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Fertile transgenic pearl millet [*Pennisetum glaucum* (L.) R. Br.] plants recovered through microprojectile bombardment and phosphinothricin selection of apical meristem-, inflorescence-, and immature embryo-derived embryogenic tissues

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Abstract Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a drought-tolerant cereal crop used for grain and forage. Novel traits from outside of the gene pool could be introduced provided a reliable gene-transfer method were available. We have obtained herbicide-resistant transgenic pearl millet plants by microprojectile bombardment of embryogenic tissues with the *bar* gene. Embryogenic tissues derived from immature embryos, inflorescences and apical meristems from diploid and tetraploid pearl millet genotypes were used as target tissues. Transformed cells were selected in the dark on Murashige and Skoog medium supplemented with 2 mg/l 2,4-D and 15 mg/l phosphinothricin (PPT). After 3–10 weeks in the dark, herbicide-resistant somatic embryos were induced to germinate on MS medium containing 0.1 mg/l thidiazuron and 0.1 mg/l 6-benzylaminopurine. Plants were transferred to the greenhouse after they were rooted in the presence of PPT and had passed a chlorophenol red assay (the medium turned from red to yellow). Transgenic plants were recovered from bombardments using intact pAHC25 plasmid DNA, a gel-purified *bar* fragment, or a mixture of pAHC25 plasmid or *bar* fragment and a plasmid containing the enhanced green fluorescent protein (*gfp*) gene (p524EGFP.1). Analyses by the polymerase chain reaction, Southern blot hybridization, GFP expression, resistance to herbicide application, and segregation of the *bar* and *gfp* genes confirmed the presence and stable integration of the foreign DNA. Transformed plants were recovered from all three explants, although transformation conditions were optimized using only the tetraploid inflorescence. Time from culture initiation to rooted transgenic plant using the tetraploid inflorescence ranged from 3–4 months. Seven independent DNA/gold precipitations were used to bombard 52 plates, 29 of which produced an average of 5.5 herbicide-resistant plants per plate. The number of herbicide-resistant plants recovered per successful bombardment ranged from one to 28 and the frequency of co-transformation with *gfp* ranged from 5% to 85%.

Keywords GFP · Herbicide resistance · Pearl millet · Phosphinothricin · Transformation

Abbreviations *BA:* 6-Benzylaminopurine · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *GFP:* Green fluorescent protein · *GUS:* β-Glucuronidase · *INF:* Inflorescence · *IS:* Immature seed · *PAT:* Phosphinothricin acetyl transferase · *PPM:* Plant preservative mixture · *PPT:* Phosphinothricin · *S:* Seed · *TDZ:* Thidiazuron

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Introduction

Millets, which rank as the world's sixth most important tropical food cereal (see FAOSTAT at www.fao.org), are grown mostly in semi-arid West Africa and India. Pearl millet is the only major cereal that reliably produces both grain and forage on poor, sandy soils under hot, dry conditions. In the drier regions of Africa and Asia, this crop is a staple food grain. Also, pearl millet has been extensively utilized as a summer annual grazing crop in the southern United States and tropical and subtropical

regions of the world. Development of semi-dwarf, disease-resistant, high-quality forage cultivars has been a primary focus for breeders in the United States, resulting in the release of cultivars such as 'Tifleaf 3' (Hanna et al. 1997). Breeding programs also are incorporating the development of high-yielding, dwarf, grain-producing hybrids (Andrews et al. 1996). The release of new high-quality forage and grain cultivars, combined with pearl millet's natural drought resistance are gradually increasing the U.S. commercial growers' interest in this under-exploited, multi-purpose crop.

Traditional breeding has been the main avenue for crop improvement in pearl millet. A transformation system to incorporate otherwise inaccessible traits, such as certain forms of disease, insect, and herbicide resistance, would be a valuable tool to add to a pearl millet breeding program. Additionally, the effect of putative apomixis gene(s) from a wild relative (*Pennisetum squamulatum*; Ozias-Akins et al. 1998) could be tested by transformation of sexual pearl millet, especially tetraploid genotypes. Most major cereal crops have been transformed (Repellin et al. 2001). Cereal transformation protocols usually involve bombardment of embryogenic tissues derived from immature zygotic embryos or inflorescences, followed by selection of transformed callus and plant regeneration. Although there is a body of literature on tissue culture and plant regeneration of pearl millet (Vasil and Vasil 1981, 1982; Botti and Vasil 1983; Morrish et al. 1990; Taylor and Vasil 1996; Oldach et al. 2001), studies on transformation are limited. For example, Taylor and Vasil (1991) and Taylor et al. (1993) showed transient β- glucuronidase (GUS) expression after bombardment of immature embryos; however, plants were not regenerated. Pearl millet callus was transformed and confirmed by Southern blot analysis for integration of the hygromycin resistance gene although plants were not recovered (Lambe et al. 1995). A limited number of T_0 transgenic pearl millet plants containing the hygromycin resistance gene were recovered and confirmed by Southern blot analysis (Lambe et al. 2000); however, molecular confirmation of transgene transmission to progeny was not shown and apparently only three plants produced progeny that segregated for hygromycin resistance. Lambe et al. (2000) also compared hygromycin and phosphinothricin (PPT) selection but were not able to recover PPT-resistant plants. Most recently, Girgi et al. (2002) recovered a limited number of PPT-resistant plants by microprojectile bombardment of scutellar tissue. Their transformation frequency was less than 0.18%, and 99% (2/455) of the regenerants that survived selection were reported to be escapes. The objectives of this study were (1) to establish conditions for moderately efficient transformation of pearl millet embryogenic tissues using microprojectile bombardment and selection for herbicide resistance, (2) to test the ability to co-transform a herbicide-resistance gene and a *gfp* gene introduced on separate DNA molecules, and (3) to regenerate transgenic pearl millet plants efficiently and document the transmission of introduced genes to progeny.

Materials and methods

Plant materials and tissue culture

Source tissue was obtained from field- and greenhouse-grown plants of a diploid F_1 pearl millet hybrid, HGM100, and an induced, partially inbred tetraploid, IA4X. Explants included: (1) immature embryos (F_2 generation for HGM100) harvested 6–10 days after pollination, (2) spikelets (F_1 generation for HGM100) excised from inflorescences approximately 5–10 mm in length, and (3) apical meristems (F_1 generation for HGM100) from germinated seeds. The same explant types were used for IA4X. Spikelets were harvested multiple times from the same inflorescence by shaving from the cut-end (base) toward the apex and then rotating the rachis. The rachis was discarded after the majority of florets had been removed. Apical meristems were excised from surface-sterilized seeds plated on 5 g/l agar plus 2 ml/l plant preservative mixture (PPM; Plant Cell Technologies, Washington, D.C.) and germinated at 28°C in the light (average of 100 μmol m^{-2} s^{-1} from cool white fluorescent lamps for a 16-h per day photoperiod). Excision times began as soon as the seed coat had swelled and softened and continued until a small shoot had emerged (24–72 h). Source tissues were surface sterilized in ethanol [immature seed (IS) sterilized in 95% ethanol for 1 min; inflorescence (INF) 70% for 30 s; seeds (S) 70% for 1 min], then Clorox (containing 5.25% sodium hypochlorite) plus 0.5 g/l Alconox detergent (IS and S treated at 20% for 20 min; INF 10% for 20 min), followed by four rinses with sterile deionized water. All tissues were aseptically removed under a stereo dissecting microscope. Embryos were plated scutellum side up. Apical meristems were either exposed or included the first leaf primordium, and spikelets that accumulated on the edge of a no. 10 blade while shaving were spread onto the medium. Embryo, spikelet, and apical meristem cultures were initiated in the dark at 26°C on Murashige and Skoog (MS) (1962) medium containing 30 g/l sucrose, 7.5 g/l agar (A1296, Sigma, St. Louis, Mo.) plus 2, 2.5, or 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), respectively. For all media, the pH was adjusted to 5.8 before autoclaving (20 min, 121°C, 105 kPa). Once smooth, pale-yellow, nodular tissue developed from the apical meristems and inflorescences, it was transferred to 2 mg/l 2,4-D. For regeneration, cultures containing well-developed somatic embryos were transferred to MS medium supplemented with thidiazuron (TDZ) (0.1 mg/l) and 6-benzylaminopurine (BA) (0.1 mg/l, added to autoclaved medium from filter-sterilized stock) and moved into the light (average of 100 µmol m^{-2} s^{-1} from cool white fluorescent lamps for a 16-h per day photoperiod) at 28°C. Shoot and root elongation from germinated somatic embryos were promoted on basal MS medium in 20×100 mm Petri dishes.

DNA constructs used for transformation

Plasmid pAHC25 (Christensen and Quail 1996), a gel-purified *Hin*dIII fragment of pAHC25, and p524EGFP.1 (Fleming et al. 2000) were used for transformation. Plasmid pAHC25 contains the selectable *bar* gene, encoding the enzyme phosphinothricin acetyltransferase (PAT), and the reporter gene (*uidA*) encoding GUS, both under control of separate maize ubiquitin promoters (*Ubi1*) and its first intron and the *nos* terminator. The *Hin*dIII linearized fragment of pAHC25 used for bombardment eliminates the *uidA* expression cassette, resulting in the *bar* expression cassette and the vector backbone (5.5 kb). Plasmid p524EGFP.1 contains the double cauliflower mosaic virus 35S promoter sequence followed by the alfalfa mosaic virus enhancer sequence (Datla et al. 1993) controlling expression of an enhanced green fluorescent protein (*gfp*) gene (Clontech, Palo Alto, Calif., USA).

DNA delivery

Microprojectile bombardments were performed with the PDS-1000 He biolistic device (Bio-Rad, Hercules, Calif.). The tissue to be

bombarded was arranged within a 2.5-cm-diameter circle. Embryos were bombarded 5–7 days after culture initiation, and the apical meristem- and inflorescence-derived tissues were bombarded 2–4 weeks after culture initiation. Tissues were incubated on osmotic medium containing 2 mg/l 2,4-D and 0.25–0.35 *M* sucrose, 1–4 h pre- and 1–16 h post-bombardment. DNA (100–500 ng/shot) was precipitated onto 0.75 μm gold particles (Analytical Scientific Instruments, El Sobrante, Calif., USA) (30–90 μg/shot) or onto 0.6 μm gold particles (Bio-Rad) following a modified Bio-Rad protocol. Two microcentrifuge tubes, one with the DNA (0.5–2.5 μl of 200 ng/μl)/shot and gold (1–3 μl of 30 μg/μl)/shot and another with the spermidine (0.5–2.5 μl of 0.1 *M*)/shot and calcium chloride (1–3 μl of 2.5 *M*)/shot were mixed together and immediately vortexed gently for 5 min. The mixture was then centrifuged at low speed to create a loose pellet. The undisturbed pellet was rinsed once with 100–500 μl 100% ethanol with a slow spin. The pellet then was resuspended in 100% ethanol (volume equal to 8 times the number of planned shots + 20 μl) by breaking up with a pipette tip. The DNA-coated gold was vortexed at high speed before aliquoting 8 μl suspension onto the center of each macro-carrier. Gold particles for co-transformation with *gfp* were prepared the same way with the addition of 1 μg p524EGFP.1 to achieve approximately a 1:1 molar ratio of the two DNA molecules. The stopping screen was placed 6 cm below the rupture disk and the target tissue 6 cm below the stopping screen. The gun was fired when the vacuum pressure reached 71 cm Hg. Bombardment pressures ranged from 6,205–10,690 kPa (900–1,550 psi), and some samples were bombarded twice.

Selection of transformed plants and transgene expression

Transient GUS assays were performed 48 h after bombardment using a freshly prepared staining solution of 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, 0.5% Triton X-100, and 0.05% X-Gluc in sodium phosphate buffer (pH 7.2). Tissue was selected from the center and one or two random locations within the bombardment circle for GUS staining and incubated for up to 24 h at 37°C. Stained tissues were cleared by replacing the stain with 70% ethanol. The tissue was photographed using a digital camera (Zeiss Axiocam; Carl Zeiss, Thornwood, N.Y., USA). Three to 10 days after bombardment, tissues were transferred to MS + 2 mg/l 2,4-D + 15 mg/l PPT (diluted from 60% glufosinate ammonium, a gift from AgrEvo USA, Pikeville, N.C.) for selection of herbicide resistant tissue in the dark at 26°C. Tissue containing well-developed somatic embryos was transferred to the TDZ+BA regeneration medium described above, without PPT. Germinated somatic embryos then were moved to basal MS + 3–10 mg/l PPT in deep dishes. Surviving plantlets were moved to polypropylene round bottom tubes (17×100 mm) or magenta vessels containing basal MS + 8–10 mg/l PPT + 50 mg/l chlorophenol red (Kramer et al. 1993). Plants that remained unaffected by the herbicide and turned the medium yellow were transplanted to soil and acclimatized under a 250 W metal halide lamp before being moved to the greenhouse. Liberty (AgrEvo, Wilmington, Del.), a commercial herbicide formulation containing 182 g/l PPT, was diluted to 250–500 mg/l PPT with water and sprayed on putative transformed plants. A standard mist-type spray bottle was used, and plants were sprayed until all leaf surfaces appeared wet. Expression of GFP was monitored in callus, roots, and pollen of putative T_0 transformants and segregating progeny using a epifluorescence stereomicroscope (Zeiss SV11) equipped with a 100 W mercury bulb light source, a 480±30 nm excitation filter, and a 515 nm long-pass emission filter (Chroma Technology, Brattleboro, Vt., USA).

Segregation of transgenes

Herbicide-resistant T_0 plants were cross- and self-pollinated. Pollen from a non-transformed tetraploid plant (Tift23BR), homozygous for a dominant allele causing a red phenotype detectable in young seedlings, was used for tetraploid crosses. Diploid plants were pollinated with the male inbred parent (Tift8677). Progeny from crosses with Tift23BR were sprayed after the red phenotype was visible in order to ensure that segregation ratios did not contain self pollination events (identified as green progeny) that were susceptible to the herbicide. Segregation of GFP expression was observed in roots of germinated seeds. Chi-square goodness of fit was used to test the significance of observed versus expected ratios.

Molecular confirmation of transformed plant recovery

A subset of plants that expressed PAT alone or GFP and PAT were subjected to Southern blot hybridization analysis. Genomic DNA was isolated from 1 g of fresh T_1 or frozen T_0 (-80°C) tissue which was ground in liquid nitrogen and extracted according to Tai and Tanksley (1990) with some modifications (Ozias-Akins et al. 1993). Genomic DNA (10 μg) was digested with either *Hin*dIII or *Sac*I. Digestion with *Hin*dIII releases the ca. 5.5 kb *bar* gene cassette plus the vector backbone from pAHC25. Digestion with *Sac*I cuts pAHC25 once and should indicate insert copy number. Digested genomic DNA was subjected to electrophoresis overnight at 25 V on a 0.8% agarose gel in 1×TBE buffer (Sambrook et al. 1989), and then transferred to GeneScreen Plus nylon membrane (NEN Research Products, Boston, Mass.) using 0.4 *N* NaOH (Sambrook et al. 1989). PCR primers specific for the *bar* open reading frame (*bar* forward primer 5′ GCC AGT TCC CGT GCT TGA AGC CGG C 3′; *bar* reverse primer 5′ GGC GGT CTG CAC CAT CGT CAA CCA C 3′) were used to create ^{32}P-labelled probes. Probes were synthesized in a 20 μl PCR reaction containing 1× reaction buffer (50 m*M* KCl; 10 m*M* Tris-HCl, pH 9.0; 0.1% Triton X-100), 1.5 m*M* $MgCl_2$, 10 μ*M* dATP, dGTP, and dTTP, 10 pmol each primer, 2.5 U *Taq* DNA polymerase (Promega, Madison, Wis.), 0.825 μ*M* dCTP-α^{32}P (NEN LifeScience Products, Boston, Mass.), and template DNA, which was a gel-purified PCR product (400 bp *bar* fragment). PCR conditions in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) were as follows: initial denaturation at 94°C for 10 min followed by 40 cycles of amplification (50 s at 95°C, 50 s at 60°C and 2 min at 72°C) and a final extension of 10 min at 72°C. Unincorporated label was removed by spinning the reaction mix through Sephadex G-50 (Sigma) in an Amicon Ultrafree-MC 0.45 μm centrifugal filter device (Millipore, Bedford, Mass.). The probe was eluted in STE (100 mM NaCl; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA). After a 2-h pre-hybridization in a solution containing 7% SDS, 0.25 *M* $NaPO_4$, 1 m*M* EDTA, and 1% bovine serum albumin at 65°C, hybridization was performed overnight in the same solution plus the labeled probe. Blots were washed as follows: first wash in 50 ml 2× SSPE/1.0% SDS for 20 min at 65°C; second wash in 50 ml 0.5× SSPE/1.0% SDS for 20 min at 65°C, and final wash in 50 ml 0.1× SSPE/1.0% SDS for 20 min at 65°C. Hybridization signals were detected using the Cyclone Imaging System with OptiQuant software (Packard, Meriden, Conn.).

Results and discussion

Initiation of embryogenic cultures and plant regeneration

Of the three explant types, immature embryos were the most easily obtained in large numbers. Diploid embryos were more developmentally uniform at the time of harvest (6–10 days after pollination) than tetraploid embryos. Older embryos (more than 10 days old using the diploid) were usually white and easier to isolate and culture; however, they produced more watery, non-embryogenic tissue and browned more easily than embryos that were still translucent when isolated. The frequency of pale-yellow, embryogenic tissue that formed on the scutellar

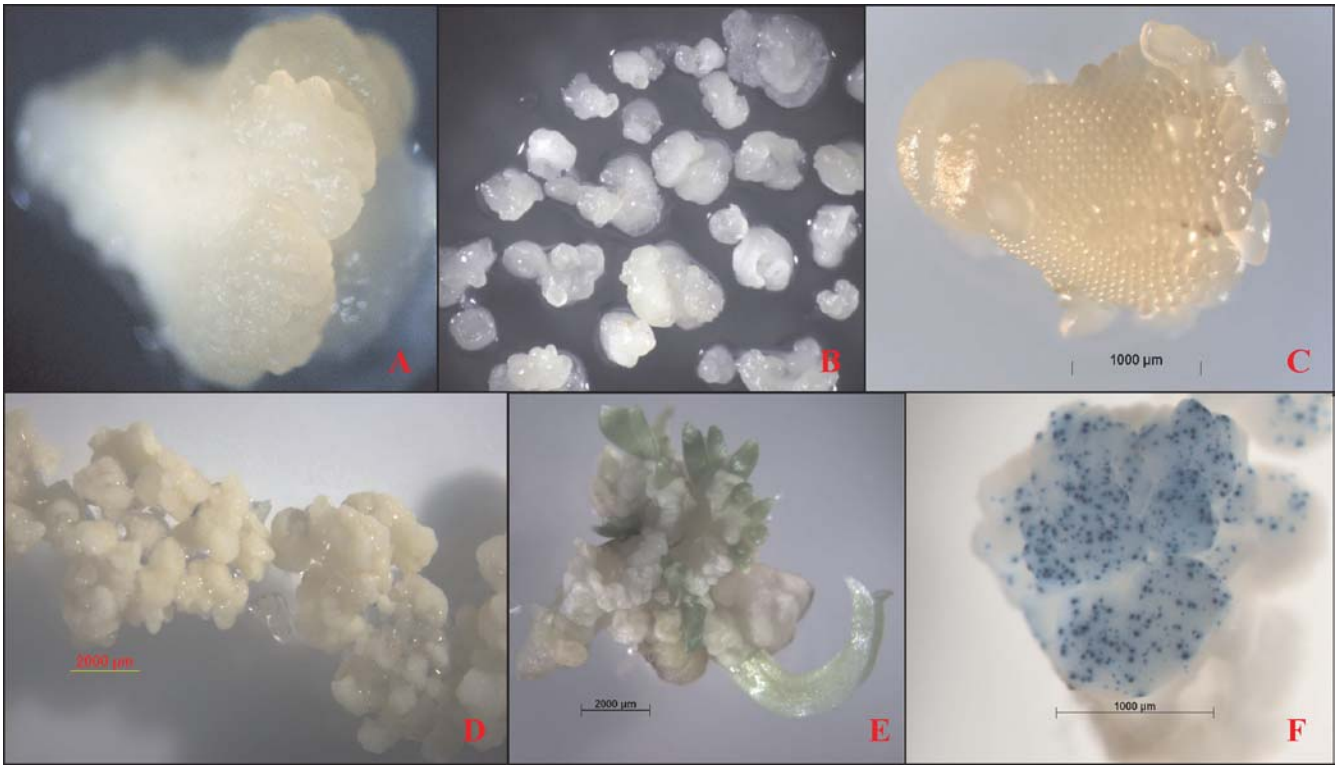

Fig. 1. **A** Pearl millet immature embryo 10 days after culture, forming pale-yellow embryogenic tissue on the edge of the scutellum. **B** Tetraploid apical meristems 14 days after culture, arranged for bombardment. **C** Inflorescence sheet showing embryogenic tissue forming at the medium/explant junction. **D** Shaved florets 15 days after bombardment. **E** Germinating somatic embryos after stimulation on medium containing thidiazuron (0.1 mg/l) and 6-benzylaminopurine (0.1 mg/l). **F** Transient β-glucuronidase expression 48 h after bombardment

Table 1 Frequency of pearl millet embryogenic tissue production using the apical meristem

Tissue[a]	No. plated[b]	No. forming embryogenic tissue	% Response	Data collected (days)
2x	76	37	49	21
2x	77	51	66	22
2x	67	29	43	21
2x	77	42	55	22
2x	84	41	49	22
2x	68	41	60	22
2x	60	29	48	20
2x	46	27	59	20
4x	54	40	74	20
4x	37	29	78	20

[a] 2x=diploid, 4x=tetraploid
[b] Number of apical meristems plated

surface of undamaged, translucent cultured embryos was commonly 100% (Fig. 1A).

Embryogenic callus production ranged from 43–78% when the apical meristem, exposed or including the first leaf primordium, was dissected from germinated seeds and cultured (Table 1, Fig. 1B). In addition to embryogenic tissue, friable watery tissue was more likely to grow when the first leaf was left on the explant. Although intact immature inflorescences produced embryogenic callus, it formed mainly at the medium/explant interface (Fig. 1C).

Shaving the spikelet primordia from the inflorescence was an effective way to increase the amount of tissue responding per explant. If the shave was too deep, spikelets remained intact on a thin sheet of rachis and continued to be capable of embryogenic tissue production but reduced the total amount of bombardment grade tissue available per inflorescence. By rotating the rachis axis, inflorescences could be shaved multiple times, allowing large quantities of responsive spikelets to be harvested and used for bombardment (Fig. 1D). One to 3 weeks after culture initiation, florets began to swell and develop embryogenic callus and somatic embryos. The large number of spikelets produced from a single shaved inflorescence, literally hundreds with good technique, did

not all respond at the same rate of growth and development, nor in their degree of browning; therefore, the most rapidly growing tissues were transferred to fresh medium prior to bombardment. Smooth, pale-yellow or white embryogenic tissues could be obtained from each of the three different explants.

Small scale experiments indicated that TDZ (0.1 mg/l) in combination with BA (0.1 mg/l) could cause rapid germination of somatic embryos (Fig. 1E). TDZ alone frequently caused the zygotic embryo still present in the primary cultures of immature embryos to germinate preferentially rather than the somatic embryos on the scutellar surface.

Microprojectile bombardment and herbicide selection

Microprojectile bombardment conditions were adjusted based on results of transient GUS expression and evidence of PPT resistance after 2–4 weeks under selection. Initial conditions, tested only on the embryo, involved a 0.4 *M* sorbitol, 4 h pre-, 16 h post-bombardment osmotic treatment and a bombardment pressure of 7,584 kPa (1,100 psi). Since transient GUS expression was observed to increase with osmotic treatment, such treatment was routinely included, although the osmoticum was changed to sucrose (Goldman et al. 2000). Consistent transient expression was observed with an osmotic treatment of 0.25–0.35 *M* sucrose and double bombardments at 7,584 kPa (1,100 psi) or single at 9,308–10,690 kPa (1,350–1,550 psi) (Fig. 1F).

Initial selection conditions on the embryo used 3–5 mg/l PPT. These conditions produced plants that were escapes that either were killed during chlorophenol red assay or did not turn the medium yellow but remained alive and were killed in the greenhouse when Liberty was applied. In order to decrease the chance of escapes, the concentration of PPT was increased to 15 mg/l and contact between the bombarded tissue region and herbicide-containing medium was improved by fragmenting the scutellar-derived embryogenic tissue. Although the fragmentation increased contact between tissue and the herbicide-containing medium, it was not compatible with results from regeneration experiments, which indicated that the best plant regeneration occurred when the embryo was left intact throughout the culture pathway. A combination of high PPT concentration (15 mg/l) and the bombardment conditions that supported the best transient expression was effective for producing three zygotic embryo-derived transgenic plants (Table 2).

Further transformation attempts were conducted with apical meristem and shaved inflorescence explants since orientation and size of the explant were not as critical for producing regenerable embryogenic tissue as for the embryo. Tissues were maintained in the dark under herbicide selection until actively growing, herbicide-resistant, pale-yellow embryogenic callus had formed. Upon transfer of the herbicide-resistant tissue to 0.1 mg/l TDZ + 0.1 mg/l BA in the light, somatic embryos matured and began to germinate within 7–10 days. Germinating embryos and surrounding tissues were moved to basal MS + 8–10 mg/l PPT where shoot elongation and rooting occurred. When immature or ungerminated somatic embryos remained in the cultures, they were cycled from TDZ+BA to basal MS + 8–10 mg/l PPT multiple times in order to induce somatic embryo germination. Even at high levels of PPT (8–10 mg/l), some escapes still produced roots and a shoot; however, the shoot usually did not continue to elongate and the roots lacked root hairs and grew slowly. These escapes could not be identified on medium containing chlorophenol red and low levels of PPT (3–5 mg/l) because medium colors ranging from deep purple to pale yellow occurred. A PPT concentration of 8–10 mg/l in chlorophenol red-containing medium was effective for eliminating escapes since the color tended to become either deep purple as the plant was killed or bright yellow while the plant remained vigorous (Fig. 2A). In most cases, regenerated plants that turned the medium bright yellow (3–7 days) and produced a pure white root system and a vigorous, green, elongating shoot on basal medium plus 8–10 mg/l PPT were resistant to a lethal dose of Liberty herbicide. Plants in the greenhouse were sprayed with 250–500 mg/l PPT in the form of Liberty to confirm expression of the *bar* gene. Control plants or segregating seedlings showed symptoms in 2–3 days and were dead within 2 weeks (Fig. 2B). The only exceptions appeared to be mixed plants that must have arisen from two somatic embryos that could not be separated at the time of plant formation. Unlike a chimera, mixed plants contained one resistant plant and one or two adjacent plants that were not resistant to a lethal dose of Liberty (Fig. 2C). It is possible that a non-transformed somatic embryo(s) was able to survive due to inefficient contact with PPT-containing medium and/or detoxification of the medium by the adjacent transformed tissue. A single bombardment (469) resulted in a plant that may have been a true chimera. When sprayed with Liberty, this plant responded with leaves that contained resistant and susceptible sectors separated by the main leaf vein.

Based on T_0 plants that were resistant to a lethal dose of Liberty, 20 DNA/gold precipitations produced 39 separate bombardments from which plants were recovered (Table 2). The overall time frame from culture initiation to herbicide-resistant, rooted plant in soil ranged from 3–8 months.

Co-transformation with GFP

GFP-expressing plants were recovered from two co-bombardments, one with pAHC25, and the other with the *Hin*dIII fragment containing the *bar* gene. Both shots used tetraploid inflorescence tissue that was selected for PPT resistance. Without PPT in the medium, GFP was not observed after transient expression had ended and none of the plants regenerated were herbicide resistant or expressed GFP. After about 1 week on PPT medium and 3 weeks after bombardment, larger sectors of GFP-

Table 2 Herbicide-resistant pearl millet plants regenerated from bombarded diploid and tetraploid tissues

Precip.[a]	Freq.[b]	DNA[c]	Shot no.	Explant[d]	Pressure (psi)	Sucrose (*M*)	Age[e]	PPT[f]	DNA	Plants[g]	Seed[h]
53	1/3	400	327	2x-AM	1,100	0.25	12	1	pAHC25	1	N
54	1/8	250	330	2x-AM	1,100	0.25	17	2	pAHC25	2	Y
56	1/9	267	351	4x-EM	1,100	0.35	7	nt	pAHC25	2	Y
60	1/8	300	380	2x-AM	1,100	0.25	19	nt	pAHC25	1	Y
67	2/6	286	420	2x-INF	1,350	0.25	32	5	pAHC25	1	Y
67	nt	286	422	2x-INF	1,350	0.35	32	5	pAHC25	1	N
69	1/4	320	426	2x-EM	1,350	0.25	7	4	pAHC25	1	Y
89	3/7	429	458	4x-AM	1,550	0.35	14	6	pAHC25	2	Y
89	nt	429	459	2x-AM	1,550	0.5	18	6	pAHC25	1	N
89	nt	429	462	2x-AM	1,550	0.35	19	6	pAHC25	3	Y
92	1/4	400	469	4x-AM	1,550	0.25	33	nt	pAHC25	1	Y
94	3/3	400	474	2x-AM	2x1,100	0.25	15	5	pAHC25	1	Y
94	nt	400	475	2x-AM	2x1,100	0.25	16	5	pAHC25	2	Y
94	nt	400	476	2x-AM	2x1,100	0.25	16	5	pAHC25	2	Y
95a	1/5	300	478	2x-AM	2x1,100	0.25	14	6	pAHC25	1	Y
95b	3/5	200	482	2x-AM	2x1,100	0.25	15	6	bar frag	2	Y
95b	nt	200	485	2x-AM	1,550	0.25	23	6	bar frag	1	Y
95b	nt	200	486	2x-AM	1,550	0.25	23	6	bar frag	1	Y
96	5/8	200	487	2x-AM	1,350	0.25	12	5	pAHC25	2	Y
96	nt	200	488	2x-AM	1,350	0.25	12	5	pAHC25	5	Y
96	nt	200	490	2x-AM	1,350	0.25	13	5	pAHC25	1	Y
96	nt	200	493	2x-AM	1,350	0.25	15	5	pAHC25	1	Y
96	nt	200	494	2x-AM	1,350	0.25	15	5	pAHC25	5	Y
97	2/7	200	498	2x-AM	1,350	0.25	12	4	pAHC25	1	N
97	nt	200	500	2x-AM	1,350	0.25	13	4	pAHC25	1	Y
98	1/6	267	505	2x-INF	1,100	0.25	7	5	pAHC25	1	N
100	1/7	286	514	4x-INF	1,350	0.25	14	6	pAHC25	1	Y
101	2/6	333	519	2x-INF	1,350	0.25	24	9	pAHC25	1	N
101	nt	333	522	2x-INF	1,350	0.4	19	9	pAHC25	2	N
102	4/6	400	526	4x-INF	1,550	0.35	16	7	pAHC25	6	Y
102	nt	400	527	2x-INF	1,550	0.25	19	7	pAHC25	1	Y
102	nt	400	528	2x-INF	1,550	0.35	19	7	pAHC25	3	Y
102	nt	400	529	4x-INF	1,550	0.35	21	7	pAHC25	4	Y
104a	2/5	200	542	4x-INF	1,550	0.35	20	10	gfp+frag	8	Y
104b	2/5	400	543	4x-INF	1,550	0.35	20	10	gfp+p25	6	N
104a	nt	200	546	4x-INF	1,550	0.35	20	10	gfp+frag	6	Y
104b	nt	400	547	4x-INF	1,550	0.35	25	10	gfp+p25	21	Y
105	2/6	500	553	4x-INF	1,550	0.35	38	5	gfp+p25	6	Y
105	nt	500	554	4x-INF	1,550	0.35	32	5	gfp+p25	3	N

[a] Identity of DNA/gold precipitation reaction
[b] Number of shots/total in precipitation reaction that produced herbicide-resistant plant(s); *nt* not tracked
[c] DNA concentration in nanograms used per shot
[d] 2x=diploid, 4x=tetraploid, *EM* embryo, *AM* apical meristem, *INF* inflorescence
[e] Age=days from culture initiation to bombardment
[f] *PPT* Days from bombardment until phosphinothricin (PPT) added to medium; *nt* not tracked
[g] Number of plants recovered
[h] Seeds=seeds produced via self or cross pollination (*Y* yes, *N* no)

expressing tissue could be seen. These stable GFP-expressing tissues that were also PPT resistant were used to regenerate herbicide-resistant, GFP-expressing T_0 plants. Two out of six co-bombardments produced herbicide-resistant plants that also expressed GFP. Both of those bombardments plus four others (542, 543, 553, 554) also produced herbicide-resistant plants that did not express GFP. Segregation data (see below) indicated that these bombardments produced plants that expressed only PPT resistance in addition to plants that expressed both PPT resistance and GFP. Expression of GFP could be seen in roots, immature embryos, callus, shoots germinating from callus, and stems from mature plants (Fig. 2D–G). Although chlorophyll masked the green fluorescence of GFP, expression was strong enough for transformed leaves to appear pink and non-transformed leaves to appear brick red. Not all GFP-expressing plants produced pollen that fluoresced, and based on preliminary crossing experiments, at least one plant from shot 546 contained an insert that may permit *gfp* to be transmitted only via the female gamete when self-pollinated.

Integration of the transgenes into genomic DNA

Southern blot analysis confirmed the genomic integration of *bar* from pAHC25 and the *Hin*dIII fragment. In the *Hin*dIII digest, multiple bands were observed; the 5.5 kb

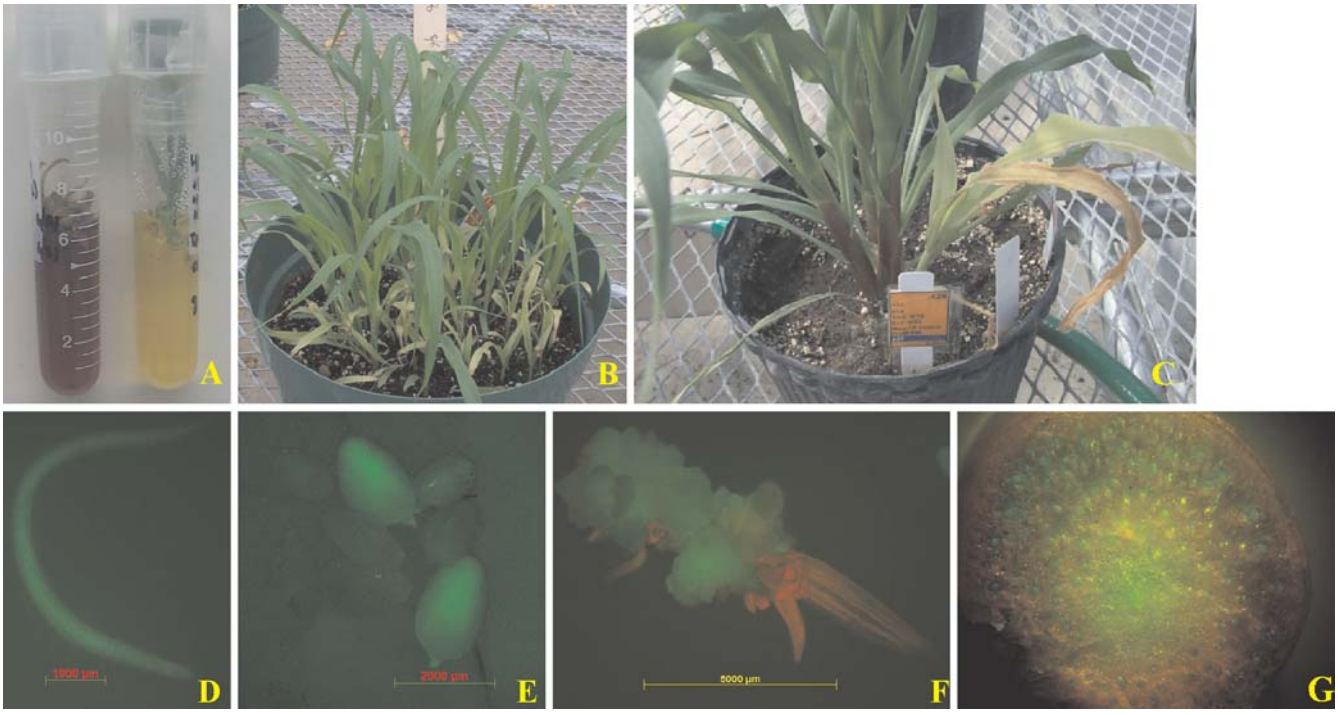

Fig. 2 **A** Chlorophenol red assay showing a dead plant in purple medium and a healthy herbicide-resistant pearl millet plant in yellow medium. **B** Segregation of resistant and sensitive seedlings (426×Tift8677) after herbicide application. **C** A mixed plant (no. 426), derived from an immature embryo, 1 week after application of 250 mg/l AI Liberty. **D** Green fluorescent protein (GFP) expression in root. **E** GFP expression in immature embryo. **F** GFP expression in callus and germinating shoots. **G** GFP expression in the cross-section of a stem

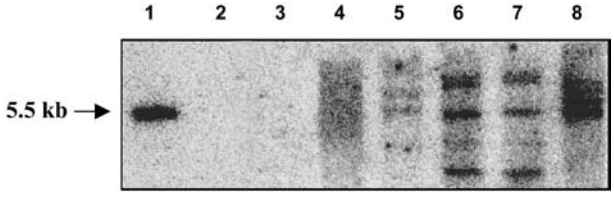

Fig. 3 Southern blot hybridization of *Hin*dIII-digested DNA from two herbicide-resistant T_0 plants and two T_1 progeny plants for the *bar* gene. Bombardment numbers are described in Table 2. *Lane 1* pAHC25, *lane 2* blank, *lane 3* p524EGFP, *lane 4* non-transformed control, *lane 5* T_1 546-1, *lane 6* T_0 494, *lane 7* T_1 494, *lane 8* T_0 487

bar-containing *Hin*dIII fragment was present as well as larger and smaller bands (Fig. 3). These could represent rearrangement events and multiple copies at a single locus or at multiple loci. Transmission of all hybridizing bands from 494–4 to its progeny is evidence of integration at a single locus. Deletions, duplications, and other physical alterations of the transforming DNA are common when using the biolistic approach and are probably the cause of the additional hybridizing bands seen here. Digestion with *Sac*I also indicated that most plants contained multiple copies and possibly more than one unique insertion site (Fig. 4). Stripping the blot and re-probing with *gfp* confirmed genomic integration of p524EGFP.1 in T_1 546–1 (data not shown).

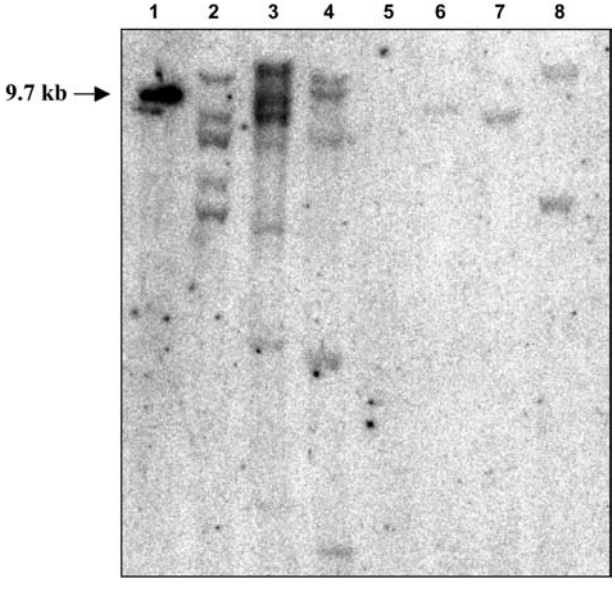

Fig. 4 Southern blot hybridization of *Sac*I-digested DNA from six T_0 transgenic plants for the *bar* gene. Bombardment numbers are described in Table 2. *Lane 1* pAHC25, *lane 2* 529-1, *lane 3* 547-2, *lane 4* 527, *lane 5* non-transformed control, *lane 6* 547B, *lane 7* 547-4, *lane 8* 542-2

Table 3 Segregation of herbicide resistance and green fluorescent protein (*GFP*) expression from diploid and tetraploid primary transformants of pearl millet

ID	Explant[c]	Cross	DNA	Ratio of expressing to non-expressing	Expected	χ^2	*P**
426	2x-EM	Tift8677	pAHC25	20:39	1:1	6.1	0.014
426	2x-EM	self	pAHC25	11:9	3:1	2.9	0.089*
458	4x-AM	Tift23BR	pAHC25	20:32	1:1	2.7	0.100*
478	2x-AM	Tift8677	pAHC25	29:23	1:1	0.69	0.406*
487	2x-AM	Tift8677	pAHC25	20:31	1:1	2.3	0.129*
490	2x-AM	self	pAHC25	28:11	3:1	0.21	0.647*
494	2x-AM	Tift8677	pAHC25	10:6	1:1	1.0	0.317*
494-1	2x-AM	Tift8677	pAHC25	22:25	1:1	0.19	0.890*
494-4	2x-AM	Tift8677	pAHC25	27:16	1:1	2.9	0.089*
514	4x-INF	Tift23BR	pAHC25	3 red, *bar*+:3 red, *bar*-30 green, *bar*+:9 green *bar*-	1:13:1	0.076	0.783*
542	4x-INF	Tift23BR	*bar* frag+GFP	24:15	1:1 (no gfp)[a]	2.1	0.147*
546 feb12	4x-INF	Tift23BR	*bar* frag+GFP	19:13	1:1 linked[b]	1.1	0.294*
546 feb15	4x-INF	Tift23BR	*bar* frag+GFP	18:17	1:1 linked	0.029	0.865*
547 feb 22	4x-INF	Tift23BR	pAHC25+GFP	21:17	1:1 (no gfp)	0.42	0.517*
547 mar 7	4x-INF	Tift23BR	pAHC25+GFP	8 GFP+/*bar*+:3 GFP-/*bar*-:3 GFP-/*bar*+	1:1 for bar	2.27	0.132*

[a] No GFP expression observed in progeny
[b] GFP expression linked to *bar* expression
[c] 2x=diploid, 4x=tetraploid, *EM* embryo, *AM* apical meristem, *INF* inflorescence
* If $P \geq 0.05$, the observed segregation ratio is not significantly different from the expected ratio

Fertility of primary transformants and transmission of transgenes to progeny

The fertility of herbicide-resistant plants varied between lines. Seeds were harvested from 69 of the 110 herbicide-resistant plants. Most plants were female fertile when cross-pollinated. The greenhouse environment can have a negative influence on pollen viability and fertilization and may explain why some self- and cross-pollinations with the same plant produced different fertility levels. At least four shots produced completely sterile off-type plants (488, 459, 519, 522). Transmission and segregation of the novel genes were observed with both diploid and tetraploid primary transformants (Table 3). Most ratios fit a 1:1 (resistant to sensitive) segregation ratio expected for a test-cross with the transgene at a single locus. Some self-pollination events such as no. 490 (2×-AM) and no. 514 (4×-INF) fit a 3:1 ratio. Even though no. 514 was crossed with red pollen, it set mainly selfed seeds as indicated by the 6 red plants and 39 green plants germinated from a single seed head (Table 3). GFP expression could be observed in roots of germinated seeds and was an effective screening tool to determine segregation ratios prior to red color formation in out-crosses or Liberty application.

Frequency of transformed plant recovery using the tetraploid inflorescence

Initial experiments indicated that transgenic plants could be recovered using all three explant sources. Since the shaved inflorescence produced the largest quantity of bombardment-grade tissue per explant, seven additional experiments were conducted to test the reproducibility of transformation with this tissue and to estimate a transformation frequency (Table 4). The shaved inflorescence was efficient for generating 2.5-cm-diameter circles of target tissue, ranging from a single inflorescence producing four targets to one inflorescence per target. The seven DNA/gold precipitations resulted in 52 bombardments and the recovery of 155 transgenic plants. All precipitations resulted in transgenic plant recovery; however, the efficiency ranged from two plants (precipitation D) to 82 plants (precipitation E). On a per bombardment basis, 29 of the 52 bombardments (55%) produced transgenic plants, and the number of plants recovered per successful bombardment ranged from 1 to 28 with an average of 5.5. All of the DNA/gold precipitations that included p524EGFP.1 produced plants that were herbicide resistant and expressed GFP. Of those bombardments that resulted in co-transformed plants (13 total), the frequency of co-transformation ranged from 5% to 85%.

While transformation systems for more popular cereals such as rice and wheat are continually being modified and improved, limited information has been available on methods to recover fertile, transformed pearl millet plants effectively. We have developed a protocol that can use three different explant sources (apical meristems, immature embryos and immature spikelets) to initiate suitable tissue cultures for microprojectile bombardment followed by transformed plant regeneration. Apical meristem culture has the advantage of not requiring plants to be grown and maintained. The responsive tissue was very similar to individual florets; however, only one apical meristem was available per seed. Originally, we thought that the immature embryo would be the best choice for transformation because of the high response rate and large numbers of embryos available per seed head. On the contrary, shaving the spikelets proved to be an excellent

Table 4 Transformation experiments using the tetraploid inflorescence for bombardment

Precipitation ID	No. of plates bombarded (No. producing transgenic plants)	DNA	Gold (μm)	Osmotic treatment[a]	Pressure (psi)	No. of herbicide resistant plants[b]	No. of co-transformed plants
A	4 (3)	pAHC25+GFP[d]	0.6	None	1,100[c]	3	17
B	8 (4)	pAHC25+GFP[d]	0.75	0.35 *M* sucrose	1,550	5	6
C	8 (4)	pAHC25+GFP[d]	0.75	0.35 *M* sucrose	1,550	20	1
D	8 (2)	pAHC25	0.75	0.35 *M* sucrose	1,550	2	–
E	12 (9)	pAHC25+GFP[d]	0.6	0.35 *M* sucrose	1,550	63	19
F	6 (5)	pAHC25	0.6	0.35 *M* sucrose	1,550	16	–
G	6 (2)	pAHC25	0.6	0.35 *M* sucrose	1,550	4	–

[a] Tissue was incubated 2–4 h pre- and post-bombardment
[b] Herbicide selection was initiated 2 weeks after bombardment, and a plant was considered transformed if it was completely resistant to a lethal application of Liberty (500 mg/l AI) and co-transformed if the roots fluoresced
[c] Two bombardments of each plate at 1100 psi
[d] Green fluorescent protein

way to increase dramatically the amount of tissue from a single inflorescence that was capable of embryogenic tissue production. In this study, up to 4 bombardment circles, each 2.5 cm in diameter, were created from a single inflorescence. This amount of tissue derived from the apical meristem would have required at least 4 h of meristem isolation and culture.

Girgi et al. (2002) recently reported a transformation frequency of 0.18% with immature embryos. Since we did not extensively test embryo explants after optimized conditions of osmotic treatment and bombardment were established for inflorescence tissues, it is not clear if the embryo would be an efficient explant using our optimized conditions. Therefore, only a crude comparison of transformation frequencies can be made between our study and that of Girgi et al. (2002) on a per plate/bombardment basis. If we assume that Girgi et al. (2002) plated 30–40 embryos per bombardment with the PDS instrument, the total number of embryos reported would have resulted in 54–72 bombardments. According to our transformation frequency with inflorescence tissues, 55% of the bombardments should have resulted in at least one transformation event or a minimum total of 27–36 events should have been recovered by Girgi et al. (2002) to match the minimum transformation frequency we achieved with inflorescence tissues. Only 4 events using the PDS instrument were reported by Girgi et al. (2002). This difference in transformation frequency between the two studies could be due not only to explant type, but also genotype, bombardment and selection conditions.

Herbicide selection was not always obvious even with a concentration of 15 mg/l PPT in the dark. Callus from all source tissues occasionally survived multiple transfers in the dark before being killed in the light. When fescue plants (Cho et al. 2000) were selected with bialaphos or hygromycin it was found that chimeric plants or escapes were possible with bialaphos and the selection pressure with hygromycin was much stronger, with sensitive tissue not surviving multiple transfers to fresh medium. Lambe et al. (2000) tested both *hph* and *bar* as selectable marker genes with pearl millet and were only able to recover transgenic plants using *hph*. Although Girgi et al. (2002) were able to recover a few transgenic plants containing the *bar* gene, most regenerated plants were escapes, probably due to the low level of selection imposed. The timing of post-bombardment selection should be further investigated for pearl millet transformation. In our study, waiting 10–14 days prior to PPT selection was most effective for recovery of herbicide-resistant plants. Unpublished observations (senior author) involving hybrid bermudgrass and a 2-week delay before adding PPT, along with results presented here, may indicate that delaying selection could be a key to maximizing transformed plant recovery.

In the present study, we only recovered GFP-expressing tissues that were also herbicide resistant. Lack of GFP expression from 2–3 weeks after bombardment followed by the appearance of discrete sectors of expression with the addition of herbicide is similar to what Vain et al. (1998) described with co-transformation of *gfp* and a hygromycin-resistance gene on separate plasmids. They also found that without hygromycin selection, GFP sectors could not be maintained or amplified easily in a clonal manner using consecutive cycles of visual selection. Nevertheless, non-destructive GFP selection has been used effectively in some cases to recover transformed plants (Fleming et al. 2000). Non-destructive, visual, GFP selection for transformed tissue was not effective in our study and led to the generation of large amounts of non-transformed tissue that otherwise would have been eliminated via herbicide selection.

Silencing of the *gus* gene in monocots has been shown to occur, but in most cases flanking selectable marker genes remained functional (Iyer et al. 2000). Lambe et al. (1995) indicated that methylation-based transgene silencing occurred in pearl millet callus and later observed that GUS expression was silenced during in vitro culture but restored in regenerated plants (Lambe et al. 2000). Although the reasons are unclear in our experiments, besides transient GUS expression, stable expression of GUS was not detected in any diploid or tetraploid herbicide-resistant plants. This result is in contrast to

the expression of GUS in most of the transgenic plants of Girgi et al. (2002).

The frequency of pearl millet transformation in initial experiments involving all three explants was low, with an average of three bombardments required to produce a single transformation event. Additional experiments involving only the tetraploid inflorescence were more efficient in recovering transformed plants. With minimal culture initiation time, enough tissue was obtained to create 52 bombardment circles, each 2.5 cm in diameter, from which 155 transgenic plants were recovered. Since diploid and tetraploid tissues were not always bombarded under the same conditions it is not possible to conclude whether there were differences between the ploidy levels for transformation frequency. In some cases, such as *gfp* co-bombardments in which both diploid and tetraploid tissues were tested under the same conditions, only tetraploid tissue produced transformed plants.

Southern blot analysis was used to confirm integration of the transgenes from independent events and to examine copy number. Although multiple plants were produced from several successful bombardments, the independent origin of each plant was not thoroughly investigated. It is unlikely that all 21 herbicide-resistant plants regenerated from shot 547 are independent events, although a combination of phenotypic, segregation, and Southern data indicated that at least some of these plants were from different transformation events. Regarding the complexity of integration pattern, Fu et al. (2000) reported that linearized plasmids minus the vector backbone predominantly produced transgenic plants with low-copy-number inserts and a low frequency of transgene rearrangements compared to supercoiled plasmid DNA. As shown by Southern blots, linearizing the plasmid did not always reduce the complexity of the integration event in our study, and, due to the low yield in the gel purification process, this approach does not seem practical. In this experiment, the linear *Hin*dIII fragment from pAHC25 still contained the vector backbone and was co-bombarded with a plasmid, both of which may have hindered simple transgene integration.

The relatively rapid time frame from culture initiation to plant recovery (3–8 months in all initial experiments; 3–4 months with tetraploid inflorescence) may reduce the amount of somaclonal variation that is known to occur in pearl millet (Morrish et al. 1990). Thidiazuron has been effective for regeneration in other monocots (Chengalrayan and Gallo-Meagher 2001) and the TDZ-based germination medium used here stimulated prolific embryo germination, which was beneficial for reducing in vitro culture time. Although there is room for further optimization of this transformation protocol, it was effective for producing fertile transgenic plants from a variety of explant types and genome configurations (diploid F_1 hybrid, diploid F_2 segregating population, tetraploid genotypes). This protocol should be a valuable tool in any pearl millet breeding program or as a model system when evaluating putative apomixis regulatory genes.

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References

Andrews DJ, Hanna WW, Rajewski JF, Collins VP (1996) Advances in grain pearl millet: utilization and production research. In: Janick J (ed) Progress in new crops. ASHS Press, Alexandria, Va., pp 170–177

Botti C, Vasil IK (1983) Plant regeneration by somatic embryogenesis from parts of cultured mature embryos of *Pennisetum americanum* (L.) K. Schum. Z Pflanzenphysiol 111:319–325

Chengalrayan K, Gallo-Meagher M (2001) Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cell Dev Biol Plant 37:434–439

Cho MJ, Ha CD, Lemaux PG (2000) Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly regenerative tissues. Plant Cell Rep 19:1084–1089

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:1–6

Datla R, Bekkaoui F, Hammerlindl JK, Pilate G, Dunstan DI, Crosby WL (1993) Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci 94:139–149

Fleming GH, Olivares-Fuster O, Fatta Del-Bosco S, Grosser JW (2000) An alternative method for the genetic transformation of sweet orange. In Vitro Cell Dev Biol Plant 36:450–455

Fu X, Duc LT, Fontana S, Bong BB, Tinjuangjun P, Sudhakar D, Twyman RM, Christou P, Kohli A (2000) Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Res 9:11–19

Girgi M, O'Kennedy MM, Morgenstern A, Mayer G, Lörz H, Oldach KH (2002) Transgenic and herbicide resistant pearl millet (*Pennisetum glaucum* L.) R. Br. via microprojectile bombardment of scutellar tissue. Mol Breed 10:243–252

Goldman JJ, Ozias-Akins P, Hanna WW (2000) GUS transient gene expression in bombarded pearl millet tissue. Int Sorghum Millet Newsl 41:74–76

Hanna WW, Hill GM, Gates RN, Wilson JP, Burton GW (1997) Registration of 'Tifleaf 3' pearl millet. Crop Sci 37:1388

Iyer LM, Kumpatla SP, Chandrasekharan MB, Hall TC (2000) Transgene silencing in monocots. Plant Mol Biol 43:323–346

Kramer K, DiMaio J, Carswell GK, Shillito RD (1993) Selection of transformed protoplast-derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator chlorophenol red. Planta 190:454–458

Lambe P, Dinant M, Matagne RF (1995) Differential long-term expression and methylation of the hygromycin phosphotransferase (hph) and β-glucuronidase (GUS) genes in transgenic pearl millet (*Pennisetum glaucum*) callus. Plant Sci 108:51–62

Lambe P, Dinant M, Deltour R (2000) Transgenic pearl millet (*Pennisetum glaucum*). In: Bajaj YPS (ed) Transgenic crops. Biotechnology in agriculture and forestry, vol 46. Springer, Berlin Heidelberg New York, pp 84–108

Morrish FM, Hanna WW, Vasil IK (1990) The expression and perpetuation of inherent somatic variation in regenerants from embryogenic cultures of *Pennisetum glaucum* (L.) R. Br. (pearl millet). Theor Appl Genet 80:409–416

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Oldach KH, Morgenstern A, Rother S, Girgi M, O'Kennedy M, Lörz H (2001) Efficient in vitro plant regeneration from

immature zygotic embryos of pearl millet [*Pennisetum glaucum* (L.) R. Br.] and *Sorghum bicolor* (L.) Moench. Plant Cell Rep 20:416–421

Ozias-Akins P, Lubbers EL, Hanna WW, McNay JW (1993) Transmission of the apomictic mode of reproduction in *Pennisetum*: co-inheritance of the trait and molecular markers. Theor Appl Genet 85:632–638

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes. Proc Natl Acad Sci USA 95:5127–5132

Repellin A, Baga M, Jauhar PP, Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: new challenges. Plant Cell Tissue Organ Cult 64:159–183

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Tai TH, Tanksley SD (1990) A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. Plant Mol Biol Rep 8:297–303

Taylor MG, Vasil IK (1991) Histology of, and physical factors affecting, transient GUS expression in pearl millet [*Pennisetum glaucum* (L.) R. Br.] embryos following microprojectile bombardment. Plant Cell Rep 10:120–125

Taylor MG, Vasil IK (1996) The ultrastructure of somatic embryo development in pearl millet (*Pennisetum glaucum*; Poaceae). Am J Bot 83:28–44

Taylor MG, Vasil V, Vasil IK (1993) Enhanced GUS expression in cereal/grass cell suspensions and immature embryos using the maize ubiquitin-based plasmid pAHC25. Plant Cell Rep 12:491–495

Vain P, Worland B, Kohli A, Snape JW, Christou P (1998) The green fluorescent protein (GFP) as a vital screenable marker in rice transformation. Theor Appl Genet 96:164–169

Vasil V, Vasil IK (1981) Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum* and the *Pennisetum americanum* ×*Pennisetum purpureum* hybrid. Am J Bot 68:864–872

Vasil V, Vasil IK (1982) Characterization of an embryogenic cell suspension culture derived from cultured inflorescences of *Pennisetum americanum* (pearl millet; Gramineae). Am J Bot 69:1441–1449