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Transgenic and herbicide resistant pearl millet (*Pennisetum glaucum* L.) R.Br. via microprojectile bombardment of scutellar tissue

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Abstract

Four different pearl millet breeding lines were transformed and led to the regeneration of fertile transgenic plants. Scutellar tissue was bombarded with two plasmids containing the *bar* selectable marker and the β-glucuronidase reporter gene (*gus* or *uidA*) under control of the constitutive CaMV 35S promoter or the maize *Ubiquitin*1 promoter (the CaMV 35S is not a maize promoter). For the delivery of the DNA-coated microprojectiles, either the particle gun PDS 1000/He or the particle inflow gun was used. The calli and regenerants were selected for their resistance to the herbicide Basta (glufosinate ammonium) mediated by the *bar* gene. Putative transformants were screened for enzyme activity by painting selected leaves or spraying whole plants with an aqueous solution of the herbicide Basta and by the histochemical GUS assay using cut leaf segments. PCR and Southern blot analysis of genomic DNA indicated the presence of introduced foreign genes in the genomic DNA of the transformants. Five regenerated plants represent independent transformation events and have been grown to maturity and set seed. The integration of the *bar* selectable and the *gus* reporter gene was confirmed by genomic Southern blot analysis in all five plants. All five plants had multiple integrations of both marker genes. To date, the T_1 progeny of three out of four lines generated by the PDS particle gun shows co-segregating marker genes, indicating an integration of the *bar* and the *gus* gene at the same locus in the genome.

Abbreviations: PDS – Particle gun PDS 1000/He, PIG – Particle inflow gun, GUS – β-glucuronidase, 2,4-D – 2,4-diclorophenoxyacetic acid, MS – Murashige and Skoog (1962), IAA – indole acetic acid, X-Gluc – 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Introduction

Pearl millet [*Pennisetum glaucum* (L.) R.Br.] also cited as *P. americanum* (L.) Leeke, is the most important of a number of unrelated millet species that are cultivated as a staple food mainly in Africa and India. There, in agroclimatic zones where heat, low and erratic rainfall, poor soil quality and the lack of fertilizers are predominant, grain yields from pearl millet are higher and more reliable than those from sorghum or maize (Andrews et al. 1993). In 1998, millet was sixth in production amongst all cereals with 29 million metric tons harvested on 25 million ha, an area bigger than that used for wheat production in the USA (24 million ha) (FAO 1998).

Since the 1980's several reports described the regeneration of pearl millet plants using immature inflorescences (Vasil and Vasil 1981), shoot apices (Devi et al. 1999; Lambé et al. 1999), mature (Botti and Vasil 1983) and immature embryos (Lambé et al. 1999; Vasil and Vasil 1981) as explants. Pearl millet (Vasil and Vasil 1981) and sorghum (Gamborg et al.

1977) were the first cereals in which embryogenic in vitro culture systems were established, starting from immature embryos as explants. Their research provided the basic facts for later successful transformation systems in cereals. In the early 1990's genetic transformation using immature embryos as explants was successful in rice (Christou et al. 1991), sorghum (Casas et al. 1993) and wheat (Vasil et al. 1992). Yet, the genetic transformation of pearl millet was not reported until 2000, when Lambé et al. described the stable transfer of the selectable and the visible marker gene *hph* (hygromycin phosphotransferase) and *gus*, respectively. This delay in generation of fertile transgenic pearl millet plants might be due to its formerly limited importance in the industrialized world and its more difficult manipulation in culture compared to other cereals.

Recently, we have described an *in vitro* culture system for sorghum and pearl millet initiating from immature zygotic embryos (Oldach et al. 2001a). In this paper, we present the usefulness of this in vitro culture system for obtaining transgenic pearl millet plants by using the PDS particle gun (Sanford et al. 1987) for biolistic-mediated transfer of the *bar* and the *gus* gene as selectable and visible marker.

Another aim of this study was to develop an additional reliable transformation system for pearl millet by using a simple and inexpensive device such as the particle inflow gun (PIG) (Finer et al. 1992). The development of a low-cost method will form the basis for future genetic enhancement of this crop for the benefit of India and Sub-Saharan Africa. Five genetically independent transgenic plants were identified by selection with the herbicide Basta and by subsequent genomic Southern blot analysis of resistant plants. The inheritance of the introduced maker genes *bar* and *gus* was verified by Basta spraying, histochemical GUS-staining and genomic Southern blot analysis. The inheritance and expression of both marker genes in the T_1 generation was confirmed in three independent pearl millet lines to date.

Materials and methods

The material and methods used for generating the transgenic plants differ among the plants produced using the PIG (Finer et al. 1992) and the particle gun PDS 1000/He (BioRad, München, Germany). In the following we will describe first the transformation procedure for the PIG and subsequently for the PDS particle gun.

Plant material, tissue culture and selection

For the PIG transformation seed samples were kindly provided by ICRISAT, Zimbabwe (pearl millet genotype 842B), and Savanna Aricultural Research Institute SARI in Ghana (pearl millet genotypes Bonga Nara and Manga Nara). 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. Covering the flowers with paper bags prevented cross-pollination. Greenhouse-grown florets of pearl millet containing immature embryos (10–14 days post-pollination) were soaked in 70% ethanol for one minute and sterilised for 15 minutes in a 2.5% sodium hypochlorite solution containing one drop, per 100 ml, of the surfactant Tween 20, before being thoroughly rinsed with sterile distilled water. Immature zygotic embryos (0.5–1 mm in size) were aseptically excised from the florets using a dissecting microscope and placed with their axes in contact with the callus induction medium. All tissue culture was performed under aseptic conditions. PM1 callus induction medium is described by Pinard and Chandrapalaiah (1991), and contains MS salts (Murashige and Skoog 1962), 2 mg l^{-1} 2,4 D, 30 g l^{-1} sucrose as carbon source and 8 g l^{-1} agar as solidifier. Cultures initiated on PM1 induction medium were further cultured on regeneration medium as described by Pinard and Chandrapalaiah (1991), containing the hormones IAA (0.2 mg l^{-1}) and kinetin (0.5 mg l^{-1}), but the medium was modified by the addition 10 mg l^{-1} silver nitrate (for genotype 842B) or 20 mg l^{-1} silver nitrate (for genotypes Manga Nara and Bongo Nara). These cultures were subsequently transferred to rooting medium, which was identical to the regeneration medium, except that both hormones and silver nitrate were omitted. The successful transformation event of pearl millet genotype 842B was obtained by the following protocol: immature zygotic embryos were pre-cultured for five days on PM1 induction medium, bombarded at 900 kPa, and transferred to selection medium 24 hours after bombardment. Transgenic tissue was selected on induction medium containing 1 mg l^{-1} bialaphos (weeks 1–4), followed by 2 mg l^{-1} (weeks 5–6). Thereafter, remaining tissue was transferred to regeneration medium supplemented with 0.5 mg l^{-1} (weeks 7–9) fol-

lowed by 0.1 mg l^{-1} bialaphos (weeks 10–15). Subsequently, putative transgenic regenerants were transferred to rooting medium supplemented with 0.1 mg l^{-1} bialaphos for additional 8–10 weeks. Thereafter the plant was hardened-off to the greenhouse. 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 pearl millet plants as described by (O'Kennedy et al. 1998). Glufosinate and its commercial formulation, Basta® and the tripeptide, bialaphos, or its commercial formulation Herbiace® are both phosphinothricin (PPT)-based selective agents. The total duration in tissue culture after bombardment on bialaphos selection was therefore approximately five and a half months. Cultures on callus induction and regeneration media were incubated at 24–25 °C, under low-light conditions (1.8 μE m^{-2} s^{-1}), whereas regenerating shoots ($\geqslant 1$ cm) were incubated under dim light (18 μE m^{-2} s^{-1}).

For the plants produced by the PDS particle gun, the culture conditions of the three pearl millet genotypes (Manga Nara, Bongo Nara from SARI in Ghana; 7042 from International Crop Research Institute for the Semi-Arid Tropics ICRISAT in India), isolation of zygotic immature embryos and compositions of callus induction and regeneration media are as described before (Oldach et al. 2001a). Briefly, after isolation the immature zygotic embryos were kept on callus induction medium G (modified L3 medium containing 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D); 0.1 mg l^{-1} 6-benzylaminopurine (BAP); 30 g l^{-1} sucrose and 5.0 mg l^{-1} $AgNO_3$) for 2–4 days in the dark at 26 °C. For 5 h before and 19 h after the bombardment, the explants were incubated on a hormone-free MS medium (Murashige and Skoog 1962) with high osmotic potential (0.7 M sucrose). After the osmotic treatment, the explants were transferred to the original callus induction medium and incubated for 3–4 weeks without herbicide selection. The calli were transferred to selective regeneration medium R (modified L3 medium containing 1.0 mg l^{-1} BAP, 30 g l^{-1} sucrose and 2.0 mg l^{-1} Basta) and incubated in the light for 4–12 weeks (70 ± 5 μE m^{-2} s^{-1}, 16 h per day). Regenerating tissue was transferred fortnightly to fresh medium. Rooting was supported without any selection as described before. Regenerated plantlets with a shoot length of 3–10 cm were put into soil for further growth in the greenhouse. About 2–3 weeks of culture in the greenhouse, herbicide selection of the whole plantlets began. All regenerated and progeny plants were selected for the expression of an active phosphinothricin acetyl transferase by spraying them twice within two weeks with an aqueous solution of Basta (100 and 150 mg l^{-1} phosphinothricin).

Expression vectors, microprojectile bombardment, detection of GUS activity and molecular analysis

Plasmid pAHC25 was used for PIG transformation and is a dual expression vector which contains the *uidA* reporter gene that encodes the β-glucuronidase (GUS) enzyme, and for selection the *bar* gene, which encodes the enzyme phosphinothricin acetyl transferase (PAT), which confers herbicide resistance. Both the *uidA* and *bar* genes are under the control of the maize *Ubi*1 promoter, first exon and first intron, and the nopaline synthase terminator (Christensen and Quail 1996). PIG-bombarded tissue was assayed for GUS activity 48 h to 28 days after bombardment by staining the tissue according to Jefferson (1987). Genomic DNA was extracted from putative transgenic pearl millet leaf material using the mini extraction procedure of Dellaporta et al. (1983). PCR analysis was carried out by using the *bar* specific primers BAR_L: 5′-CATCGAGACAAGCACGGTCAACTTC-3′ and BAR_R: 5′-CTCTTGAAGCCCTGTGCCTCCAG-3′ to amplify a 0.28 kb fragment from genomic DNA preparations of putative transgenic pearl millet plantlets. Five micrograms of pearl millet genomic DNA either undigested or digested with restriction enzymes were separated on an agarose gel and analysed by Southern blotting as described by O'Kennedy et al. (1998). The *bar* gene of pAHC25 was labeled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche) using primers BAR_L and BAR_R.

In contrast to a single dual vector for the PIG transformation experiment, plasmid p35SAcS and pubi.gus were used in co-transformation experiments with the PDS particle gun. The plasmid p35SAcS containing the *bar* selection marker gene under control of the cauliflower mosaic virus 35S promoter and terminator was kindly provided by Dr. P. Eckes (Aventis CropScience). The expression vector pubi.gus was constructed by inserting the *Pst*I-*Sst*I-*gus* fragment from pDMC204 (CAMBIA TG0027) into the correspondingly restricted (*Pst*I and *Sst*I) plasmid pubi.cas (Oldach et al. 2001b). The expression of the *gus* gene is regulated by the *Ubiquitin*1 promoter from maize (Christensen et al. 1992) and the nopaline synthase terminator (T*nos*) from *Agrobacterium*

tumefaciens. The coating of the plasmid DNA onto gold particles and the parameters for particle bombardment of scutellar tissue of pearl millet were based on the conditions used for the transformation of scutellar tissue of wheat (Becker et al. 1994). Modifications were: the osmotic treatment 5 h before and 19 hours after bombardment, the smaller average size of microprojectiles (0.4–0.8 μm) instead of 0.4–1.2 μm and a higher helium gas pressure of 1550–1800 psi instead of 900–1550 psi. Transient GUS activity in bombarded embryos and stable GUS activity in leaves of transgenic plants was determined histochemically using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as described by Becker et al. (1994). Total genomic DNA was isolated from leaf tissue using the extraction protocol of Dellaporta et al. (1983). 20 μg per lane of restriction enzyme-digested genomic DNA and 30 pg of both used restricted plasmid DNA (positive control) were separated in a 0.8% agarose gel, blotted onto a nylon membrane (Hybond NX, Amersham, Braunschweig, Germany) and hybridised for 16 h at 65 °C. The *bar*- and *gus*-specific probes were synthesised using the non-radioactive digoxigenin chemiluminescent method (Roche, Mannheim, Germany). The primer pairs bar3 and bar4 being 5′-GAGACCAGTTGAGATTAGGCC-3′ (position 20 $\rightarrow$ 40) and 5′-ATCTGGGTAACTGGCCTAACT-3′ (position 533 $\leftarrow$ 553) for *bar* amplification and gus1 and gus2 being 5′-CAGGAAGTGATGGAGCATCAG-3′ (position 603 $\rightarrow$ 623) and 5′-TCGTGCACCATCAGCACGTTA-3′ (position 1220 $\leftarrow$ 1240) for *gus* amplification were used. The size of the labelled probes was 500 bp for *bar* and 637 bp for *gus*. Non-incorporated nucleotides were removed (Microspin Columns HR S300, Pharmacia, Erlangen, Germany). Hybridisation was carried out at 65 °C for 16 h. Subsequently, blots were washed under the following conditions, 10 min each: 1×SSC; 1×SSC, 0.1% SDS (w/v); 0.5×SSC, 0.1% SDS; and 0.2×SSC, 0.1% SDS. Hybridisation signals were visualised on Hyperfilm MP (Amersham, Braunschweig, Germany).

Results

Genetic transformation of pearl millet

Four pearl millet genotypes (842B, 7042, Manga Nara, Bongo Nara) served as donor plants for the isolation of zygotic immature embryos that were used as target tissue for microprojectile bombardment. Either the dual vector pAHC25 was bombarded using the PIG or the two expression vectors pubi.gus and p35SAcS were co-transformed using the PDS particle gun (Figure 1). Single immature embryos and calli were histochemically stained 2 days (Fig. 2A) and several weeks, i.e. 4 weeks (Fig. 2B), after bombardment. Whereas hundreds of scutellum cells are showing a transient GUS expression (Fig. 2A), single somatic embryos (dark blue embryo, indicated by arrow) amongst non-transgenic white compact callus are still expressing the transgene weeks later (Fig. 2B).

Analysis of regenerated plants (T_0 generation)

Using the PIG, a total number of 2738 immature zygotic embryos of genotype 842B were bombarded as well as 1332 and 1150 of the Ghanaian genotypes Manga Nara and Bongo Nara, respectively. A total of 2165 immature pearl millet embryos were bombarded in seven experiments using the PDS particle gun. Nearly equal numbers of immature zygotic embryos were taken from the three genotypes 7042, Manga Nara and Bongo Nara (Table 1).

Putatively transformed callus tissue was identified by its vigorous growth on selection agent-containing medium, an indication of the stable integration and functionality of the *bar* gene in the pearl millet genome. Non-transformed tissue turned brown and watery on selection media. Regenerated putative transgenic plantlets were hardened-off in the greenhouse and screened for the presence of the transgenes.

To date, two regenerants, designated as BB206.10A and BB206.10I, of the 455 regenerants screened and coming from the PIG-mediated transformation system were positive for the *bar* and *gus* gene. Digests of genomic DNA from both plants BB206.10A and BB206.10I with *Sac* I, *Hind* III and *Pst* I appear identical in integration pattern of the *bar* transgene (Fig. 3A), which hints that these two plants represent clones of the same transformation event. The hybridisation signals after *Eco*RI digest, a restriction enzyme that releases the *bar* gene, vary slightly between the two plants. This might be due to a different methylation status in these plants. The actual transformation rate using the PIG device is 0.02% (Table 1).

The regenerants originating from the PDS bombardment were completely sprayed with Basta in the green house. This whole plant spraying was repeated within 8–14 days. Subsequently, surviving plantlets

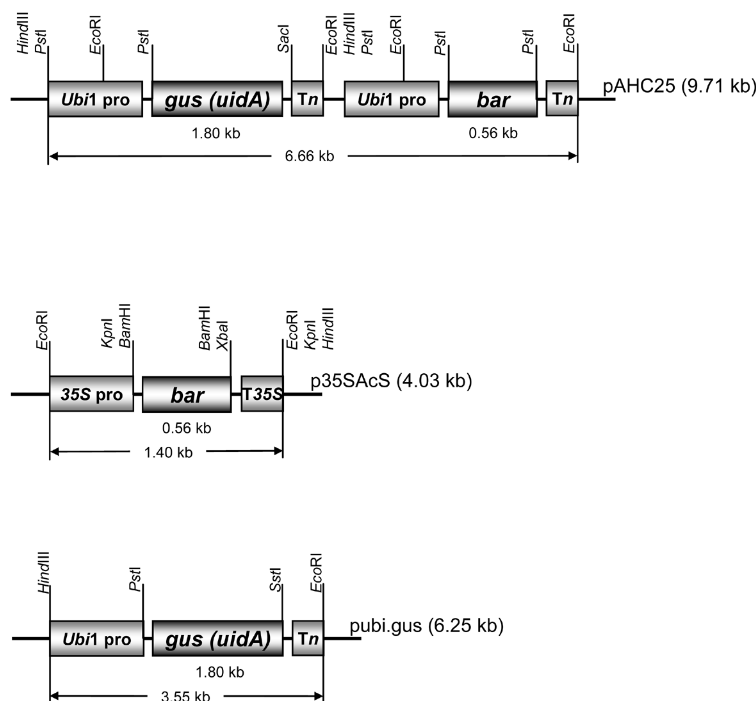

Figure 1. Constructs used for pearl millet transformation. The plasmids pAHC25, p35SAcS and pubi.gus. pAHC25 is a dual expression vector and was used for PIG transformation experiments. The *gus* (*uidA*) and *bar* gene in pAHC25 and in pubi.gus are under the control of the maize *Ubiquitin*1 promoter (*Ubi*1 pro), first exon and first intron, and the nopaline synthase terminator (T*n*) from *Agrobacterium tumefaciens*. Expression of the *bar* gene in vector p35SAcS is regulated by *35S* promoter (*35S* pro) and 35S terminator (T*35S*) from Cauliflower mosaic virus. Vectors p35SAcS and pubi.gus were used in co-transformation experiments with the PDS particle gun. Restriction sites relevant to presented Southern blot analysis, sizes of coding sequences and expression cassettes are indicated.

were investigated by genomic Southern blot analysis to verify the integration of the co-transformed marker genes *bar* and *gus*. Genomic DNA was digested with *Hind* III, a restriction enzyme linearising the transferred plasmids p35SAcS and pubi.gus (Figure 1 and Fig. 3B,C lane 9), blotted onto a nylone membrane and hybridised to a DIG-labelled *bar* probe (Fig. 3B). After visualising of the *bar*-specific hybridisation signals, the membrane was stripped and hybridised to a DIG-labelled *gus* probe (Fig. 3C). Four regenerants were identified having a different integration pattern of the transgenes, corresponding to an overall transformation rate of 0.18% when the PDS particle gun was used (Table 1).

The genotypes 7042 (70) and Manga Nara (MN) yielded in one transgenic plant each, two genetically independent transformation events (B1 and B2) came from the genotype Bongo Nara (Fig. 3B, C); (Table 1). The transgenic line B2 is represented by three clonal plants in Figure 3B, C showing an identical integration pattern for both transgenes (lanes 5–7). No hybridisation signal could be detected in the non-transgenic wild-type control (lane 1).

These six transgenic T_0 plants, representing five independent transformation events and coming from two different bombardment methods, were all phenotypically normal. Their flowering and setting of seeds was comparable to seed-derived pearl millet plants of the corresponding genotypes.

Mature pollen grains of the transgenic plant B1 were stained histochemically to determine the segregation of the *gus* gene. Pollen grains from randomly chosen anthers of three different flowers were counted. A 1:1 segregation of stained and unstained pollen (Fig. 4A) hints to a mendelian segregation of the *gus* gene in plant B1. The male gametophyte, the germinated pollen, shows GUS-activity on an unstained stigma of a control plant after adding the GUS substrate X-Gluc (Fig. 4B).

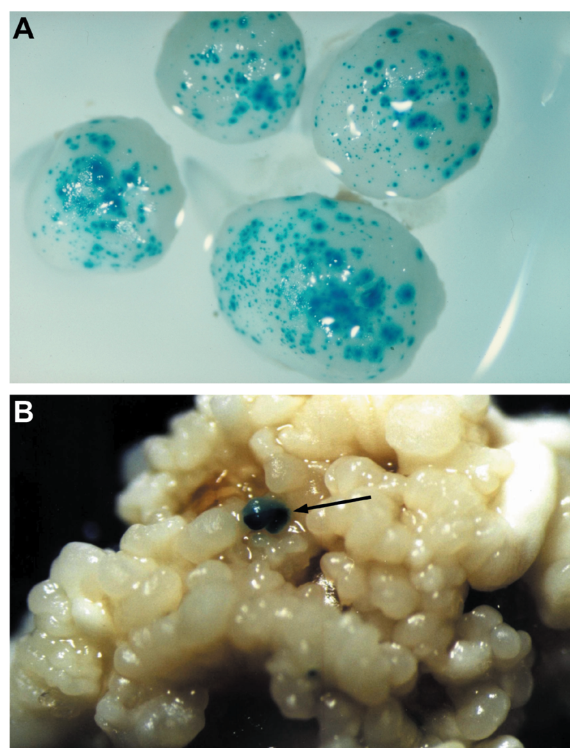

Figure 2. (**A**) Transient GUS activity in scutellum cells of immature zygotic embryos of genotype 7042, precultured for 2 days and stained for GUS activity 48 h after bombardment using the PDS particle gun. (**B**) Transgenic somatic embryo expressing the *uidA* gene (blue embryo, indicated by arrow) amongst non-transgenic white compact calli, 4 weeks after PIG bombardment; genotype 842B.

Analysis of T_1 generation

The PIG-produced transgenic plants (BB206.10A and BB206.10I) are used as pollen recipients and pollinated with untransformed pearl millet line 842B. To date, no transgenic T_1 progeny has been generated from these two clonal lines.

The T_1 generation of the PDS-produced transgenic plants (MN, 70, B1 and B2) was generated by removing 25–32 immature embryos from seeds of each T_0 plant and culturing them on regeneration medium R without selective agent for about three weeks. Thereby, the time-consuming seed dormancy could be circumvented and the T_1 generation could early get hardened-off in the greenhouse for the analysis of presence and expression of the transgenes.

Enzyme activity was verified by Basta spraying and histochemical GUS-staining (Fig. 5A, B). At least eight Basta-resistant progeny plants were analysed by

Table 1. Transformation rates per pearl millet genotype and transformation method

Genotypes	Number of bombarded embryos	Number of independent transgenic lines	Transformation rate (%)
PIG-mediated transformation method			
842B	2738	1	0.04
Manga Nara	1332	0	0
Bongo Nara	1150	0	0
Total	5220	1	0.02
PDS particle gun-mediated transformation method			
7042	719	1	0.14
Manga Nara	728	1	0.14
Bongo Nara	718	2	0.28
Total	2165	4	0.18

genomic Southern blot to verify the integration and its pattern of the inherited transgenes (Fig. 6A, B).

The progeny of three from the four T_0 plants showed a segregation ratio for Basta resistance consistent with a 3:1 segregation of a dominant single copy gene (Table 2), at a significance level of 5% according to the χ^2-test. GUS activity was detected in the T_1 progeny of the lines 70, MN and B1, whereas neither Basta resistance nor GUS-staining was detectable in the examined T_1 plants of the line B2. No transgenic progeny plants could be identified in this line.

Discussion

In this study, two transformation protocols for pearl millet are reported using alternatively the particle inflow gun PIG or the particle gun PDS 1000/He for the delivery of plant expression vectors into embryogenic cells. Immature zygotic embryos were used as explant material for stable integration of two different marker genes into the genome of pearl millet.

Genomic Southern blot analysis revealed five independent transformation events. The transgenic plants are all containing multiple copies of the *gus* and *bar* marker genes. The approximate number of integrated gene copies in the four T_0 plants produced by the PDS particle gun ranged from 4–7 for the *bar* and 2–7 for the *gus* gene. The transgenic plant produced using the PIG is represented by the two clonal

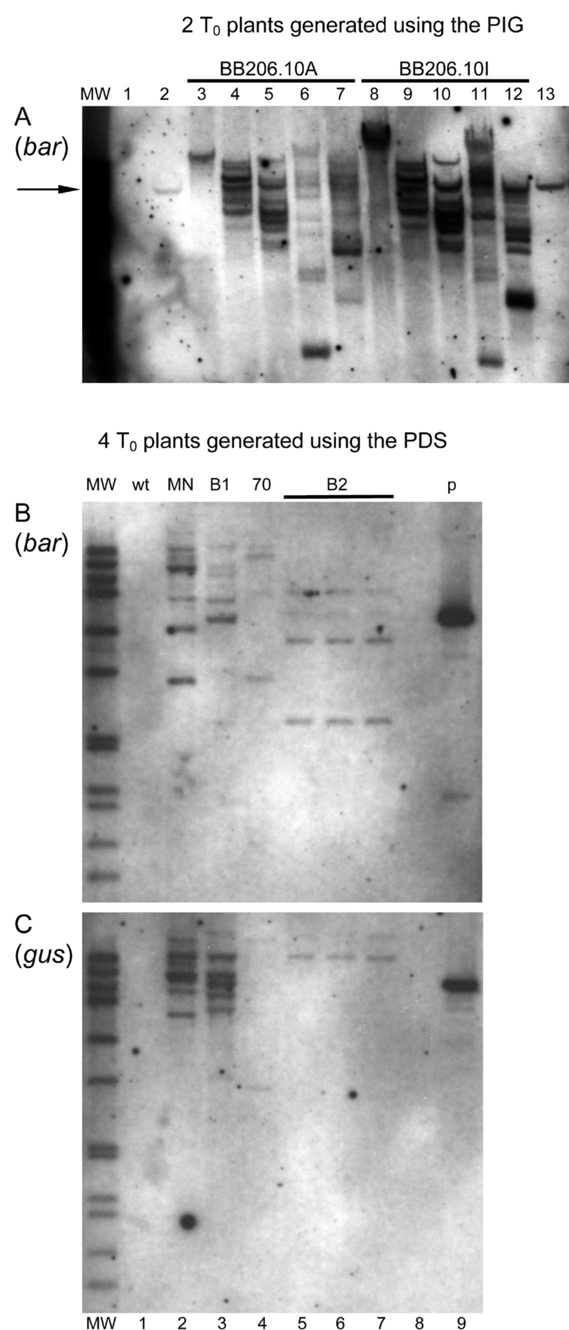

Figure 3. Genomic Southern blot analysis of (**A**) two clonal T_0 plants, genotype 842B, (**B, C**) four T_0 plants, genotypes Manga Nara, Bongo Nara and 7042 and of wild-type, non-transgenic controls (lanes 1). The blots were hybridised to DIG-labelled PCR probes using PCR primers (**A**) BAR_L and BAR_R, (**B**) bar3 and bar4 and (**C**) gus1 and gus2 to label *bar* or *gus* gene fragments. (**A**) Lane 1: wt control, genotype 842B; lanes 2 & 13: as lane 1 but spiked with 2 and 10 transgene copies, respectively; lane 3: 1 μg undigested DNA from plant BB206.10A; lanes 4–7: BB206.10A DNA digested with *Sac* I, *Hind* III, *Pst* I and *Eco* RI; lane 8: 5 μg undigested DNA from plant BB206.10I; lanes 9–12: BB206.10I DNA digested with *Sac* I, *Hind* III, *Pst* I and *Eco* RI. The arrow indicates the size of *Sac*I-linearised pAHC25, which is 9.7 Kb. (**B**,**C**) Lanes 1–7: Genomic DNA of wt control, genotype 7042, of the transgenic plants MN, B1, 70 and the three clonal plants of line B2, all after *Hind* III digest. *Hind* III linearises both co-transformed plasmids p35SAcS and pubi.gus (30 pg of each mixed in lane p). (**B**) Hybridisation signals after applying the DIG-labelled *bar* probe and (**C**) the *gus* probe.

plants BB206.10A and BB206.10I and possesses about a dozen of copies of the *bar* gene.

The 1:1 ratio of stained and unstained pollen (Fig. 4A) and the 3:1 segregation of Basta-resistant T_1 progeny plants as well as the identical integration pattern between T_0 and T_1 plants demonstrate that the multiple transgene copies are integrated at a single site into the genome and co-segregate comparable to a single dominant gene. Three of the four lines produced by the PDS particle gun are stably expressing both transgenes *bar* and *gus*. The T_0 plant B2 must have been chimeric because it did not inherit its transgenes.

The transgenic plants neither showed phenotypic abnormalities nor reduced fertility. Besides this report on transgenic pearl millet plants, recently, Lambé et al. (2000) presented the generation of transgenic pearl millet plants expressing the hygromycin phosphotransferase gene *hph* from E. coli mediating resistance to the antibiotic hygromycin B. In contrast to our findings, their *bar*-transformed calli could not be regenerated to plants. Further essential differences exist between the two systems. Here we describe a system using immature embryos as primary explants that are bombarded 2–5 days after isolation whereas Lambé et al. report on a system using calli and embryogenic units that are bombarded 7–8 weeks after isolation of shoot tips as primary explants. Different devices, protocols and genotypes were used for bombardment in the two cases. Common in both systems is that the transgenic lines contain multiple transgene copies, an undesirable feature that might cause transgene silencing in the progenies (Kumpatla et al. 1998).

The availability of different transformation systems presumably reflects the increasing economical importance of pearl millet in the industrialized world. Although the presented and the mentioned systems still need improvement because of multiple copy numbers in the transgenic lines, a relatively low transformation rate and the anomalies concerning phenotype and pattern of inheritance observed by Lambé et al., they show that genetic engineering of pearl millet is possible.

Weeds compete with crop plants for nutrients and light and thus reduce crop yields by an average of 10–15%. They can efficiently be controlled by the application of herbicides making herbicide resistance as one of the most desirable breeding traits in pearl millet cultivars (Andrews et al. 1996). To distinguish between crop plants and weeds, herbicide resistance can be genetically engineered in crop plants. Glufo-

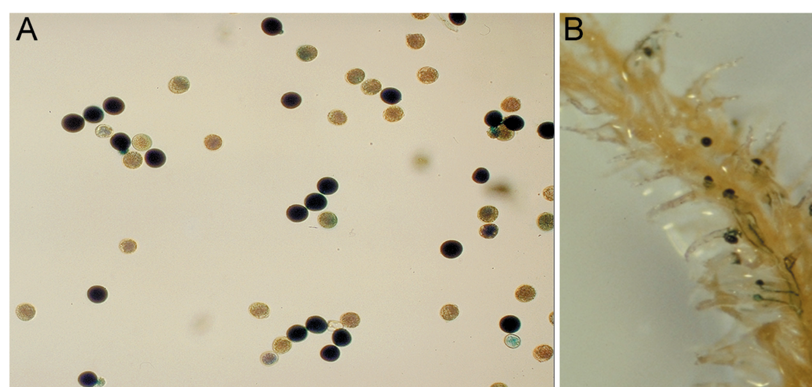

Figure 4. (**A**) Mature pollen grains of T_0 plant B1 are showing a 1:1 segregation of stained and unstained pollen in the histochemical GUS-assay. (**B**) Fresh pollen of plant B1 germinated on a stigma of a wild-type plant of the same genotype after histochemical GUS staining.

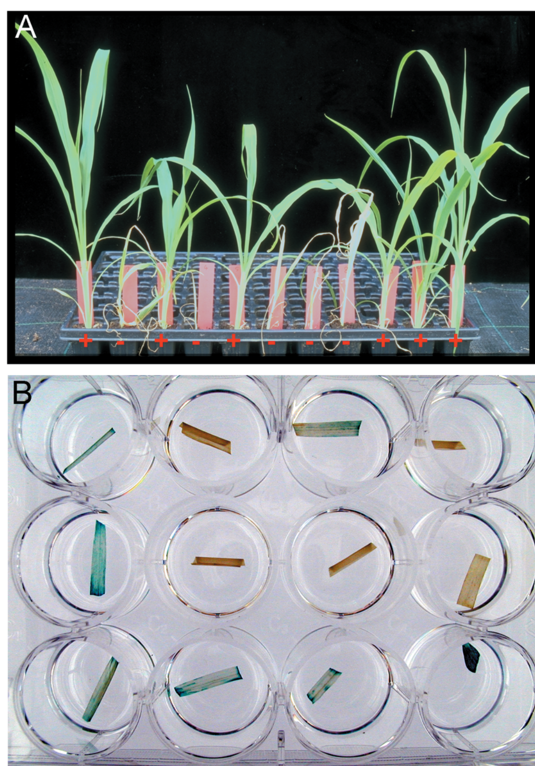

Figure 5. (**A**) Segregation of Basta resistance in the T_1 progeny of line 70, genotype 7042. Resistant plants are marked by "+", non-transgenic plants by "–". (**B**) Segregation of GUS activity in the same transgenic line.

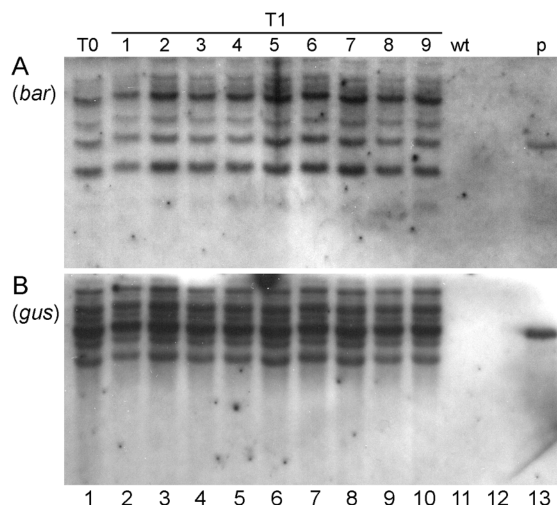

Figure 6. Southern blot analysis of T_0 plant MN and its T_1 progeny. Genomic DNA was extracted from T_0 plant MN (lane 1), from 9 of its T_1 progeny plants (lanes 2–10) and a wild-type, non-transgenic control (wt, lane 11) of the corresponding genotype Manga Nara. Identical pattern of hybridisation signals in T_0 and all examined T_1 progeny plants after restriction with *Hind* III linearising both plasmids p35SAcS and pubi.chi, as indicated in lane p (lane 13). The same blot was (**A**) hybridised to the DIG-labelled *bar* probe and (**B**) after washing of the membrane to the DIG-labelled *gus* probe.

sinate ammonium, the active agent of the herbicide Basta, was initially used to assist in genetic engineering of different desirable traits. Meanwhile, Basta-resistant maize, soybean and canola expressing the *bar* gene are already on the market. The production area of herbicide resistant maize i.e. has increased in the USA from 3% in 1996, to 18% in 1999 and 26% in 2001 (Economic Research Service (ERS)). Pearl millet as a robust cereal adaptive to sandy, acid and low fertile soils, has desirable nutritional values and therefore becomes more and more attractive to the industrialized world. In the United States pearl millet is already supplementing maize feed for poultry and swine because of its high protein content (Andrews et al. 1996; Burton et al. 1972).

As shown here, the resistance to the herbicide Basta can be used for different pearl millet cultivars

Table 2. Segregation of T_1 progeny from the four lines generated by the PDS particle gun

Transgenic line	Segregation of Basta resistance (resistant: susceptible)	Number of analysed T_1 seedlings
70	3.2: 1	25
MN	2.6: 1	29
B1	2.4: 1	31
B2	0: 32	32

for the selection of transgenic plants. Furthermore, it may be a future weed control applicable for genetically engineered pearl millet cultivars.

Before this might be achieved, various parameters still can be improved in our protocols. The low selection pressure is most possibly the reason for the numerous escapees obtained. Higher selection pressures will be employed in future pearl millet transgenic studies.

Although using a similar transformation protocol, our transformation rate for pearl millet is clearly lower (~ 0.18%) in comparison to our transformation rate for wheat (~ 1.0%) when the PDS particle gun was used (Becker et al. 1994; Oldach et al. 2001b). The reason for that might be the use of three pearl millet genotypes, that significantly varies in their regeneration frequencies (Oldach et al. 2001a) in contrast to the optimal genotype in the transformation system of wheat.

Furthermore, the parameters for DNA delivery probably have to be more adapted to the pearl millet tissue. To reduce tissue damage after bombardment, which seems to be a major problem in obtaining transgenic plants from scutellar tissue (Perl et al. 1992; Taylor and Vasil 1991), we have used microprojectiles with a small diameter (0.4–0.8 μm). On the other hand, smaller and thereby lighter microprojectiles require a higher gas pressure for acceleration to reach the necessary kinetic energy compared to heavier projectiles. Thus, the effect of increased gas pressures on the transformation rate has to be evaluated in detail for further improvement of the pearl millet transformation protocol.

The success of the stable transformation of different commercial pearl millet genotypes opens the way to transfer genes of agronomic interest. Besides herbicide resistance, disease resistance is of main interest in pearl millet. Genetic engineering of pearl millet for the control of diseases such as *Sclerospora graminicola*, causal agent of downy mildew, will have a major impact on pearl millet production in the developing world.

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