Stable transformation of Hi-II maize using the particle inflow gun

Embryogenic type II callus, initiated from cultured immature zygotic embryos of the maize line Hi-II, was transformed by microprojectile bombardment using a particle inflow gun. This highly regenerable maize line was transformed with the plasmid pAHC25, a dual expression vector containing the uidA *reporter gene and the* bar *selectable marker gene, both under the control of the maize ubiquitin promoter. Transgenic plants were obtained from 10 independent transformation events. Transmission of the* bar *and* uidA *genes to the progeny of three of these lines was demonstrated by Southern blot analysis and by transgene expression.*

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 disease or insect resistance or improved nutritional 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 to be very recalcitrant to *Agrobacterium* infection. High efficiency *Agrobacterium*-mediated transformation of maize (*Zea mays* L.) has been reported only recently.[1] The use of microprojectile bombardment-mediated transformation of embryogenic tissue culture material, with the subsequent generation of fertile transgenic plants, has overcome the regeneration problems associated with the production of plants from cereal protoplasts[2,3] and bypassed *Agrobacterium* host-specificity. In 1993 the first paper was published on the field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*.[4]

In maize callus cultures, higher transient expression was obtained using the maize ubiquitin promoter compared with the commonly used CaMV 35S promoter.[5] Similarly, the promoter region of the rice *actin 1* gene has been shown to direct high level constitutive expression in monocots, such as maize.[6,7] Plasmid pAHC25 is a dual expression vector which contains the *uidA* reporter gene that encodes the β-glucuronidase (GUS) enzyme and, for selection, the *bar* gene, that encodes the enzyme phosphinothricin acetyl transferase (PAT). Phosphinothricin acetyl transferase inactivates the herbicidal compound phosphinothricin (PPT) by acetylation. Phosphinothricin is the active ingredient of the herbicide Basta® or Ignite® (AgrEvo). Both the *uidA* and *bar* genes are under the control of the maize *Ubi1* promoter, first exon and first intron, and the nopaline synthase (NOS) terminator.[5,8]

In this paper, we demonstrate stable transformation of maize, by introducing the *uidA* and *bar* genes into Hi-II maize (A188 × B73)[9] embryonic callus tissue, using a simple and inexpensive device, the particle inflow gun (PIG)[10,11] Regenerating calli were selected on bialaphos-containing media and the presence of the transgenes in primary transformants was confirmed by the polymerase chain reaction (PCR). The presence and copy number of these genes were studied in subsequent generations. Functional expression of PAT in these plants was demonstrated by their resistance to typical necrosis associated with localised applications of herbicide to leaves.

Materials and methods

Tissue culture and plant growth conditions. Seed of partially inbred lines (Hi-II Parent A and B) from the cross A188 × B73, obtained from C.L. Armstrong (Monsanto, St Louis, Missouri), was germinated between layers of absorbent brown paper and cellulose wadding (Multa Seed). Seedlings were planted in a soil mix consisting of red soil, rough sand and compost (in the ratio 1:1:1), and were watered daily with a soluble fertiliser (Hortichem N:P:K at 3:1:5, Ocean Chemicals) until flowering.

Excised immature embryos (approximately 1.5 mm long; 10–14 days post pollination) were cultured according to Armstrong and Green[12] on N6 1-100-25-Ag medium.[13] Embryos were aseptically removed from the immature kernels and placed with the axis in contact with the medium. Cultures were incubated at approximately 25°C, under low light conditions (1.4 μE $m^{-2}s^{-1}$). Embryogenic callus cultures were subcultured every 14 days to fresh media. Maturation medium (0T6S) contained MS salts,[14] B5 vitamins,[15] 6 g l^{-1} agarose, and 60 g l^{-1} sucrose. The regeneration medium (0T2S) contained MS salts, B5 vitamins, 20 g l^{-1} sucrose, 2 g l^{-1} gelrite and 4 g l^{-1} bacto agar. The pH of the media was adjusted to 5.8 before autoclaving. Regeneration was performed at approximately 25°C under fluorescent lights at 70–80 μE $m^{-2}s^{-1}$, and with a 16 hours day:8 hours night regime.

Embryos from R_1 and subsequent transgenic generations were cultured[16] and plated with the scutellum in contact with 0T2S medium, containing 1 mg l^{-1} bialaphos.

Plasmid preparation and microprojectile bombardment. Plasmid pAHC25 DNA was extracted from overnight *E. coli* cultures using a Wizard maxiprep kit according to the supplier's recommendation (Promega). DNA delivery parameters were optimised based on transient expression of the *uidA* gene in embryogenic callus cells.

Embryonic callus tissue, initiated from immature embryos, was subjected to osmoticum treatment three hours before bombardment.[17] Embryogenic callus tissue was spread in the middle (0–1-cm diameter) of the 9-cm Petri dish, containing $N6_{10}$ medium with 8 g l^{-1} agarose, 0.2 M sorbitol and 0.2 M mannitol. A bombardment mixture was prepared by precipitating plasmid DNA on tungsten particles (M10, 0.2–2 μm, Sylvania Chemicals/Metals) as described by Vain *et al.*[11] The bombardment conditions were 600 kPa helium pressure, 15.5-cm distance from syringe filter to target, and a 500 μm nylon mesh baffle placed 9 cm above the target. Two microlitres of the bombardment mix (0.16 μg of plasmid DNA) was placed on the plastic syringe filter, a vacuum of approximately –87 kPa was applied and the particles were discharged with a 50-ms burst. All experiments were conducted with the PIG. Sixteen hours after bombardment, calli were transferred from the $N6_{10}$ media containing osmoticum to $N6_{10}$ media without osmoticum.

Selection and regeneration of transformants. Selection for PPT-resistant maize tissue was initiated 48 h after bombardment by placing the callus cultures on a solidified $N6_{10}$ medium containing 1–20 mg l^{-1} of bialaphos. Using different selection protocols (3, 5, 8 or 20 mg l^{-1} bialaphos), resistant cell colonies emerged on the selection plates approximately 6–10 weeks after bombardment. Bialaphos-resistant callus lines were maintained and expanded on selection medium. Callus was transferred to maturation medium (0T6S), containing 1 mg l^{-1} bialaphos, to form somatic embryos. 0T2S medium, containing 1–10 mg l^{-1} bialaphos, was used for regeneration of putative transformants. Developing plantlets were subcultured at 2–3-week intervals

until they were 8–10 cm in length. These were then transferred to a mix of perlite and vermiculite (1:1) and hardened-off in a mist bed. Fungal infection was prevented by dipping roots in 0.1% (m/v) Benlate-DF. In the mist bed, plantlets were watered daily with Hoagland's solution,[18] and were sprayed with water every 15 min for 20 s. Plantlets taller than 15 cm were removed to pots in the greenhouse.

Analysis of transformed tissues and plants

β-*Glucuronidase activity.* Bombarded tissues were assayed for GUS expression 48 h after bombardment. Embryogenic callus culture and immature embryo tissue were stained for GUS activity according to Jefferson *et al.*[19]

DNA extraction. Genomic DNA was extracted from maize transformants using the mini-extraction procedure of Dellaporta *et al.*[20] Briefly, approximately 2.5 g of maize leaf tissue was ground to a powder in liquid nitrogen, 15 ml of extraction buffer (0.1 M Tris-Cl at pH 8.0; 0.5 M NaCl; 50 mM EDTA; 10 mM β-mercaptoethanol) and 1 ml 20% SDS were added and incubated for 1 h at 65°C. Five millilitres of 5 M KOAc, pH 5.2, was added and the mixture incubated on ice for 10 min. After centrifugation (10 000 *g* × 10 min), nucleic acids were precipitated from the supernatant by addition of 0.1 volumes of 3 M NaOAc (pH 5.2) and 0.6 volumes of isopropanol and centrifugation as before. Pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), treated with RNase A and DNA recovered by ethanol precipitation. Final DNA pellets were resuspended in 100 µl TE buffer and the concentration was determined by comparison to known standards in gel electrophoresis.

Polymerase chain reaction (PCR) analysis. GUS (GGTGGGAAAGCGCGTTACAAG and GTTTACGCGTTGCTTCCGCCA) and *bar* (CATCGAGACAAGCACGGTCAACTTC and CTCTTGAAGCCCTGTGCCTCCAG) specific primers were used to amplify 1.2 kilobase and 0.28 kilobase fragments, respectively, from genomic DNA preparations of putative transgenic maize plantlets. PCR was performed in 10-µl reaction volumes using the following assay conditions: 10 mM Tris-Cl (pH 9.0 at 25°C); 50 mM KCl; 1.5 mM $MgCl_2$; 1% (v/v) Triton X-100; 100 µM of each dNTP; 0.5 µM of each primer; 0.5 U Taq polymerase (Promega) and approximately 50 ng of genomic DNA. Samples were overlaid with 10 µl mineral oil, denatured at 94°C for 2 min, and subjected to 35 cycles of amplification in a Hybaid thermal cycler. Each cycle consisted of a denaturing step at 94°C for 20 s, an annealing step at 64°C for 30 s and an elongation step at 72°C for 45 s. PCR products were separated in 1.4% (m/v) agarose gels and visualised by staining with ethidium bromide.

Southern blot analysis. Five microgrammes of maize genomic DNA was digested with *Sac* I and the fragments were separated in 0.8% agarose gels using TAE (0.04 M Tris-acetate, 0.002 M EDTA, pH 8.5) buffer. An adaptation of the classical Southern blot analysis (Southern, 1975) was employed to detect single copy genes and to estimate transgene copy number in maize transformants. Following gel electrophoresis, gels were sequentially soaked in denaturing (1.5 M NaCl; 0.5 M NaOH, 2 × 15 min) and neutralising (0.5 M Tris-Cl, pH 7.5; 3 M NaCl, 2 × 15 min). A standard upward capillary blotting assembly, using 20 × SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0) buffer, was set up and blotting allowed to proceed for 8 h. DNA was fixed to nylon membranes by exposing the membranes (DNA side down) for 3 min on a 312-nm transilluminator (UVP, model TM-40) and stored dry until hybridisation.

Both *uidA* and *bar* genes were excised from pAHC25 by restriction digestion, the respective gene fragments gel purified, and labelled with digoxygenin (DIG) by random prime labelling as recommended by the supplier (Boehringer-Mannheim). Effectiveness of label incorporation was estimated by comparison to supplied, known standards in mock hybridisations.

Blots were prehybridised at 42°C in hybridisation solution (5 × SSC; 2% (m/v) blocking reagent (Boehringer-Mannheim); 0.1% (m/v) sarkosyl; 0.2% (m/v) SDS; 50% (v/v) formamide, 20 ml 100-cm^{-2} membrane) for 3 h. Hybridisations were carried out overnight at 42°C in hybridisation solution (5 ml 100-cm^{-2} membrane), containing 25 ng ml^{-1} denatured DIG-labelled GUS or *bar* probe. Blots were then subjected to 2 × 5-min washes under low stringency conditions (2 × SSC, at room temperature) and 3 × 15-min high stringency washes (0.5 × SSC, at 68°C). Hybridisation signals were detected by autoradiography using alkaline phosphatase-conjugated DIG antibodies and the substrate CDP-Star, as described by the supplier (Boehringer-Mannheim).

Herbicide application. A 2% (v/v) Basta; 0.01% (v/v) Tween 20 solution was applied to both surfaces of selected leaves of young transgenic plantlets using a small sponge. Alternatively, this solution was sprayed on mature greenhouse-grown plants, which were then observed over a two-week period. The prescribed application rate for this commercial formulation is 0.5–1% (v/v), and 400 l ha^{-1}.

Results and discussion

Optimisation of bombardment conditions by transient expression. A number of conditions were optimised before high levels of transient GUS expression were obtained after bombardment of embryogenic callus cultures with pAHC25 (Fig.1). Increasing agarose concentration from 6 g l^{-1} to 8 g l^{-1} resulted in an almost 30% increase in the number of blue foci per mg of callus bombarded. A helium pressure of 600 kPa yielded the highest levels of transient expression when bombardment pressures of 400, 500, 600 and 700 kPa were compared. Bombardment of Type II callus placed 0–1 cm, 1–2 cm or 2–3 cm from the centre of the Petri dish, resulted in approximately 13, 4.2 or 0.4 blue foci per mg of callus, respectively. Replacement of spermidine (free base) with spermine tetrahydrocloride caused the number of blue foci per mg of callus to drop by 36%. Precipitation of DNA onto gold particles (Biorad, microcarrier, 1.0 µm),[21] in contrast to tungsten particles, was also performed. This resulted in a 90.4% drop in the number of blue foci obtained per mg of callus compared with bombardment as described under materials and methods.

It is apparent that the conversion rate of transient to stable transformation events is low. Optimised or maximised transient activity does not necessarily result in optimal or even any stable transformation.[22] One factor that limits the recovery of stable transformants is injury to the cells. Injured cells can transiently express a foreign gene but such cells may not be capable of further division or growth.[23] Therefore, callus cultures were treated before and after bombardment with osmoticum, since it was shown previously that osmotic treatment of maize cells resulted in a 6–7-fold increase in the number of stable transformants.[17] These authors believed that the basis of osmotic treatment resulted from the plasmolysis of cells, which may have reduced cell damage by preventing extrusion of the protoplasm from bombarded cells.[11] Vain *et al.*[11] reported damage in the centre of the blast area and that it could be eliminated by using a baffle. Similarly, in our study, a massive tissue displacement occurred in experiments when bombarding without a baffle, so we used a

Fig. 1. Transient GUS activity in embryogenic callus cells 48 h after bombardment.

500-μm nylon mesh baffle in routine bombardment experiments.

Selection of transformed tissues and regeneration of R_0 plants. Putatively transformed callus tissue was identified by its vigorous growth on bialaphos-containing medium, indicating the stable integration and functionality of the *bar* gene in the maize genome. Growth of these callus lines, which were maintained on bialaphos, was similar to that of control tissue on non-selective medium. Non-transformed controls exhibited brown, watery callus on selection media. Bialaphos-resistant callus tissue was removed 6–8 weeks after bombardment and maintained as individual cell lines on medium containing 3–20 mg l^{-1} bialaphos. Ten such transformed lines were identified, but fertile transgenic plants from only three of these could be regenerated.

Transgenic plants could be regenerated on 0T2S medium, containing 1–3 mg l^{-1} bialaphos, as long as one year after bombardment. Abnormal phenotypic characteristics, such as dwarfism, tassel ears and underdeveloped tassels and ears, were observed in some R_0 plants. Since the progeny (R_1 to R_3) of these resembled that of control plants, that were germinated from seed, these aberrations can probably be ascribed to the stress induced by tissue culture. Progeny (R_1) were obtained from transformed (R_0) plants by back-crossing with seed-derived A188 plants or by self-pollination.

Analysis of transformants

β-Glucuronidase activity. Bialaphos-resistant callus lines were tested for GUS activity by immersing a 2-mm^2 piece of callus tissue in GUS assay mix. Resistant callus displayed uniformly dark-blue staining, indicative of the stable integration of GUS (Fig. 2*A*). Control callus, however, remained yellowish-white in the presence of the substrate. Lines that were actively growing on the selection medium, but not showing any blue colouring in the GUS assay, were analysed by PCR for the presence of the *bar* gene. Although the *bar* gene was present in most of these (results not shown), we did not continue any further experimentation

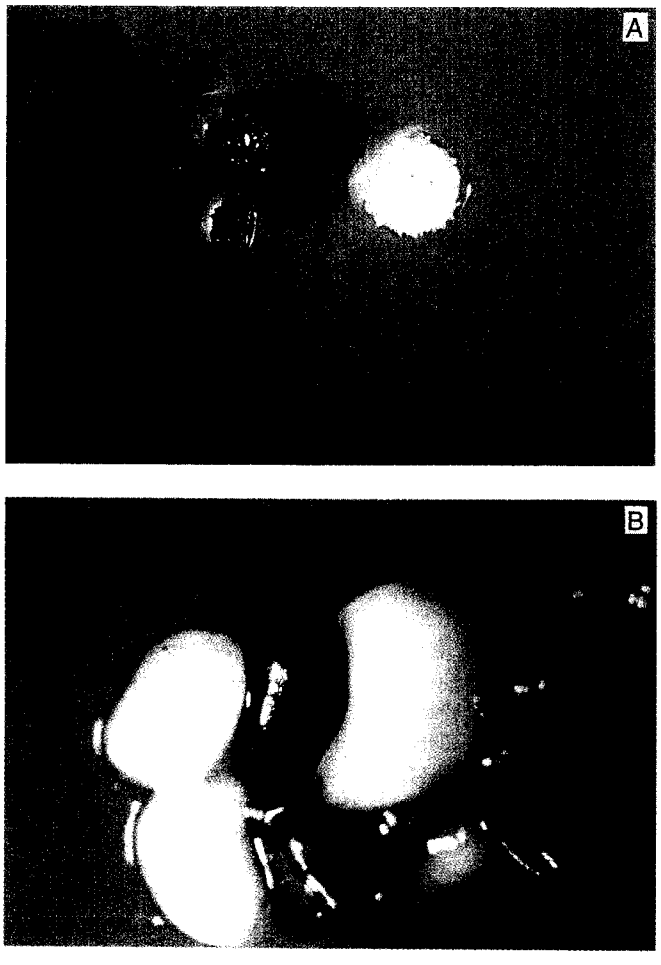

Fig. 2. GUS expression in different tissues of transgenic and control plant material. *A*, Transgenic callus (*right*) and control callus (*left*). *B*, Immature embryos of a transgenic plant cross pollinated with the line A188.

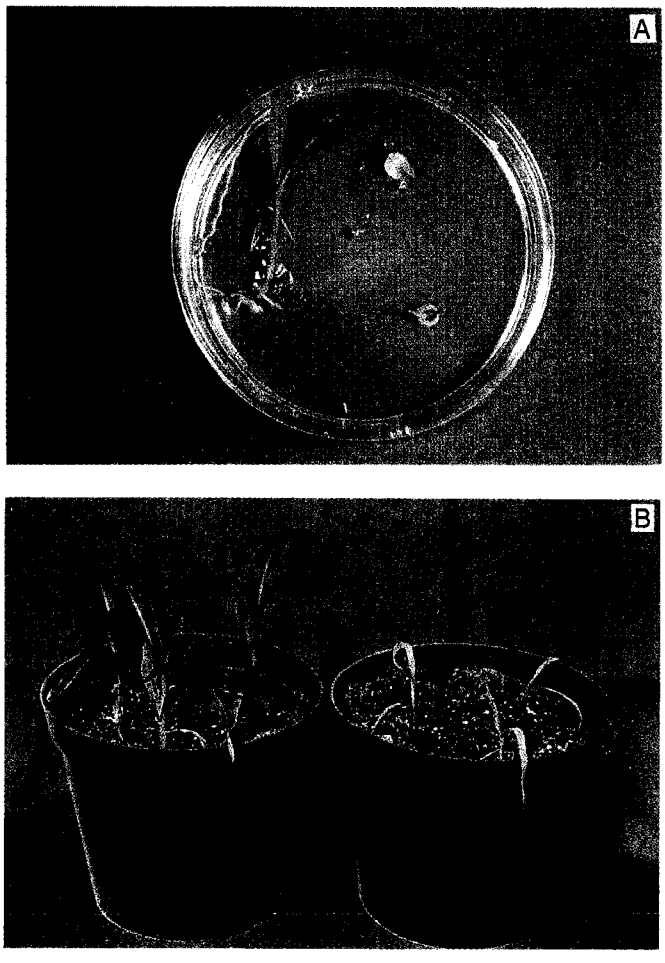

Fig. 3. Herbicide resistance test. *A*, A germination test of R_1 immature embryos of a transgenic line on medium containing 1 mg l^{-1} bialaphos. *B*, Basta®-sprayed transgenic plants (*right*) and non-transformed plants (*left*).

with these lines. Approximately half of the GUS assayed mature embryos of cross-pollinated transgenic plants stained blue, which indicated that the *uidA* gene was introduced in the maize genome, and transmitted to the next generation (Fig 2*B*). The ratio of GUS expression in the mature embryos after selfing was not determined, since the GUS assay kills the embryos and therefore eliminates valuable transgenic material. The intensity of GUS-staining in the immature embryos of transformants depended on their developmental stage, with younger immature embryos displaying a more intense blue (data not shown). This is consistent with reports of Zhong *et al.*[7]

Herbicide resistance. Functional activity of PAT was assessed by localised application of Basta to labelled leaves. No necrosis was observed on leaves of R_0 plants of lines BB13C/1, BB12C/9 and BB14C/1. Control plants, however, developed necrotic lesions within 7 days and eventually died. Similar Basta resistance was observed in R_1 and subsequent generations (Fig. 3*A* and *B*).

Polymerase chain reaction. The presence of both *bar* and GUS genes was confirmed by PCR (data not shown). Only lines containing both genes were maintained and plants regenerated from them. Ten such lines, from five different bombardment experiments, were identified.

Southern blot analysis. For the estimation of transgene copy number, the reliable non-radioactive detection of a single transgene copy in genomic maize DNA needed to be established. For this purpose, genomic DNA of untransformed maize plants was spiked with plasmid DNA in quantities simulating 1 and 10 copies of the GUS gene. These were subjected to Southern blot analysis and probed with a DIG-labelled internal fragment of the GUS gene. A reconstruction of a single copy of the GUS gene could clearly be distinguished (Fig. 4, A188).

Sac I was chosen to determine gene copy number as the enzyme has a single restriction site between the GUS and *bar* genes on pAHC25, and does not cut these genes internally. Hybridisation of *Sac* I-digested genomic DNA confirmed the presence of the GUS gene in all of the putative transformants that were tested and yielded unique transgene integration patterns for 10 of the 11 transformed lines (Fig. 4), suggesting at least 10 independent transformation events. When undigested genomic DNA was probed, hybridisation to a high molecular band was observed, indicating integration of the transgenes into maize chromosomal DNA (data not shown).

Copy number estimations indicated that no plants containing a single transgene copy were regenerated. Transformants BB12C/9, BB12C/10, BB13C/1 and BB14C/1 seemed to have between three and five copies, while BB11C/4, BB12C/5, BB12C/7 and BB14C/2 each have eight or more copies of the *bar* transgene (Fig. 4*A*). Similar results were obtained after the blot was stripped and re-probed with a DIG-labelled GUS probe (data not shown). The introduction of multiple copies of the same gene into plants often leads to a phenomenon called transgene silencing or co-suppression. The exact mechanism of this phenomenon in transgenic plants is not fully understood, but the selection of transgenic plants with one or few transgene copies has become desirable. Microprojectile-mediated transformation normally introduces multiple copies into plants, as is also evident from our results. However, no differences in resistance were observed when mature plants of lines BB12C/9 (3 copies of *bar*) and BB14C/2 (>8 copies) were sprayed with Basta.

Inheritance of the *bar* and GUS genes in the R_1, R_2 and R_3 progenies of lines BB12C/9, BB13C/1 and BB14C/1 was confirmed by Southern blot analysis. Integration patterns for the *bar* gene in the R_1 and R_3 generations remained identical (Fig. 4*B*).

Segregation of the *bar* gene was determined by embryo culture on bialaphos-containing medium as described by Weymann *et al.*[16] Table 1 presents segregation data of R_1 to R_3 progeny from four transformed R_0 plants. These initial results indicate a 1:1 segregation in crosses and a 3:1 segregation in selfing. Mendelian segregation appears to have been obtained for the progeny of lines BB12C/9, BB13C/1, and BB14C/1, but not for line BB12C/10 (Table 1). Transgenic plants were used as either the female or male parent. This germination test of immature R_1 to R_3 embryos on bialaphos-containing medium was useful for determining Mendelian segregation and for selecting individuals that were actively expressing the *bar* gene, but it was not a good indicator of the presence or absence of the *bar* gene (Fig. 3*A*).

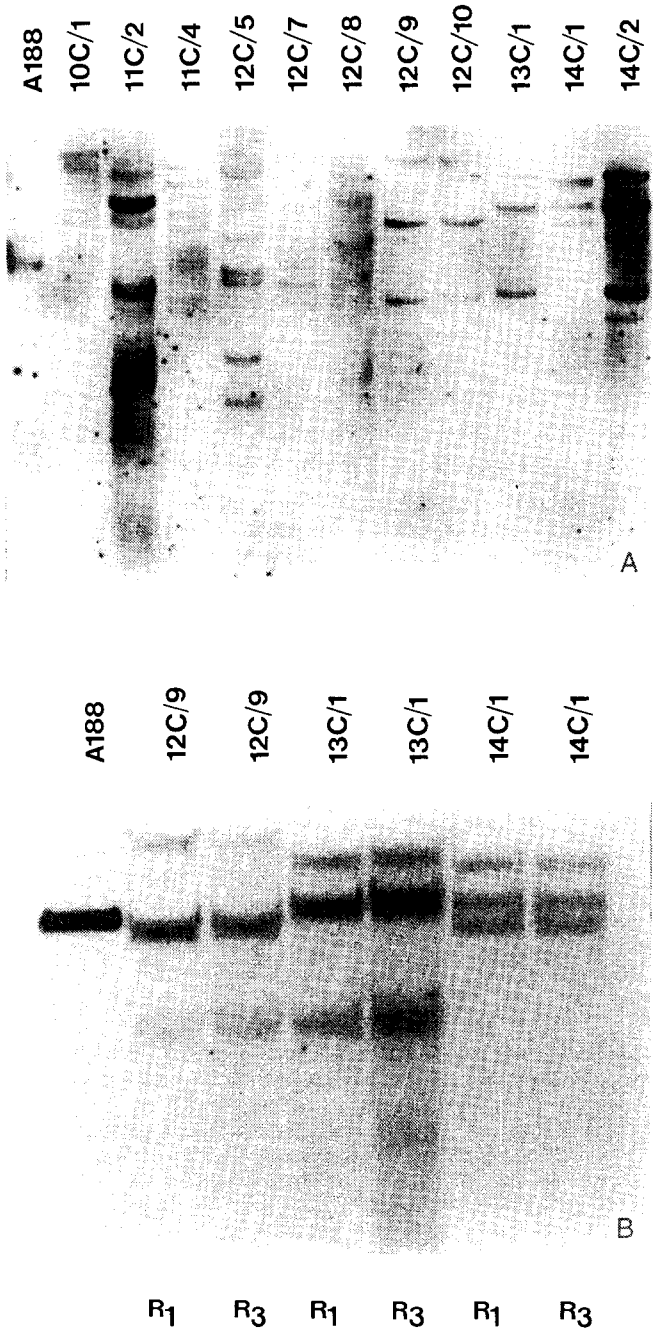

Fig. 4. DNA hybridisation analysis of callus, R_1 and R_3 transgenic plants. *A*, The independently transformed callus lines are designated 10C/1 to 14C/2, representing callus lines BB10C/1 to BB14C/2. *B*, The progeny (R_1 and R_3) of transgenic plants. Genomic DNA was digested with *Sac I* and hybridised with DIG-labelled GUS probe.

Table 1. Segregation of *bar* gene activity in R_1, R_2 and R_3 progeny of transgenic maize plants. In each fertilisation, the pollen recipient (female) is listed first.

Line	R_1 germination + : −		R_2 germination + : −		R_3 cell suspension culture + : −	
BB13C/1	9 : 16	(BB13C/1 × A188)	Not determined		20 : 27	(BB13C/1 × A188)
	11 : 17	(BB13C/1 × A188)				
BB12C/9	2 : 1	(BB12C/9 × A188)	55 : 17	(Selfed)	Not determined	
BB14C/1	1 : 1	(Selfed)	47 : 29	(Selfed)	33 : 57	(Selfed)
	20 : 2	(BB14C/1 × A188)				
BB12C/10	1 : 0	(Selfed)	0 : 223	(Selfed)		
	32 : 106	(A188 × BB12C/10)				

The data in Table 1 show that the *bar* gene was stably transmitted to the R_2 and R_3 generations in an apparent Mendelian manner. The prediction of a 1:1 segregation ratio in these out-crossed regenerants is based on the assumption that there is one expressed copy of *bar* or all expressed copies are integrated at a single locus.

Conclusion

We have provided cumulative evidence for stable transformation of Hi-II maize using the particle inflow gun, which is a simple and inexpensive device for transformation. Our study confirmed the efficient expression of *bar* and GUS genes in fertile transgenic Hi-II maize plants by the maize ubiquitin promoter element of pAHC25. Our current goal is to use the PIG for stable transformation of South African elite maize lines with a view to introducing agronomically important genes.

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M.M. O'KENNEDY, J.T. BURGER* and T.G. WATSON

Division of Food Science and Technology,
CSIR, P.O. Box 395,
Pretoria, 0001 South Africa
(e-mail: mokenned@csir.co.za)

**Current address: Department of Genetics,*
University of Stellenbosch,
Private Bag X1,
Matieland, 7602.

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