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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Transformation of elite white maize using the particle inflow gun and detailed analysis of a low-copy integration event

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Abstract Elite white maize lines W506 and M37W were transformed with a selectable marker gene (*bar*) and a reporter gene (*uidA*) or the polygalacturonase-inhibiting protein (*pgip*) gene after bombardment of cultured immature zygotic embryos using the particle inflow gun. Successful transformation with this device did not require a narrow range of parameters, since transformants were obtained from a wide range of treatments, namely pre-culture of the embryos for 4–6 days, bombardment at helium pressures of 700–900 kPa, selection-free culture for 2–4 days after bombardment and selection on medium containing bialaphos at 0.5–2 mg l^{-1}. However, bombardments with helium pressures below 700 kPa yielded no transformants. The culture of immature zygotic embryos of selected elite white maize lines on medium containing 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 20 m*M* L-proline proved to be most successful for the production of regenerable embryogenic calli and for the selection of putative transgenic calli on bialaphos-containing medium after transformation. Transgenic plants were obtained from four independent transformation events as confirmed by Southern blot analysis. Transmission of the *bar* and *uidA* genes to the T_4 progeny of one of these transformation events was demonstrated by Southern blot analysis and by transgene expression. In this event, the transgenes *bar* and *uidA* were inserted in tandem.

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Keywords Elite white maize transformation · Cereals · Immature embryos · Biolistics · Fertile transgenic plants · *pgip* gene

Abbreviations *2,4-D*: 2,4-Dichlorophenoxyacetic acid · *GUS*: β-Glucuronidase · *PIG*: Particle inflow gun

Introduction

Globally, the improvement of maize by genetic engineering has focussed mainly on yellow maize varieties, which form the bulk of the supply to the animal feed market. Of the 700 million tons of maize produced worldwide annually, more than 98% is yellow maize. However, in certain regions of the world, such as Southern Africa, white maize varieties are produced in larger quantities due to the requirements of the local market. For example, of the approximately 10 million tons harvested annually in South Africa, 65% is made up of white maize varieties used for human consumption, and the balance, yellow maize, is utilised by the animal feed industry. Yellow maize varieties transformed in the northern hemisphere with the *Bacillus thuringiensis* (Bt) gene for insect resistance are currently being grown commercially in South Africa. It would be a time-consuming exercise to introduce this transgene into local white maize varieties by backcrossing. In addition, the recovery of progeny with both the transgenic trait and suitable agronomic traits is often difficult due to incompatible heterotic groups and poor combining ability. Consequently, genetic engineering can probably be more successfully applied to maize improvement in developing countries by identifying and isolating agronomically useful genes and subsequently introducing them directly into the genome of selected elite white maize lines.

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 the field performance of elite transgenic maize plants expressing an insecticidal pro-

tein derived from *B. thuringiensis* that had been transformed using the BioRad helium-driven biolistic device (PDS-1000He). Subsequently, the stable transformation of elite maize by microprojectile bombardment of immature embryos has been performed by a number of laboratories using the BioRad PDS1000 He gun and their own proprietary elite maize lines (Armstrong and Songstad 1993; Koziel et al. 1993; Hill et al. 1995; H. Pierce, Pioneer Hi-Bred International, personal communication). Elite lines of maize are extremely diverse with respect to tissue culture characteristics and transformability response (H. Pierce, personal communication). Therefore, transformation systems need to be optimised independently for different elite maize lines, such as the white genotypes grown in Southern Africa.

Prior to the recent development of *Agrobacterium*-mediated transformation of maize (*Zea mays* L.), the production of genetically engineered maize relied on biolistic transformation. High-efficiency *Agrobacterium*-mediated transformation of maize 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).

Previously, a laboratory strain of maize, Hi-II (A188 × B73), was stably transformed in our laboratory by bombarding highly regenerable type-II maize embryogenic calli using the particle inflow gun (O'Kennedy et al. 1998). However, type-II calli are rarely produced for any of the elite maize lines when these lines are tissue-cultured.

The aim of the investigation reported here was to optimise the parameters for elite maize transformation using the particle inflow gun, targeting scutellum cells of cultured immature zygotic embryos. We demonstrate the stable transformation of elite white maize breeding lines by biolistic-mediated transformation using this simple and inexpensive device. In order to develop a reliable transformation protocol for selected elite white maize lines, we used the selectable marker gene, *bar*, and the reporter gene, *uidA*. In addition, the bean polygalacturonase inhibiting protein-1 (*pgip-1*) gene was used in some experiments in an attempt to confer fungal resistance on maize lines (Berger et al. 2000).

Materials and methods

Tissue culture and plant growth conditions

Seed of elite line M37W was germinated between layers of absorbent brown paper and cellulose wadding (Multa Seed). The seedlings were planted in a soil mix consisting of red soil, rough sand and compost (1:1:1) and were watered daily with a soluble fertiliser (Hortichem N:P:K at 3:1:5; Ocean Chemicals) until flowering. Cobs were harvested 10–14 days post-pollination for embryo excision. Seed of elite lines W506 and W508 were planted in the field, and the cobs were harvested 10–14 days post-pollination for embryo excision.

Excised immature zygotic embryos (approximately 1.5 mm long) from the harvested cobs were cultured on MS medium (Murashige and Skoog 1962) containing 2 mg l^{-1} 2,4-D, 20 m*M* L-proline, 30 g l^{-1} sucrose and 8 g l^{-1} agar (Oxoid technical agar no. 3); this medium was designated 2MS+Pro medium. Media D (Duncan et al. 1985) and N6C1SN (Bohorova et al. 1995) were also tested as callus induction media. Embryos were aseptically removed from the immature kernels and placed with the axis in contact with the medium. Between 80 and 120 embryos per maize line, in two to three independent experiments, were plated on each of the three media described above. Cultures were incubated at 25°C, under low light conditions (1.4 µE m^{-2} s^{-1}). Immature embryo cultures were subcultured every 14 days to fresh media. After 1 month, calli induced on 2MS+Pro medium were subsequently cultured on regeneration medium, designated 0MS, containing MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar. The pH of the medium was adjusted to 5.8 before autoclaving. After 1 month, calli induced on medium D or medium N6C1SN were regenerated on medium H as described by Duncan et al. (1985) or on MSR medium as described by Bohorova et al. (1995), respectively. Regeneration was performed at 25°C under a 16 h (day)/8 h (night) photoperiod with light being supplied by fluorescent tubes at an intensity of 70–80 µE m^{-2} s^{-1}. Regenerated plantlets were hardened-off as described by O'Kennedy et al. (1998).

Transgenic plants and their progeny were self-pollinated or cross-pollinated with plants originating from the same transformation event. Immature embryos of transgenic plants were isolated and germinated on bialaphos-containing medium as previously described by Weymann et al. (1993).

Plasmid constructs and microprojectile bombardment

Plasmids

Plasmid pAHC25 is a dual expression vector which contains the *uidA* reporter gene that encodes the GUS enzyme, and for selection, the *bar* gene, which encodes the enzyme phosphinothricin acetyl transferase (PAT) that confers herbicide resistance. PAT inactivates the herbicidal compound phosphinothricin (PPT) by acetylation. L-PPT is the active ingredient in several herbicide formulations, such as Basta® (Hoechst AG, Germany), which contains glufosinate ammonium, the salt of a chemically synthesized racemic mixture D,L-PPT, and Herbiace® (Meiji Seika Kaisha, Japan), which contains bialaphos, a tripeptide consisting of L-PPT and two alanine residues. Both the *uidA* and *bar* genes are under the control of the maize *Ubi1* promoter, first exon and first intron, and the nopaline synthase terminator (Christensen and Quail 1996).

The construct pUBI-PGIP contains the *pgip* gene that codes for the polygalacturonase–inhibiting protein 1 (PGIP1) from *Phaseolus vulgaris* (bean), and the selectable marker gene *bar*. Both these genes are under the control of the maize *Ubi1* promoter. pUBI-PGIP was constructed by replacing the *uidA* gene in pAHC25 with the *pgip* gene from pLD1 (Berger et al. 2000). Molecular manipulations were done according to standard protocols (Sambrook et al. 1989). Restriction and DNA modifying enzymes were supplied by Roche Biochemicals. Plasmid pLD1 was linearised by digestion with *Xba*I. The resulting 5′ single-stranded overhangs were filled in by adding dNTPs and the Klenow fragment of DNA Polymerase I. The resulting blunt end of the *pgip* fragment was required for ligation into the *Sma*I site of pAHC25. Subsequently, the *pgip* fragment was liberated from pLD1 by digestion with *Sac*I. The *pgip* fragment was separated from the vector fragment by agarose gel electrophoresis and the *pgip* fragment purified from the gel using a Nucleotrap kit (Macherey-Nagel). Yields were estimated by comparison to known DNA standards (Gibco BRL). The *uidA* gene was excised from pAHC25 by digestion with *Sma*I and *Sac*I. The vector and *uidA* fragments were separated and purified by agarose gel electrophoresis as described before.

The modified *pgip* fragment was ligated into the *Sma*I and *Sac*I sites of pAHC25 using a rapid ligation kit (Roche Biochemicals). Ligation mixes of different vector insert molar ratios were transformed into *E. coli* DH5α (Gibco BRL) competent cells, which were subsequently plated on Luria agar plates with ampicillin (100 µg ml^{-1}) for selection. Plasmid DNA was isolated from the transformants and screened for pUBI-PGIP by restriction enzyme digestion. The DNA sequence of the cloning junction point between the 5′ end of *pgip* and the ubiquitin promoter sequence in pUBI-PGIP was confirmed to be correct using a "PGIP SEQUENCING" primer 5′-gTgCAgTTCTCAAAgATAC-3′. This primer was complimentary to codons 22–27 of bean PGIP1.

Bombardment

A PIG built according to the design of Finer et al. (1992) and Vain et al. (1993a) was used for all bombardment experiments. DNA delivery parameters were optimised based on transient expression of the *uidA* gene in cultured immature embryo scutellum cells (data not shown). Cultured immature embryos were subjected to osmoticum treatment 3 h before bombardment as described by Vain et al. (1993b). Embryogenic callus tissue was spread out (0–1 cm in diameter) in the centre of a 9-cm petri dish containing 2MS+Pro medium, media D or N6C1SN, with 10 g/l agar, 0.2 *M* D-sorbitol and 0.2 *M* D-mannitol. A bombardment mixture was prepared by precipitating plasmid DNA on tungsten particles as described by O'Kennedy et al. (1998). Plasmids pAHC25 and pUBI-PGIP DNA were extracted from overnight *E. coli* cultures using a Wizard (Promega) or Qiagen (Southern Cross Biotechnologies) maxiprep kit according to the supplier's recommendation. Sixteen hours after bombardment, bombarded tissue was transferred from the media containing osmoticum to media without osmoticum.

Selection and regeneration of transformants

Selection for PPT-resistant maize tissue was initiated 2–4 days after bombardment by placing the cultured immature embryos on 2MS+Pro, medium D or N6C1SN medium supplemented with 0.5–5.0 mg l^{-1} bialaphos. The bialaphos content in the various media was increased in a step-wise manner, ranging from 2 mg l^{-1} to 5 mg l^{-1}, with each transfer to fresh selection media every 2 weeks. After 4–6 weeks on selection media, cultured embryos that produced white compact calli, presumably embryogenic, were transferred to the various selective regeneration media containing 1 mg l^{-1} bialaphos. Regenerated putative transgenic plants were subcultured at 2- to 3-week intervals until they reached 8–10 cm in height. These were then hardened-off as described by O'Kennedy et al. (1998).

Analysis of transformed tissues and plants

DNA extraction

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

Polymerase chain reaction analysis

Pgip- (5′-GCTCTAGAATGACTCAATTCAATATCCCAG-3′ and 5′-GCACGAGCTCTTAAGTGCAGGAAGGAAG-3′), *uidA*-(GUS_L: 5′-GGTGGGAA AGCGCGTTACAAG-3′ and GUS_R: 5′-GTTTACGCGTTGCTTCCGCCA-3′) and *bar*- (BAR_L: 5′-CATCGAGACAAGCACGGTCAACTTC-3′ and BAR_R: 5′-CTCTTGAAG CCCTGTGCCTCCAG-3′) specific primers were used to amplify 1-kb, 1.2-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 min, and then subjected to 35 cycles of a denaturing step at 94°C for 45 s, an annealing step at 64°C for 30 s and an elongation step at 72°C for 45 s. Amplification of the 4.6-kb *gus-bar* cassette using the GUS_L and BAR_R primers was carried out as follows. Amplification consisted of ten cycles of a denaturing step at 94°C for 10 s, an annealing step at 56°C for 30 s and an elongation step at 68°C for 2 min, in a Geneamp 2400 Perkin Elmer PCR system. The next 20 cycles consisted of a denaturing step at 94°C for 10 s, an annealing step at 56°C for 30 s and an elongation step at 68°C for 2 min which increased by 20 s at each cycle, with a final elongation step for 7 min.

Southern blot analysis

Aliquots (each 5 µg) 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*, *uidA* and *bar* genes were labelled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche Biochemicals).

Herbicide application

A 2% Basta (200 g l^{-1} of the active ingredient, glufosinate ammonium), 0.01% Tween 20 solution was applied to both surfaces of selected leaves of transgenic plants as described by O'Kennedy et al. (1998).

β-Glucuronidase activity

Bombarded tissues and progeny of transgenic plants were assayed for GUS expression according to Jefferson et al. (1987).

Results

Identification of genotypes and culture medium

Three different callus induction media, namely 2MS+Pro, media D and N6C1SN, were tested in order to identify the maize line and callus induction medium combination most suitable for elite maize transformation. Thirteen white and six yellow maize lines were screened. Elite white maize lines W506, W508, and M37W cultured on 2MS+Pro callus induction medium were determined to be superior in their regeneration potential with respect to the other lines and media combinations screened (data not shown). In the present study, only embryogenic white, compact structured calli, designated type-I calli, were produced when immature zygotic embryos of the selected elite maize lines were cultured. The type-I calli regenerated to produce fertile plants.

Although 40–63% of the immature zygotic embryos of lines W506, W508 and M37W produced white. compact calli, which we regarded as being embryogenic, fewer than two regenerants per explant were obtained. The classification of the callus as embryogenic, therefore, did not necessarily imply regenerability. The number of regenerants per immature embryo explant of the three responsive lines ranged between 0.3 and 1.2.

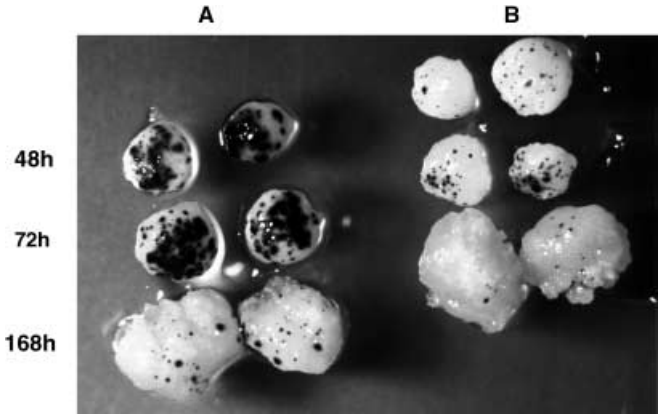

Fig. 1A,B Transient GUS activity in scutellum cells of immature zygotic embryos of line W506. The embryos were pre-cultured for 4 days before bombardment. DNA was coated on the tungsten particles by either using spermidine (free base) (**A**) or spermine tetrahydrocloride (**B**), and bombarded at a helium pressure of 600 kPa. The embryos were stained for GUS activity 48 h, 72 h and 168 h after bombardment

Optimisation of the selected bombardment conditions by transient expression

High levels of transient *uidA* expression were obtained following bombardment of cultured immature zygotic embryos with pAHC25 (Fig. 1). The number of blue foci obtained when spermidine-free base was used to precipitate plasmid DNA onto the tungsten particles was approximately double to threefold higher than the number obtained using spermine tetrahydrocloride when assayed 48 h, 72 h and even 168 h (7 days) after bombardment (Fig. 1). Therefore, it was decided to use spermidine-free base for all future bombardment experiments for the purpose of obtaining stable transformation.

Selection of transgenic tissues and regeneration of T_0 plants

A range of pre-culture times, selection conditions and bombardment pressures were applied in attempts to obtain stable transformation. Immature embryos of elite maize lines M37W (310 embryos), W506 (98 embryos) and W508 (241 embryos) were pre-cultured for 4–14 days before bombardment and transferred to selection medium containing 0.5–3.0 mg l^{-1} bialaphos 2–4 days after bombardment at 700–900 kPa (Table 1). In a separate experiment, immature embryos of maize line W506 (3,855 embryos) were bombarded at lower helium pressures (400–500 kPa) (data not shown).

Putatively transformed callus tissue was identified by its vigorous growth on bialaphos-containing medium, suggesting the stable integration and functionality of the *bar* gene in the maize genome. Non-transformed tis-

Table 1 Conditions for bombardment of elite maize immature zygotic embryos of lines M37W, W506 and W506

Experiment number	Number of embryos bombarded	Line	Helium pressure (kPa)	Days in culture before bombardment	Days after bombardment to selection	Selection conditions[a]	Plasmid	Putative transgenic[b] regenerants	Transgenic T_0 plants[c]
1	16	M37W[d]	800	4	4	0.5/14→2/14→3/14	pUBl-PGlP	2	1(M6)
	16		600	5				0	0
	9		700	5				1	0
2	15	M37W[e]	700	5	4	1/14→2/14→3/14	pUBl-PGIP	1	1(M7)
3	30	M37W[d]	700	6	2	1/14→3/30	pUBl-PGIP	5	0
	14		800					8	1(M4)
4	17	M37W[d]	800	13	2	3/14→5/30	pUBl-PGIP	15	0
5	34	M37W[e]	800	6	2	2/30→5/14	pUBl-PGIP	2	0(M12)
	41		900					1	0
6	85	M37W[e]	800	7	3	2/14→5/14	pUBI-PGIP	3	0
	20		900	7				1	0
	13		900	14				0	0
7	28	W506[e]	800	4	3	2/14→5/30	pAHC25	1	0
	48		900	4	3			1	0
	22		900	5	3			2	1(BB163.1B)
8	62	W508[e]	700	8	2	2/14→5/30	pAHC25	1	0
	65		800	8	2			0	0
	62		900	8	2			2	0
	52		800	9	2			2	0
Total numbers	649							48	4

[a] mg l^{-1}/days on bialaphos selection medium
[b] Plants regenerated from calli growing on bialaphos selection medium
[c] Confirmed by PCR and Southern blot analysis (names of transgenic plants are given in brackets)
[d] Greenhouse-grown material
[e] Field-grown material

sue turned brown and watery on selection media. Bialaphos-resistant cells or cell clumps developed embryogenic calli that matured to form somatic embryos on medium containing 0.5–3.0 mg l^{-1} bialaphos and on medium in which the levels of bialaphos were increased subsequently step-wise to 3–5 mg l^{-1} bialaphos (Table 1). These were sub-cultured on regeneration medium containing 1 mg l^{-1} bialaphos, and 4 out of the 48 regenerants were transgenic as determined below: three were derived from the transformation of M37W (named M4, M6, and M7), and one was derived from the transformation of W506 (named BB163.1B) (Table 1). Plant M12 was derived from bombarded M37W tissue.

Analysis of transgenic plants

Herbicide resistance

Functional expression of PAT was demonstrated in the leaves of transgenic plants by their resistance to typical necrosis associated with localised applications of the herbicide Basta to maize leaves. No necrosis was observed on leaves of primary transformants (T_0 plants) M4, M6, M7 and BB163.1B (data not shown). In contrast, control plants developed necrotic lesions within 7 days and eventually died. Similar herbicide resistance was observed in T_1 and subsequent generations of transformation event BB163.1B (data not shown).

Mendelian inheritance of herbicide resistance was obtained for the progeny of transformation event BB163.1.B in the T_1 and T_2 generations (Table 2). T_1 progeny derived from the self-pollinated transformant BB163.1.B exhibited a 3:1 herbicide resistance: sensitive ratio, as expected. T_2 progeny resulting from cross-pollination amongst T_1 transformants exhibited the same 3:1 ratio, indicating that the trait was inherited as a single dominant allele. T_3 progeny were obtained from cross-pollination amongst T_2 transformants. Three individual T_3 plants were self-pollinated. The progeny of two of these showed an almost 100% inheritance of herbicide resistance, indicating that the trait was homozygous in the parents, whereas the progeny of the third showed a 3:1 ratio, indicating that the parent only had one allele of the *bar* transgene integration event (Table 2).

β-Glucuronidase activity

Immature embryos in the T_3 generation of event BB163.1B displayed uniform dark-blue staining, indicative of GUS activity resulting from stable integration of *uidA* (Fig. 2). Approximately three-quarters of these T_3 embryos, derived from self-pollination of a T_2 plant, were GUS-positive, indicating that the *uidA* transgene was inherited as a single dominant trait in a Mendelian manner. In the next generation (T_4), progeny derived from cross-pollination of T_3 plants showed almost 100% inheritance of the *uidA* gene, as evidenced by subjecting leaf tips to GUS assays (Table 2).

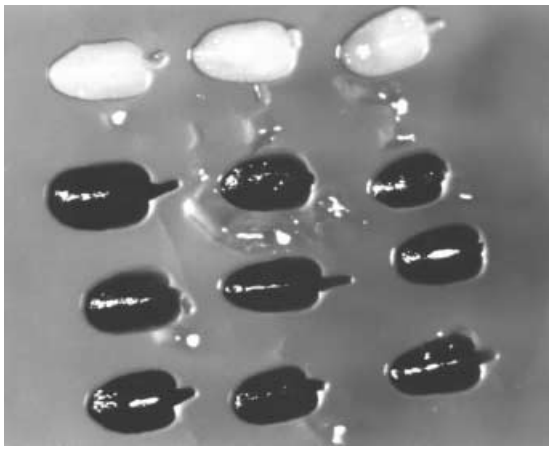

Fig. 2 GUS expression in immature zygotic embryos in the T_3 generation of transgenic event BB163.1.B. Transgenic progeny stained *blue* in the GUS assay mix, whereas non-transgenic progeny, which had lost the *uidA* gene by segregation, remained *white* in the GUS assay mix. Progeny were obtained from a self-pollinated T_2 transgenic plant

Table 2 Segregation of bialaphos resistance (*bar* gene) and GUS activity (*uid*A gene) in progeny of event BB163.1B

	Bialaphos resistance[a]			GUS activity[b]
Generation[c]	T_1	T_2	T_4	T_4
Proportion(+:–)	46:21	32:13	98:2 96:4 75:25	97:3 89:11 [d]

[a] Embryos were germinated on bialaphos-containing media
[b] Leaf material of germinated T_4 generation seedlings was assayed for GUS expression
[c] T_1 seed was derived by self pollination of the T_0 plant; T_2 seed was derived from cross-pollination of two transformed T_1 plants; T_4 seed was derived from three individual T_3 plants that were self-pollinated
[d] Not determined

PCR and Southern blot analysis

The presence of the *bar, pgip* or *uidA* genes was confirmed by PCR analysis before Southern blot analysis was carried out. For the estimation of transgene copy number, the reliable non-radioactive detection of a single transgene copy in genomic maize DNA was first established. For this purpose, genomic DNA of untransformed maize plants was spiked with plasmid DNA in quantities simulating two and ten copies of the *bar* gene. These were subjected to Southern blot analysis and probed with a DIG-labelled internal fragment of the *pgip* or *bar* gene. A reconstruction of two and ten copies of the *pgip* gene (Fig. 3, lanes 2, 7) or *bar* gene (Figs. 4, lanes 1, 9; 5, lanes 2, 13) could clearly be distinguished.

Hybridisation of *Sac* I-digested genomic DNA confirmed the presence of the *bar* or *pgip* gene in the transformants that were tested. Unique transgene integration patterns were observed for each transformation event

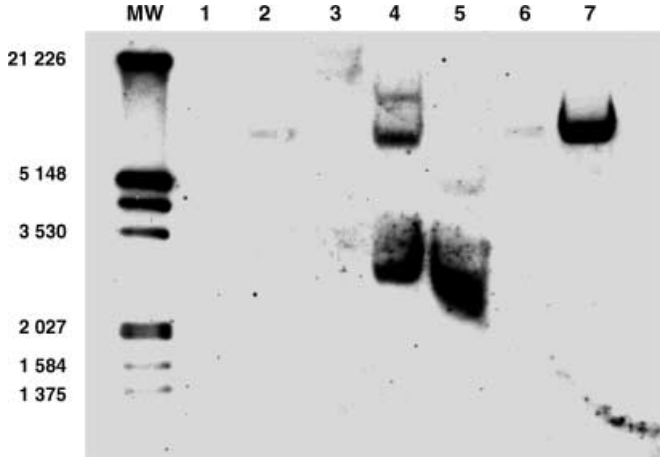

Fig. 3 Southern blot analysis of elite maize line M37W showing stable integration of the *pgip* gene in the T_0 generation. Genomic DNA was purified from plant leaf material, restricted with *Sac*I, resolved on a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal *pgip* fragment as described in the Materials and methods. *MW* DIG-labelled molecular-weight marker III (Roche); *lanes 2, 7* copy-number reconstructions representing two and ten transgene copies per haploid genome, respectively; *lane 1* untransformed M37W genomic DNA; *lanes 3–5* independently transformed elite maize lines designated M4, M6 and M7, respectively and; *lane 6* DNA from maize line M12

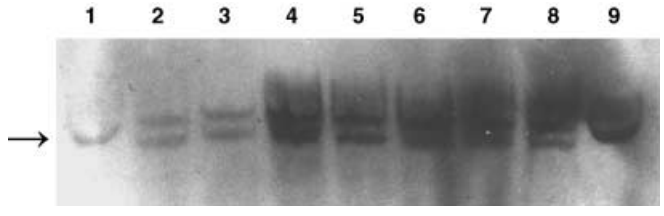

Fig. 4 Southern blot analysis of T_1 and T_2 progeny of elite maize line W506; transformation event BB163.1B. Genomic DNA was purified from plant leaf material, restricted with *Sac*I, resolved on a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal *bar* fragment as described in the Materials and methods. *Lanes 1, 9* Untransformed maize line A188 DNA spiked with two and ten transgene copies, respectively; *lanes 2, 3* two individual plants of the T_1 progeny; *lanes 4–8* five individual plants (designated i, ii, iii, iv and v, respectively) of the T_2 progeny. The *arrow* indicates the size of pAHC25 restricted once with *Sac*I, which is 9.7 kb

(Figs. 3, lanes 3–5; 4, lanes 2, 3). Copy-number estimations indicated that no plants contained a single transgene copy. However, out of five transformants, two, namely BB163.1B and M4, had no more than two integration events of each of the transgenes. In the elite line M37W, three events were shown to have integration of the *pgip* gene (Fig. 3, lanes 3–5). Hybridisation of the *pgip* probe was not observed in the untransformed sample as expected (Fig. 3, lane 1). Although a PCR amplification of the *bar* gene was obtained for M12, there is uncertainty as to whether the faint band of M12 in the Southern blot (Fig. 3, lane 6) confirms an integration of the *bar* gene into the genome of maize. It was considered to be more likely that the faint band was an overflow of lane 7. Unfortunately, no further plant material was available for DNA extraction, and a repeat Southern blot. M12 was therefore not regarded to be a transformation event in this study. Inheritance of the *bar* transgene in the progenies of transformation event BB163.1.B was confirmed by Southern blot analysis. Integration patterns for the *Sac*I digest in the T_1 and T_2 generations remained identical when probed with the *bar* gene (Fig. 4, compare lanes 4–8 with lanes 2, 3).

Subsequently, the architecture of the integration site in BB163.1B was investigated in detail by Southern blot analysis of two individual T_3 transgenic plants. For this purpose, genomic DNA was digested with *Sac*I, *Hin*dIII, *Pst*I or *Eco*RI and probed sequentially with internal fragments of the *bar* and the *uidA* genes (Fig. 5). Integration of the transgenes into high-molecular-weight genomic DNA was illustrated by positive hybridisation in lanes 3 and 4 of Fig. 5.

The *uidA-bar* cassette from pAHC25 was shown to have been integrated into the genomic DNA as an intact fragment, since a product of 4.6 kb flanking these genes could be PCR-amplified from genomic DNA of BB163.1B (Figs. 6, lanes 1, 2; 7). The *Sac*I restriction site within this fragment was preserved, since digestion of the PCR product released the expected fragments of 1.9 kb and 2.7 kb (Figs. 6, lanes 3, 4; 7).

Southern analysis of event BB163.1B revealed that the *bar* probe binds to two fragments (Fig. 5A, lanes 5–8), whereas the *uidA* probe only binds to one (Fig. 5B, lanes 5–8). Based on the PCR evidence that the *uidA-bar* cassette is intact, we conclude that one integration event has both the *uidA* and *bar* transgenes and another only the *bar* gene. To reconcile this with the data that the *Sac*I digest integration pattern remains identical throughout the T_1–T_4 generations of the transgenic plants (Figs. 4, 5), we hypothesise that these inserts are linked as tandem insertions. Our model of the transgene integration pattern is illustrated in Fig. 7. The *uidA* expression cassette (promoter, intron, gene) is intact since the *uidA* probe hybridises to the predicted 4.2-kb and 2.2-kb *Hin*dIII and *Pst*I fragments, respectively (Figs. 5B, lanes 7–10; 7), and T_3 embryos express GUS activity (Fig. 2). At least one copy of the *bar* expression cassette is intact, since transgenic plants are resistant to herbicide up to the fourth generation (Table 2). A second copy of the *bar* gene has been inserted, since the *bar* probe hybridises to two individual *Sac*I and *Hin*dIII fragments (Figs. 5A, lanes 5–8; 7) and also binds as a strongly hybridising doublet to the predicted 1.5-kb *Eco*RI and 0.6-kb *Pst*I fragments that flank each copy of the *bar* gene (Figs. 5A, lanes 9–12; 7). However, the data do not reveal whether the promoter is absent or if this copy is active.

Phenotypic characteristics of transgenic plants

Abnormal phenotypic characteristics, such as dwarfism, tassel ears and underdeveloped tassels and ears, were ob-

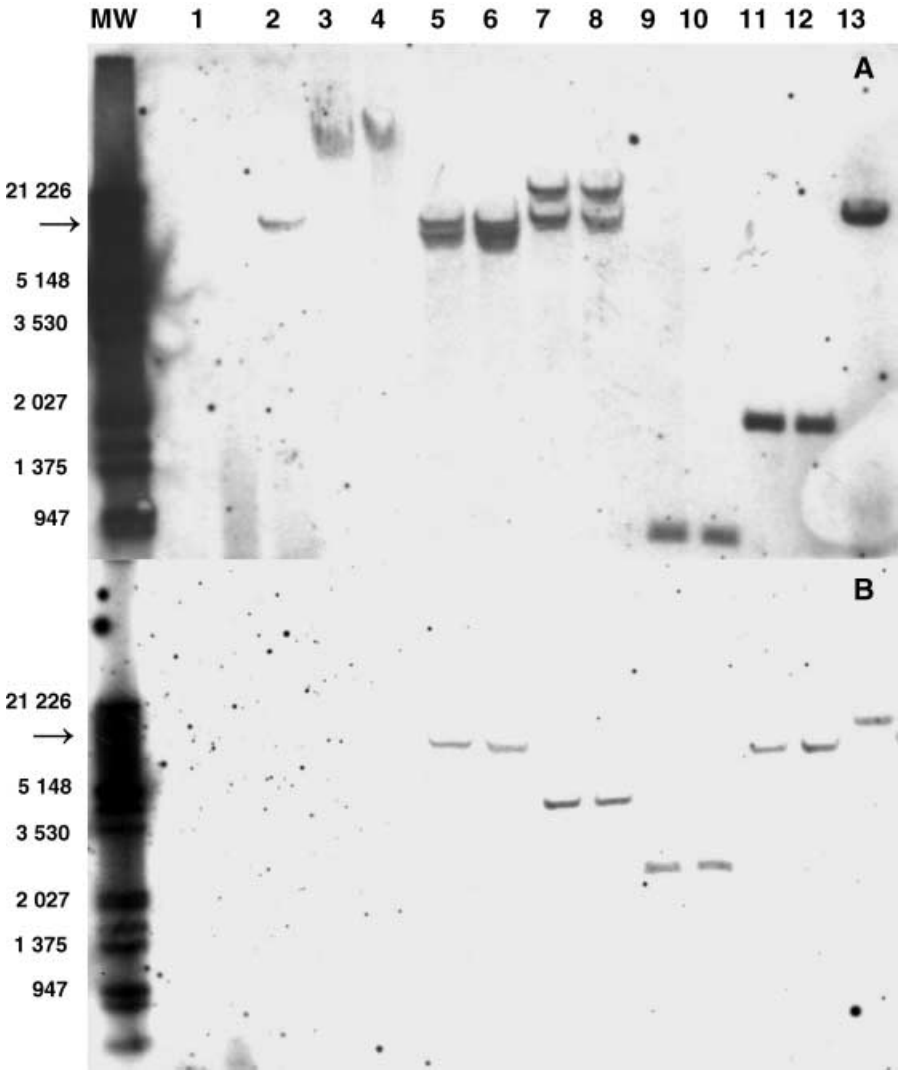

Fig. 5A, B Southern blot analysis of T_3 progeny of elite maize line W506; transformation event BB163.1B. Genomic DNA was purified from plant leaf material, restricted with *Sac*I, *Hin*dIII, *Pst*I or *Eco*RI and resolved on a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal *bar* fragment (**A**), stripped and reprobed with an internal fragment of *uidA* (**B**). *MW* DIG-labelled molecular-weight marker III (Roche); *lane 1* untransformed maize DNA; *lanes 2, 13* untransformed maize DNA spiked with two and ten transgene copies, respectively; *lanes 3, 4* undigested DNA from two individual T_3 plants; *lanes 5, 6* DNA from two individual T_3 plants digested with *Sac*I; *lanes 7, 8* DNA from two individual T_3 plants digested with *Hin*dIII; *lanes 9, 10* DNA from two individual T_3 plants digested with *Pst*I; *lanes 11, 12* DNA from two individual T_3 plants digested with *Eco*RI. The *arrows* indicate the size of pAHC25 restricted once with *Sac*I, which is 9.7 kb

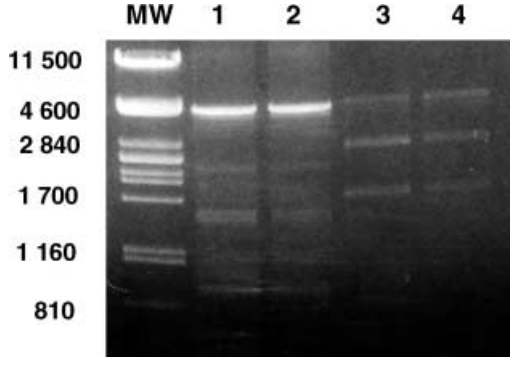

Fig. 6 Diagnostic PCR amplification and restriction enzyme digestion of transgenes from genomic DNA of transgenic maize (BB163.1B) *MW* Molecular-weight marker; *lanes 1, 2* PCR product of 4.6 kb amplified from two individual transgenic plants using the GUS_L and BAR_R primers; *lanes 3, 4* PCR products from *lanes 1* and *2* digested with *Sac*I for 2 h. The 4.6-kb fragment disappears after 4 h of digestion, whereas fragment sizes 1,900 kb and 2,700 kb intensify (data not shown)

served in the primary transformants (T_0 plants) and non-transgenic regeneration controls. Since the T_1 and T_2 progeny of BB163.1B were fertile and resembled control plants that were germinated from seed, these aberrations in the T_0 plants can be ascribed to tissue culture-induced stress. Although all three M37W events survived the hardening-off process in the T_0 generation, attempts at self-pollination were unsuccessful, and no T_1 progeny were recovered. Therefore, it was not possible to test for increased fungal resistance conferred by the *pgip* transgene. Progeny of BB163.1B (T_1–T_4) were obtained from transformed (T_0) plants by cross-pollination of T_1 and T_2 transgenic plants originating from the same transformation event and self-pollination of T_3.

Transformation frequency

Three of the putatively transformed M37W plants obtained in this study were resistant to Basta application. These three plants were also shown to be PCR-and Southern blot-positive for both the *pgip* (Fig. 3) and the

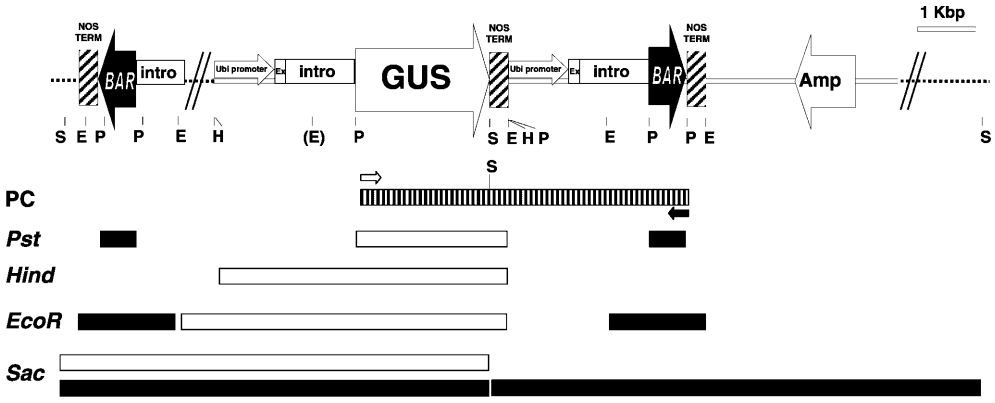

Fig. 7 Diagram of integration pattern of *bar* and *uidA* genes in transgenic maize event BB163.1.B. This diagram was interpreted from the Southern blot (Figs. 4, 5) and PCR data (Fig. 6) from which it was concluded that the transgenes were integrated as one complete copy of pAHC25 with an additional *bar* gene inserted upstream in the reverse orientation. The *bar* and *uidA* genes are indicated by *solid black* and *white arrows*, respectively. The maize ubiquitin-1 promoter, non-translated exon and non-translated intron upstream of each gene are indicated by a *white arrow* and *white boxes*, respectively. The nopaline synthetase terminator downstream of each gene is indicated by a *hatched box*. The β-lactamase gene, encoding ampicillin resistance (*AmpR*) is indicated by a *white arrow*. Restriction enzyme sites are indicated as follows: *P Pst*I, *H Hin*dIII, *E Eco*RI, *S Sac*I. The unknown sequence and the maize genomic sequence are indicated by *double forward slash bars* and *dotted lines*, respectively. The 4.6-kb PCR product obtained by amplification of genomic DNA from transgenic maize event BB163.1.B using the GUS Left ($_L$) and BAR Right ($_R$) primers is indicated as a *box with vertical bars*. The GUS_L and BAR_R primers are indicated by *open* and *closed arrows*, respectively. Restriction fragments of genomic DNA from transgenic maize event BB163.1.B, which hybridized with the *uidA* and *bar* probes in the Southern blot shown in Fig. 5, are shown as *white* and *black boxes*, respectively. The *Eco*RI restriction site within the ubi promoter intron upstream of *uid*A, which is known to be cut inefficiently (Fig. 1 of Christensen and Quail 1996), was also not cut in our study and is indicated by (*E*). The AmpR gene is shown, although it is not known whether this part of the vector was inserted, since no Southern analysis was done with the β-lactamase gene probe

bar transgenes (data not shown). Transformation events designated M4, M6 and M7 were obtained following the bombardment of 310 immature embryos of maize line M37W at helium pressures ranging from 700 kPa to 900 kPa using pUBI-PGIP. Bombardment of 98 and 241 immature embryos of maize lines W506 and W508, respectively, at similar helium pressures gave rise to only one transgenic plant that contained both the *bar* and *uidA* transgenes. In another experiment in which a large number (3,855) of immature embryos of maize line W506 were cultured on 2MS+Pro, D or N6C1SN media for 2–14 days before bombardment, bombarded at 400–500 kPa and subsequently transferred to selection medium containing 0.5–5.0 mg l^{-1} bialaphos 2–7 days after bombardment, no transformation events resulted (data not shown). The helium pressures of 400–500 kPa were probably too low.

In total, four transformants (M4, M6, M7 and BB163.1B; Table 1) were obtained in this study from the total of 649 immature embryos of maize lines M37W, W506 and W508 that were cultured under selection on 2MS+Pro and subsequently 0MS media, giving a transformation efficiency of 0.9%, 1% and 0% for maize lines M37W, W506 and W508, respectively. Three events contained the *pgip* and *bar* transgenes and one event, BB163.1.B, contained the *bar* and *uidA* transgenes and produced fertile progeny up to the fourth generation of plants.

Discussion

In this study, only 4 of the 48 putative transgenic plants recovered proved to be stably transformed, and only one fertile transformation event was produced. Several factors can account for the low transformation efficiency. Low bialaphos concentrations of 1–5 mg l^{-1}, short selection procedures of 4–6 weeks and cross-protection of untransformed cells by transformed cells in the callus cluster may have led to the regeneration of untransformed embryogenic calli on selection medium. A large proportion of the regenerants may have been escapes due to the shorter selection period. In previous experiments, the selection of elite maize lines for longer periods (8–10 weeks) on bialaphos-containing medium produced only plants with stress-related abnormalities such as tassel ears, unsynchronised flowering, multiple flowering parts that were often sterile and dwarfism. Shorter selection periods produce fertile plants which have the potential to produce transgenic seed.

It is well-documented that L-proline enhances the frequency of somatic embryoid formation (Armstrong and Green 1985; Pareddy and Petolino 1990) and, consequently, it is commonly used in maize tissue culture media. Dennehey et al. (1994) reported that bialaphos is a more potent selective agent for maize than glufosinate ammonium and that L-proline interferes significantly with glufosinate or Basta selection, but not with bialaphos or Herbiace selection. Therefore, since 20 m*M* L-proline was added to the callus induction medium, bialaphos was used as selective agent in the tissue culture media in this study.

Christou (1995) observed that optimised or maximised transient activity does not necessarily result in opti-

mal or even any stable transformation. Therefore, in this study, a range of parameters involving the helium pressure, days in culture before and after bombardment before the onset of the selection pressure, the concentration of the selection agent and the step-wise changes in the level of the selection agent were used to obtain stable transgenic plants. Our approach was to employ this range of conditions rather than a single set of conditions that gave maximal transient expression.

Christou (1995) reported that 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 (Russell et al. 1992). Therefore, in this study callus cultures were treated with osmoticum before and after bombardment. Vain et al. (1993b) showed that osmotic treatment of maize cells resulted in a six- to sevenfold increase in the number of stable transformants. These authors hypothesised that the osmotic treatment alleviated plasmolysis of cells, which may have reduced cell damage by preventing extrusion of the protoplasm from bombarded cells (Vain et al. 1993a). Vain et al. (1993a) reported damage in the centre of the blast area and that it could be totally eliminated by using a baffle. Similarly, in our study, a massive tissue displacement occurred in experiments when bombardment occurred without a baffle. Therefore, a 500-µm nylon mesh baffle was used in routine bombardment experiments.

We obtained the stable transformation of elite white maize lines by pre-culturing immature zygotic embryos for 4–6 days, bombarding them at helium pressures ranging from 700 kPa to 900 kPa and then culturing them for another 2–4 days before transfer to selection medium containing 0.5–2.0 mg l^{-1} bialaphos. Thereafter, a stepwise increase of the bialaphos concentration was applied to a final concentration of 3–5 mg l^{-1}. Four transgenic plants were produced after transformation of the target cells in the scutellum of the cultured immature zygotic embryos with bombardment at helium pressures of 700–900 kPa. Not a single transformation event was obtained at helium pressures of 400–500 kPa. Immature embryos from both field-grown and greenhouse-grown plants yielded transformants. Our results indicate that maize transformation with the PIG can be achieved over a wide range of conditions.

The introduction of multiple copies of the same gene into plants often leads to a phenomenon called transgene silencing or co-suppression (Register et al. 1994; Lakshminarayan et al. 2000). Therefore, the selection of transgenic plants with one or few transgene copies has become desirable. Microprojectile-mediated transformation of maize often results in multiple copies, up to 50 transgene insertion events using either the PDS 1000/He gun (Koziel et al. 1993; Register et al. 1994; Hill et al. 1995; Wan et al. 1995) or the PIG (O'Kennedy et al. 1998). Low-copy integration (1–8 copies) in maize has been reported using the PDS 1000/He gun (Brettschneider et al. 1997). In our study, a low-copy number integration of the transgenes, one to two copies, was obtained using the PIG, and the inserted transgenes were successfully transferred in a Mendelian fashion to the progeny.

Transformant BB163.1B, which was the only one to produce fertile herbicide-resistant progeny up to at least the fourth generation, was found to contain two linked integration events, one containing the *uidA* and *bar* transgenes, and one with a potentially non-functional *bar* gene only (Fig. 7). Therefore, it is likely that the intact bar gene would not be subject to transgene silencing, enabling herbicide resistance to be expressed and inherited in a stable manner.

Several of the transgenic events obtained in this study were sterile. Phenotypic abnormalities and reduced fertility are most likely a consequence of prolonged tissue culture periods. Future ways to overcome this would be to shorten the tissue culture period but simultaneously increase the selection pressure to avoid escapes. Alternatively, a positive selectable marker gene, such as the phosphomannose isomerase gene, could be used (Negrotto et al. 2000). Another option would be to target white compact type-I calli from elite maize lines instead of immature embryos (Wan et al. 1995). This permits the selection of only proliferating, regenerable tissue as target material.

We have therefore provided cumulative evidence for stable maize transformation of elite white maize using the PIG. This is the first report of transgenic elite white maize being obtained using a simple and inexpensive device for transformation. The results presented in this paper open the way to introduce various agronomically useful genes into the genome of elite white maize lines.

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