0.8% agarose gel, transferred to a nylon membrane, and then probed with an internal *pgip* fragment. Approximately five microgram of genomic DNA were loaded per lane. MW, DIG labeled molecular weight marker III (Roche Molecular Biochemicals); lane 1 and 14, represent untransformed maize A188 spiked with 2 and 10 transgene copies, resepectively; lane 2; plant 72/1; 3, 72/4; lane 4, 72/6; lane 5, 72/9; lane 6, 72/11; lane 7, 72/12; lane 8, 72/14; lane 9, 72/17; lane 10, 72/18; lane 11, 74/6; lane 12, 74/7; lane 13, 74/8. The arrow indicates the size of pUBI-PGIP restricted once with *Sac* I, which is 9.5 Kb.

Task 3: RT-PCR analysis of selected T_5 transformants

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OBJECTIVES

To confirm the expression of the bean *pgip1* transgene in selected T_5 progeny of transgenic maize event BB87C/2 by RT-PCR analysis.

SUMMARY

Total RNA was isolated from seven T_5 generation plants which were shown to possess the *bar* and *pgip* genes by PCR and Southern blot analysis. The RNA was subjected to a two-step RT-PCR analysis to determine whether the *pgip* transgene was expressed. Results indicated that one of the plants analysed expressed the *pgip* gene (plant#7 in row 162). However, the *pgip* transcript appeared to be unstable, and over time, the RNA sample yielded negative *pgip* RT-PCR results.

RESULTS AND DISCUSSION

Selected transformed plants containing the *pgip* gene can be analysed by RT-PCR to determine whether the *pgip* transgene is expressed. This will greatly assist us in the final outcome of the field trial, since the gene could be present as confirmed by PCR and Southern blot analysis, but the gene might not be expressed. It is essential for us to know that the *pgip* gene is not only integrated into the genome of the transgenic maize, but also expressed.

All RNA isolations from leaves of T_5 generation plants were undertaken using a Promega SV RNA isolation kit. Transgenic plants analysed in this study are denoted in Table 1. RNA samples were tested for the presence of contaminating DNA by subjecting the samples to PCR amplification of the *bar* transgene. Visualisation of the PCR products on an agarose gel indicated that the *bar* gene was indeed amplified, and DNA was therefore present in the RNA sample. We therefore treated the RNA samples with RNase free DNase (Promega), and then attempted to PCR amplify the *bar* gene from the samples. After treatment with DNase I, no *bar* gene PCR products were obtained.

Initially, two T_5 generation plants in a field trial, plant 7 from row 162 (162/7) and plant 1 from row 171 (171/1), were chosen to optimise RT-PCR analysis of the *pgip* transgene. Both of these plants tested positive for both the *bar* and *pgip* transgenes in PCR and Southern Blot analysis (Tasks 1 and 2, Table 3). We initially performed RT-PCR of the *pgip* transgene using the Titan RT-PCR kit (Roche Biochemicals). However, we were unable to obtain *pgip* gene RT-PCR products with this kit, and therefore decided to undertake a reverse transcriptase reaction with C. therm polymerase (Roche Biochemicals) followed by a separate PCR amplification step using Taq DNA polymerase (BioTaq). C. therm polymerase has distinct advantages over the Titan One-Step RT-PCR system as reverse transcription can proceed at higher than normal temperatures (60-70°C as opposed to 50-60°C) which minimizes secondary and hairpin structures in the RNA.

Examination of RT-PCR products, of samples 162/7 and 171/1 subjected to the C.therm Polymerase two step RT-PCR system, on an agarose gel, indicated that the *pgip* gene is expressed in transgenic maize line 162/7 but not in line 171/1 (Figure 1). Thereafter, a two-step RT-PCR was performed on all RNA samples isolated from T_5 generation plants indicated in Table 1. However, analysis of the RT-PCR products on an agarose gel indicated that none of the samples expressed the *pgip* gene (results not shown). Furthermore, sample 162/7, which had previously been shown to express the *pgip* gene (Figure 1), also tested negative. We therefore repeated the two step RT-PCR reaction of 162/7, but this time we used 10 μl of the RT reaction in the PCR amplification step as opposed to 5 μl as was used previously. This resulted in amplification of the *pgip* gene in 162/7, but the amount of *pgip* RT-PCR product was very low and extremely difficult to visualize with the naked eye on an agarose gel (results not shown). It therefore appeared that the RNA samples were unstable and were degrading over time even though samples were stored as aliquots at -70°C.

It is also possible that negative results obtained for the other T_5 generation plants could be due to instability and degradation of the *pgip* transcript prior to RNA isolation. Leaf samples were taken from T_5 generation plants in Potchefstroom and transported to Pretoria on ice, where they were frozen in liquid nitrogen and stored at -70°C. Thereafter, total RNA was isolated from leaf samples over a period of one week. Communication with Dr Jason Pickering (Promega, UK) led us to believe that it would be

better to cut leaf samples, immediately freeze and grind samples in liquid nitrogen on site, and add SV RNA lysis buffer containing guanidium thiocyanate before transporting the samples to the CSIR. This is necessary because as soon as leaf samples are cut from the plant, the leaf produces RNAses, which cause RNA degradation.

CONCLUSION

Results from this study indicated that six of the seven T5 generation plants analysed did not express the *pgip* gene. Although plant 7 from row 162 yielded positive results in *pgip* gene RT-PCR analysis, over time, RNA from this sample no longer produced positive *pgip* expression results. This could be due to degradation of the RNA sample over time, or due to an unstable *pgip* gene transcript. It is also possible that negative results obtained for the other six samples could be due to instability and degradation of the *pgip* transcript prior to RNA isolation.

Table 1: RT-PCR analysis of T5 generation plants in field trial

T_4 generation	T_5 generation		
Plant number	Row number	Plant number	*pgip* gene expression
7/14/5	70	1	-
9/6/3	103	6	-
9/13/22	162	7	+
9/13/22	162	11	-
9/13/37	171	1	-
17/2/6	212	8	-
17/5/3	216	4	-

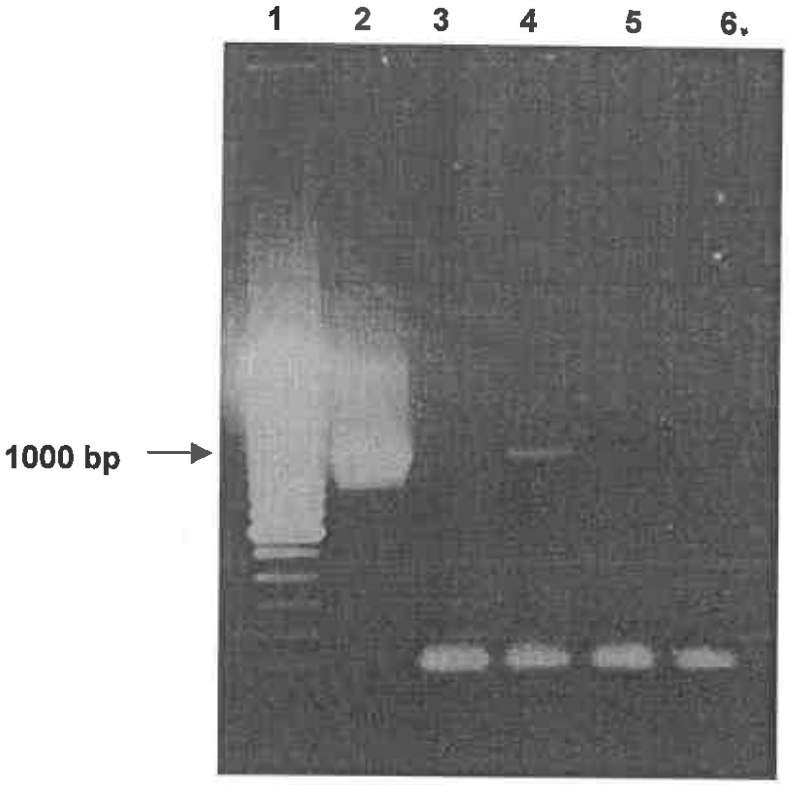

Figure 1. RT-PCR of *pgip* gene from T_5 generation transgenic maize plants. Lane 1, 100 bp molecular marker; lane 2, PCR amplification of *pgip* gene from pAct-pgip (positive control); lane 3, RT-PCR of *pgip* transgene from maize plant 1 in row 171; lane 4, RT-PCR of *pgip* gene from maize plant 7 in row 162; lane 5, RT-PCR of *pgip* gene from A188 (negative control); lane 6, water control.

Task 4: Western blots and PGIP assays of selected T_5 transformants

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SUMMARY

Western blot data indicated the presence of a protein band of approximately 40kDa insize (at the same level as the bean PGIP1 band) in the extracts of the T_5 progeny of transgenic event BB87C/2. The progeny analysed were plant #8 from row 212 (212/8), plant #6 from row 158 (158/6), plant #8 from row 216 (216/8) and plant #5 from row 292 (292/5). However, the same band is observed in the extract from the untransformed maize line A188. The Western blot results did thus not conclusively prove the expression of bean PGIP1 in the transgenic maize lines. In reducing sugar assays, the extract from leaves of the untransformed control plant (A188) and the extracts from the transgenic maize showed no inhibition of the *A. niger* polygalacturonases (PG) and some degree of *S. maydis* PG inhibition. The lack of PGIP activity was not due to the presence of a compound in the maize extracts which blocked a PGIP:PG interaction. This was concluded from control experiments in which bean PGIP was added to the maize extracts and retained its PGIP activity ie. bean PGIP added to the extract of the untransformed control plant (A188) or the extracts from the transgenic maize, showed almost complete inhibition of the *A. niger* PGs and up to 67% inhibition of the *S. maydis* PGs. In parallel to these experiments, CSIR-Biochemtek showed by PCR and Southern blot analysis that the *pgip1* gene was present in the same plant samples. They also demonstrated by RT-PCR that the *pgip1* mRNA was synthesized, although it appeared to be unstable. Taken together, these results indicate that the bean PGIP protein is not being synthesized in an active form in the transgenic maize plants.

AIM

This work was carried out to determine PROTEIN expression of the bean *pgip1* transgene in T_5 progeny of the maize transgenic event BB87C/2 using Western blot and PGIP assays.

It was reported to NAMPO in 1999, that despite the fact that the PGIP extracts from tomato leaves of the tomato cultivar UC82B transformed with the bean *pgip1* gene inhibited the PGs of *Aspergillus niger* and *Stenocarpella maydis* (Berger *et al.*, 2000), the maize PGIP extracts from the maize lines transformed with the bean *pgip1* gene were not able to inhibit the PGs of *A. niger*. The only time that the PGIP extracts from the transgenic maize lines inhibited the

A. niger PGs, was when the extracts were not dialysed. However, the non-dialysed extracts from the untransformed control maize lines also inhibited the *A. niger* PG activity. This demonstrated that there was some other inhibitory compound present in the extracts since dialysis of the same extracts would result in the loss of this inhibitory compound. This inhibitory compound has to be smaller than 10 000 kDa, since dialysis was performed using a 10 000 kDa Mwt cutoff dialysis membrane.

An explanation for the lack of PGIP activity could be that the bean*pgip1* transgene mRNA is not being expressed in the transgenic maize lines. Therefore, it was proposed that CSIR Biochemtek carry out RT-PCR analysis of the lines (see accompanying report, Task 3). Another expalnation could be that the bean PGIP protein is not being synthesized in an active form in the transgenic maize plants. It was therefore proposed that ARC-Roodeplaat perform Western blots and further PGIP assays on the transgenic maize lines as reported here.

RESULTS AND DISCUSSION

PGIP extractions from maize leaf material

The maize PGIP extracts had the following protein concentrations:

Maize Line Event BB87C/2, T_5 generation	Protein concentration (ng/μl)
212/8	307
158/6	358
216/8	299
292/5	370
103/6	323
A188 (untransformed)	400

SDS-PAGE and silver staining of gels of purified bean *pgip1*, bean *pgip2* and the bean *pgip1* extracts from transformed maize lines

The PGIP extracts were separated by SDS-PAGE and visualised with silver staining (results not shown). As positive controls, 300ng of bean PGIP1 purified from transgenic tomato (kindly provided by Professor Cervone) and 2μg bean PGIP2 (crude extract) after PVX-

directed expression in *Nicotiana benthamiana* as reported in Desiderio *et al.* (1997). A protein band of about 40kDa indicated the presence of the PGIP1 and PGIP2 proteins. The maize PGIP extracts yielded typical total protein extract profiles (data not shown).

Western Blots of purified bean *pgip1*, bean *pgip2* and the bean *pgip1* extracts from transformed maize lines

The Western blot results can be seen in Figures 1 and 2. The results in Figure 1 clearly show the presence of the purified bean PGIP1 protein (lane 2), and a fainter signal in lane 3 indicates the presence of the purified bean PGIP2 protein. A protein band of approximately 40kDa (present at the same level as the bean PGIP1 band) is present in the extracts of the transformed maize lines 212/8 (lane 6), 158-6 (lane 7), 216/8 (lane 8, faint band) and 292/5 (lane9, faint band). The positively hybridizing band at 40kDa is especially prominent in the transformed maize line 158/6 (lane 7). However, the same band is observed in the extract obtained from the untransformed maize line A188 (lane 5). Unfortunately, lane 10, containing the extract from the transformed maize line 103/6, was notvisible on the western blot. It was, thus, decided to repeat the western blot, and to include a second extract obtained from the untransformed maize line A188 in order to confirm the presence of the 40kDa protein in these extracts.

The western blot results in Figure 2 clearly show the presence of the purified bean PGIP1 protein (lane 2). A protein band of approximately 40kDa (present at the same level as the bean PGIP1 band) is present in the extracts of the transformed maize lines 212/8 (lane 6), 158/6 (lane 7), 216/8 (lane 8, faint band), 292/5 (lane 9, faint band) and 103/6 (lane 10, faint band). The band is again extremely prominent in the transformed maize line 158/6 (lane 7). However, as before, the same band is also observed in the two extracts obtained from the untransformed maize line A188 (lanes 4 and 5). The Western blot results did thus not conclusively prove the expression of bean PGIP1 in the transgenic maize lines.

An explanation for these results may be that the antibodies raised against bean PGIP cross-react with another maize protein at 40kDa - this could possibly be an endogenous PGIP. However, Southern blots of maize with the bean *pgip1* probe indicate no cross-reaction at the DNA level (see O'Kennedy reports) and attempts at isolation of a maize *pgip* gene by degenerate PCR were unsuccessful (Berger, unpublished). Further experiments could be carried out with a more specific PGIP antibody, however the antibody used here was the best available. Earlier attempts to improve PGIP antbodies by pre-absorbtion against untransformed maize extracts only yielded a less sensitive antibody (Oelofse and Berger, data not shown).

Since Western blots could not give a definitive answer on the presence of PGIP in an active or inactive form, it was decided to perform PGIP:PG inhibition studies with the maize PGIP extracts.

Inhibition of *A. niger* and *Stenocarpella maydis* PGs by the bean *pgip1* transgenic maize PGIP extracts using the Quick PAHBAH assay protocol

PGIP extracts were prepared from the leaves of field grown transgenic maize using a high salt extraction buffer. The decrease in *A. niger* PG activity by bean PGIP, the positive control, was 94% (Figure 3, compare column 2 with 1). The extract from leaves of the untransformed control plant (A188) and the extracts from the transgenic maize showed no inhibition of the*A. niger* PGs (Figure 3, compare columns 3 - 8 to column 1). These extracts were also used to test for inhibition of *Stenocarpella maydis* PG extracts. The decrease in*S. maydis* PG activity by bean PGIP, the positive control, was 55% (Figure 4, compare column 2 with 1). The extract from leaves of the untransformed control plant (A188) and the extracts from the transgenic maize lines all showed some decrease in *S. maydis* PG activity, although it is not known if the reductions are statistically significant (Figure 4, compare columns 3 - 8 to column 1).

These results indicate that the bean PGIP protein is not being synthesized in an active form in the transgenic maize plants. Similar assay results were reported in the 1999 NAMPO report. The data is suggestive that there may be some endogenous maize PGIP activity which inhibits *S. maydis* PG activity (Figure 4) but does not inhibit *A. niger* PG activity (Figure 3). However, this would have to be verified statistically.

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 hypocotyl PGIP extract 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 PGIP inhibiting compound was possibly present in the maize leaf extracts. The decrease in *A. niger* PG activity by bean PGIP, the positive control, was 99% (Figure 5, compare column 2 with 1). All the bean PGIP spiked extracts, the extract from leaves of the untransformed control plant (A188) and the extracts from the transgenic maize, showed almost complete inhibition of the *A. niger* PGs (Figure 3, compare columns 3 - 8 to column 1). The same experiment was repeated with the *S. maydis* PGs. The decrease in *S. maydis* PG activity by bean PGIP, the positive control, was 67% (Figure

6, compare column 2 with 1). All the extracts spiked with bean PGIP, namely the extract from leaves of the untransformed control plant (A188) and the extracts from the transgenic maize, showed up to 67% inhibition of the *S. maydis* PGs (Figure 6, compare columns 3 - 8 to column 1). These results indicate that the PGIP extracts from the maize lines do not contain a PGIP inhibiting compound. These control experiments indicate that if bean PGIP protein was not synthesized in an active form in the transgenic maize plants, PGIP activity should be detectable.

CONCLUSION

Western blot data indicated the presence of a protein band of approximately 40kDa in size (at the same level as the bean PGIP1 band) in the extracts of the transformed maize lines 212/8, 158/6, 216/8 and 292/5. However, the same band is observed in the extract from the untransformed maize line A188. The Western blot results did thus not conclusively prove the expression of bean PGIP1 in the transgenic maize lines.

The extract from leaves of the untransformed control plant (A188) and the extracts from the transgenic maize showed no inhibition of the *A. niger* PGs and some degree of *S. maydis* PG inhibition. Control experiments indicated that the lack of PGIP activity was not due to the presence of a compound in the maize extracts which blocked a PGIP:PG interaction. These results indicate that the bean PGIP1 protein is not being synthesized in an active form in the transgenic maize plants.