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GENETIC TRANSFORMATION AND HYBRIDIZATION

Efficient, reproducible *Agrobacterium*-mediated transformation of sorghum using heat treatment of immature embryos

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Abstract A number of parameters related to *Agrobacterium*-mediated infection were tested to optimize transformation frequencies of sorghum (*Sorghum bicolor* L.). A plasmid with a selectable marker, phosphomannose isomerase, and an *sgfp* reporter gene was used. First, storing immature spikes at 4°C before use decreased frequency of GFP-expressing calli, for example, in sorghum variety P898012 from 22.5% at 0 day to 6.4% at 5 days. Next, heating immature embryos (IEs) at various temperatures for 3 min prior to *Agrobacterium* infection increased frequencies of GFP-expressing calli, of mannose-selected calli and of transformed calli. The optimal 43°C heat treatment increased transformation frequencies from 2.6% with no heat to 7.6%. Using different heating times at 43°C prior to infection showed 3 min was optimal. Centrifuging IEs with no heat or heating at various temperatures decreased frequencies of all tissue responses; however, both heat and centrifugation increased de-differentiation of tissue. If IEs were cooled at 25°C versus on ice after heating and prior to infection, numbers with GFP-expressing cells increased from 34.2 to 49.1%. The most optimal treatment, 43°C for 3 min, cooling at 25°C and no centrifugation, yielded 49.1% GFP-expressing calli and 8.3% stable transformation frequency. Transformation frequencies greater than 7% were routinely observed using similar treatments over 5 months of testing. This reproducible frequency, calculated as numbers of independent IEs producing regenerable transgenic tissues, confirmed by PCR, western and DNA hybridization analysis, divided by total numbers of IEs infected, is several-fold higher than published frequencies.

Keywords *Agrobacterium* · Heat treatment · Phosphomannose isomerase · Sorghum · Transformation

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Introduction

Biotechnology, specifically genetic engineering, is one of the tools that can be used to improve sorghum for the future. Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important cereal crops, ranking fifth in production among cereals worldwide. Given its relative tolerance to drought, sorghum is an ideal grain for human and animal consumption in areas with extreme temperatures and minimal precipitation, and thus, it is popular as a grain and forage crop in the semi-arid regions of Asia and Africa. Attractiveness of dryland crops, such as sorghum, will surely increase elsewhere as global climate changes lead to dramatic changes in temperatures and water availability. With these likely changes, efforts to increase yield and quality parameters of crops like sorghum will be of critical importance.

Successful efforts aimed at in vitro culturing and regeneration and ultimately transformation of grain

sorghum have been reported (Masteller and Holden 1970; Cai et al. 1987; Cai and Butler 1990; Hagio et al. 1991; Casas et al. 1993, 1997; Kaeppler and Pederson 1997; Zhu et al. 1998; Zhao et al. 2000; Emani et al. 2002; Gao et al. 2005a, b; Howe et al. 2006; Nguyen et al. 2007). As with several other monocot crops, the first fertile transgenic sorghum plants were obtained using particle bombardment (Casas et al. 1993), followed 7 years later by the first report of *Agrobacterium*-mediated transformation (Zhao et al. 2000). The first reports of sorghum plants transformed with agronomically important genes were the use of the HT12 gene for higher grain lysine content (Zhao et al. 2003) and the *cry1Ac* gene for insect resistance (Girijashankar et al. 2005). Other studies mainly focused on improving transformation and regeneration frequencies (Tadesse et al. 2003; Carvalho et al. 2004; Gao et al. 2005a, b; Howe et al. 2006) reported transformation efficiencies of sorghum via *Agrobacterium* routinely ranged from 1 to 2%. In the most recent study, Howe et al. (2006) reported that in only one of 17 experiments a 4.5% frequency (4 events in 89 explants) was achieved; however, frequencies in the other 16 ranged from 0.3 to 1.9%. Results like these underscore the recalcitrance and lack of reproducibility of such technologies with sorghum (O'Kennedy et al. 2006; Zhao 2006).

In optimizing transformation strategies, the choice of selectable marker is important. Two such markers have been used frequently for sorghum, the herbicide resistance gene, *bar*, and the antibiotic resistance gene, hygromycin phosphotransferase, *hpt* (Casas et al. 1993; Zhu et al. 1998; Zhao et al. 2000; Emani et al. 2002). Environmental and consumer concerns regarding the possible impact of these markers have been raised and therefore new selection strategies offering alternatives to antibiotic and herbicide resistance genes have been developed. These approaches include genes encoding, for example, phosphomannose and xylose isomerase (PMI, XI), that enable selection by giving transgenic cells a metabolic advantage over non-transgenic cells (Penna et al. 2002). In addition, screenable or visual marker genes, encoding green (GFP; Sheen et al. 1995) or yellow (YFP; Nagai et al. 2002) fluorescence proteins have been used to identify transgenics, particularly when coupled with an effective selection system. For example, use of GFP, coupled with PMI selection, was used successfully for the first time with sorghum by Gao et al. (2005b). Expression of PMI encoded by *E. coli man*A enables transgenic PMI-expressing plant cells to convert mannose-6-phosphate into the easily metabolizable fructose-6-phosphate, which serves as a carbon source that improves the energy status of transgenic tissue (Joersbo et al. 1998). Another important aspect of the system is that the allergenicity potential for PMI was evaluated and found to lack many attributes associated with known oral allergens (Privalle 2002).

Recently, transformation efficiency via *Agrobacterium* was improved in several plant species by the use of heat and centrifugation treatments of the explants used for transformation. Khanna et al. (2004) reported that viability of banana suspension cells increased after cells were heat-shocked before *A. tumefaciens* infection, doubling post-infection viability of the suspension cells and increasing transformation efficiency. A fourfold increase in transformation frequency was also observed when suspension cells were centrifuged together with *Agrobacterium.* More recently, Hiei et al. (2006) reported a several-fold enhancement of transformation frequency of rice and maize by treating immature embryos (IEs) with both heat and centrifugation before infecting with *A. tumefaciens*.

In the present study, we analyzed, for the first time in sorghum, effects of heat and cold pretreatment and centrifugation of IEs prior to *Agrobacterium* inoculation. We perform quantitative evaluation of the effects of these treatments on transient GFP expression by determining the frequency of IEs that contain GFP-expressing cells and of stable transformation by determining the number of calli from independent events that survived mannose selection and were regenerable.

Materials and methods

Plant materials and embryo isolation

Four sorghum lines were screened initially for transient expression of GFP: P898012, an inbred variety developed at Purdue University (Jenks et al. 1994), Tx430, an inbred line developed at Texas A&M (Miller 1984), 296B, a publicly held Indian seed parent line from All-India Coordinated Sorghum Improvement Program (Seetharama et al. 2000) and C401, an inbred line from China, obtained from G. Liang, Kansas State University (Gao et al. 2005b). P898012 and Tx430 were subsequently chosen for cold pretreatment experiments and the remaining experimentation on effects of centrifugation and heat treatment was carried out on P898012. Sorghum plants were grown in a walk-in growth chamber (Kolpak Walk-In Chamber, River Falls, WI, USA) under a day/night temperature of 28/18°C, using a combination of sodium vapor and metal halide lighting at $\sim$700 μmol s^{-1} m^{-2} and fertilized twice weekly with Hoagland's solution. During winter months, water used for donor plants was pre-warmed in the growth chamber before use. Spikes were collected from plants $\sim$14 days post-pollination when embryos were $\sim$1.5 mm in size, and appeared not transparent and just turning cloudy. Isolated seeds were surface-sterilized with 70% ethanol for 5 min, then with 20% bleach (containing 5.25% NaOCl) for 20 min, and finally rinsed several times with sterile distilled water. Immature

embryos (IEs) were aseptically isolated from either freshly harvested spikes or, for cold pretreatment experiments, from spikes which were kept at 4°C for 1–5 days before embryo isolation. During isolation, IEs were stored before *Agrobacterium* inoculation in sterile 1.5 mL Eppendorf tubes containing liquid inoculation medium. All culture media used for inoculation, co-cultivation, callus induction, selection, regeneration and rooting were as described (Gao et al. 2005b), with the following exceptions. (1) In all media 3.0 gm L^{-1} Phytagel was used. (2) In the regeneration medium, mannose alone was used. (3) 1% (w/v) polyvinylpyrrolidone (PVP) was added to the medium throughout all stages to reduce tissue blackening,

Pretreatment with heat and/or centrifugation

Medium covering the IEs was removed before heating, leaving just enough to cover the isolated embryos, and then incubated at 37, 40, 43 and 46°C in a carefully controlled water bath; either before inoculation with *A. tumefaciens* carrying the pGFP-PMI plasmid (see Sect. "Plasmids, *Agrobacterium* strains and sorghum varieties") or before centrifugation that preceded *Agrobacterium* inoculation. Duration of the heat treatment was either (1) 3 min for all temperatures or (2) 5, 10 and 30 min for 43°C with and without centrifugation. After heat treatment, tubes were cooled for 2 min either on ice (0°C) or in a water bath at 25°C. Eppendorf tubes containing 30–55 isolated IEs were centrifuged in a fixed angle rotor at 16,000 × *g* at 4°C or room temperature (RT) for 10 min before inoculation. In another experiment, different centrifugation speeds (1,000, 8,000, 16,000 and 20,000 × *g*) for 10 min at RT were used following heat pretreatment at 43°C for 3 min, followed by cooling at 25°C for 2 min.

Plasmids, *Agrobacterium* strains and sorghum varieties

A binary vector, pPZP201-GFP-PMI (pGFP-PMI) (Gao et al. 2005b) which contains *sgfp* encoding GFP (Chiu et al. 1996) and *pmi* encoding PMI, was kindly provided by G. Liang, Kansas State University. Both genes were driven by the maize ubiquitin1, *ubi*1, promoter (Christensen and Quail 1996) and terminated with *A. tumefaciens* nopaline synthase 3′ termination signal, *nos*. The pGFP-PMI plasmid was mobilized into two *A. tumefaciens* strains EHA 101 (Hood et al. 1986) and LBA4404 (Hoekema et al. 1983) via the freeze-thaw transformation method (An et al. 1988).

Bacterial growth, inoculation and co-cultivation

Methods used were modified from Zhao et al. (2000) and Gao et al. (2005a). *Agrobacterium* cells were inoculated into Luria-Bertani (LB) medium supplemented with 100 and 150 mg L^{-1} spectinomycin (Sigma-Aldrich, St. Louis, MO, USA) for EHA101 and LBA4404, respectively, and incubated ON at 28°C, shaking at 250 rpm. Bacteria were centrifuged at 5,000 rpm at 4°C for 5 min and diluted to $OD_{550} = 0.4$ with liquid co-culture medium. Inoculation medium was removed from previously isolated IEs, subsequently covered completely with the *Agrobacterium* suspension for 15 min and then cultured scutellum side up on co-cultivation medium in the dark for 2 days at 24°C. IEs were transferred to callus-induction medium (CIM) with 100 mg L^{-1} carbenicillin (Agri-Bio, Bay Harbor, FL, USA) to inhibit growth of *Agrobacterium.* IEs remained on CIM for 4 weeks and explants were subcultured once every other week or sooner (≤1 week), if heavy phenolic production occurred. During that time IEs that produced no callus or died were discarded; the remaining calli were transferred to selection medium, as described below. Despite the use of PVP to mitigate effects of phenolic production, culture material was frequently and closely monitored and transferred, sometimes daily, to fresh medium when any sign of blackening occurred, removing blackened or dead cells or tissues. Callus from individual IEs was tracked to aid in establishing independence of transgenic events.

Mannose selection and regeneration

For the first 2 weeks, culturing was on selection medium with 3.0% sucrose plus 2.0% mannose; for the following 2 weeks it was on 2.0% sucrose plus 1.5% mannose and for the remainder of the selection period on medium with 1.5% sucrose plus 1.0% mannose. Cultures remained in the dark during selection. Thus, calli were kept on selection medium for a total of 6 weeks or, for slower growing calli, they were kept on selection medium with 1% mannose for an additional 2 weeks, taking care to transfer tissues at the first sign of blackening to keep the tissues healthy. Tissues were transferred to regeneration medium with 0.5 mg L^{-1} kinetin (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mg L^{-1} indole-acetic acid (IAA, Sigma-Aldrich) and kept at low light intensity (85 µmol s^{-1} m^{-2}) with a photoperiod of 16/8 h light/dark for 4 weeks. Regenerated shoots were transferred to rooting medium with 0.2 mg L^{-1}α-naphthaleneacetic acid (NAA, Sigma-Aldrich), 0.2 mg L^{-1} IAA and 0.2 mg L^{-1} indole-3-butyric acid (IBA, Sigma-Aldrich) and kept at a light intensity of 100 µmol s^{-1} m^{-2} with a photoperiod of 16/8 h light/dark. All regeneration and rooting media contained 1% mannose and 100 mg L^{-1} carbenicillin to eliminate *Agrobacterium*. After 4–6 weeks on rooting medium, well-rooted shoots were transferred to pots containing Supersoil (Rod McClellan Co., South San Francisco, CA, USA), grown initially in a Conviron E-15 growth chamber

(Pembina, ND, USA) with a photoperiod of 16/8 h light/dark period under fluorescent and incandescent lighting (250 μmol s^{-1} m^{-2}) and day/night temperatures of 25/20°C and then transferred to the Kolpak walk-in growth chamber (see Sect. "Plant material and embryo isolation") or to the greenhouse.

Visual screening for GFP expression

Immature embryos were observed using a Zeiss Lumar.v12 epifluorescence stereoscope attached to a QImaging color camera to detect GFP fluorescence. Transient expression was scored at 5 and 10 days post-infection with *A. tumefaciens*; however, visual monitoring of fluorescence was regularly performed up to 75 days and during selection and regeneration stages.

PCR analysis

Genomic DNA was isolated from shoots of putative transgenic and non-transgenic control sorghum plants. To confirm presence of *pmi*, fragments were amplified from genomic DNA using pmi-specific primers: PMI-1: 5′-ACA GCCACTCTCCATTCA-3′ and PMI-2: 5′GTTTGCCAT CACTTCCAG-3′ (Syngenta Biotechnology Inc., Research Triangle Park, NC, USA). PCR analyses were performed in 25 μl reaction mixtures, each containing 100 ng template DNA, 1× PCR buffer (Promega, Madison, WI, USA), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1 μM primer, 1% DMSO and 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA). PCR reactions were performed with an initial denaturation at 94°C for 2 min, followed by 35 cycles, with each cycle consisting of 94°C for 30 s, 60°C for 60 s, 72°C for 60 s and a final 5-min elongation step at 72°C. PCR products were analyzed by gel electrophoresis in 0.8% agarose gels.

DNA hybridization analysis

Aliquots of ~10 μg of total genomic DNA were digested with the restriction enzyme, *Hin*dIII (New England Biolabs, Ipswich, MA, USA), electrophoretically separated on 0.8% agarose gels and transferred to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were hybridized with ^{32}P-labeled probes. The 694 bp probe, made by digesting *pSP-Ds-Ubi-bar-nos* (McElroy et al. 1997) with *Kpn*I and *Hpa*I, contained 300 bp of the *nos* 3′ terminator sequence, 50 bp of the 3′ end of *bar*, 254 bp of maize *Ds* sequence and 90 bp of pSP vector backbone (Singh et al. 2006), and was labeled according to manufacturer's instructions using Ready-To-Go™ DNA Labeling Beads (Amersham Bioscience, Buckinghamshire, England).

Protein extraction

Leaf tissue was frozen in liquid N_2 and ground to powder with a Geno/grinder (model MM300, SPEX CertiPrep, Metuchen, NJ; Mixer Mill, Qiagen, Valencia, CA). A 0.5 mL aliquot of 10% (w/v) trichloroacetic acid (TCA) was added and the mixture ground thoroughly with a plastic pestle. After adding an equal volume of acetone, the suspension was mixed, incubated for 20 min at −20°C and centrifuged at RT at 20,000 × *g* for 10 min. Supernatant was discarded and pellet was washed three times with 1 mL acetone and dried briefly. Forty microliters extraction buffer [50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 50 mM DTT] was added per mg of dried powder and vortexed briefly. To extract protein, samples were immediately heated at 100°C for 4 min, vortexed vigorously for 10 s, heated for 4 min and vortexed for 15 s. Suspension was centrifuged at 20,000 × *g* for 10 min. Protein concentration of supernatant was determined by a dye binding method using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Equal amounts of protein (20 μg) were solubilized in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol and 0.01% bromophenol blue) plus 2.5% 2-mercaptoethanol, boiled for 5 min, and then run on 10–20% linear gradient Criterion gels (Bio-Rad, Hercules, CA) for 1 h 20 min at 150 V constant. Protein was then transferred to nitrocellulose membrane (Bio-Rad) at 4°C for 90 min at 60 V. The membrane was processed for blotting with rabbit polyclonal anti-GFP antibody (Immunology Consultants Laboratory, Newberg, OR) at 1:5,000 dilution in 5% powdered milk in Tris-buffered saline (TBS) at 4°C overnight. Secondary antibody, goat anti-rabbit IgG (H + L)-HRP Conjugate (Bio-Rad, Hercules, CA, USA), was used at a 1:2,500 dilution in 5% powdered milk and incubated for 1 h at 25°C. Blot was washed between antibody treatments 2× with Tris-buffered saline (TBS) containing 0.05% Nonidet P40. Protein complexing with antibody was visualized by chemiluminescence with a Renaissance kit (Perkin-Elmer Life Sciences, Waltham, MA, USA).

Data analysis

Each experiment was repeated three times and several parameters were analyzed including % of IEs expressing GFP at 10 days post-inoculation, % of IEs surviving, producing callus and blackening at 22 or 25 days post-inoculation, % survival of callus following mannose selection (i.e., % selection) and % regenerable calli (i.e., %

stable transformation). For the data presented in Figs. 2a through 5, the mean and standard deviation (SD) values were calculated for each treatment. For the data presented in Table 1, analysis of variance was carried out using MSTATC program and the differences between the treatment means were shown by using Duncan's multiple range test (MSTATC 2004).

Results

Visual screening for GFP expression

Immature embryos from four different sorghum genotypes, P898012, Tx430, 296B and C401, were used in transient expression assays using *Agrobacterium*-mediated transformation with a dual-marker plasmid containing *ubiquitin*-driven GFP and PMI. GFP expression was monitored with an epifluorescence stereomicroscope. Transient assays produced similar results in all four genotypes in terms of transient GFP expression. Therefore, two genotypes, Tx430 and P898012, were chosen for the cold pretreatment experiments. After observing similar trends for both genotypes following cold pretreatment, the P898012 genotype was selected for the centrifugation and heat treatment optimization.

Low-level GFP expression was observed 2–3 days after inoculation with *Agrobacterium*; normally higher levels of expression were observed after 5–7 days. Scoring for calli expressing GFP was, therefore, done at 5 and 10 days post-inoculation; however, data collected at 10 days was used for comparison of effects of different treatments. GFP expression was detected at 5 days (Fig. 1a) and, by 10 days, only very slight increases (i.e., IEs not showing GFP at 5 days showed expression at 10 days) or decreases (i.e., IEs showing usually weak GFP expression at 5 days, showed none at 10 days) in the frequency of IEs showing GFP expression were observed. GFP expression levels rapidly increased at 10 days (Fig. 1b) and increased expression was stable over several weeks (Fig. 1c) or even months (Fig. 1d). Nearly all GFP-expressing cells developed from the embryo's scutellum, the side away from the agar during culturing; this side grew rapidly, forming large masses of callus. During selection, fluorescence microscopy was used to monitor putative transgenic and non-transgenic cells. Non-transgenic cells first turned dark brown then black and failed to survive on mannose selection medium, whereas putative transgenic cells did not discolor, maintained an opaque, white coloration and continued to express GFP strongly. On regeneration medium, most putative transgenic shoots, developing from callus that survived selection, expressed GFP strongly (Fig. 1e, f).

Cold pretreatment

Four to five sorghum heads from different plants of P898012 and Tx430 were divided longitudinally into four sectors. To prevent possible plant-to-plant variation and differences in the physiological ages of seeds from the top, middle and bottom parts of the heads, immature seeds from the entire sector of each head were removed and sterilized. Embryos of $\sim$1.5 mm were isolated immediately for the 0-day cold pretreatment and heads to be used for the remaining sectors were kept at 4°C for the 1, 3 and 5 days cold-pretreatment time points. Isolated IEs were infected with *Agrobacterium* strains, EHA 101 and LBA 4404 with pGFP-PMI, and the numbers of GFP-expressing IEs were determined at 10-day post-inoculation for both sorghum genotypes (Table 1; Fig. 2a). Also, numbers of IEs surviving *Agrobacterium* infection, those giving rise to callus and those experiencing blackening were determined at 25 days post-inoculation (Fig. 2b).

Although during the course of the cold pretreatment regimen, variations in frequency of IEs expressing GFP were observed between sorghum genotypes, P898012 and Tx430 (most not significant), and bacterial strains, LBA4404 and EHA101 (none significant), a clear trend was observed. In all treatments, prolonged cold storage significantly decreased the percentage of IEs showing GFP expression (Table 1; Fig. 2a). While 22.5 and 21.2% of freshly isolated IEs of P898012 inoculated with LBA4404 and EHA101, respectively, showed GFP expression, the percentage was reduced to 6.4 and 6.3% when the heads were kept at 4°C for 5 days. A similar response was evident for IEs from Tx430. Mean % of GFP-expressing Tx430 IEs was 19.5 and 20.4% at day 0 versus 4.2 and 5.8% at day 5, for LBA4404 and EHA101 inoculations, respectively. Means of all four combinations (both genotypes and both *Agrobacterium* strains) for 0 day was 20.9%, while 1, 3 and 5 days cold pretreatments produced 17.3, 11.3 and 5.7%, respectively. Decrease in expression frequencies after 1-day pretreatment was not significant for P898012 compared to 0 day; however, for Tx430 there was a significant decrease after 1 day of cold storage. Frequencies at 3 and 5 days were also significantly reduced compared to 0 day for both genotypes and both bacterial strains.

When mean values of all four treatments, including both genotypes, P898012 or Tx430 and both bacterial strain, LBA 4404 or EHA 101, were compared with regard to IE survival, callus formation capacity and presence of blackening (Fig. 2b), the pattern was very similar to the frequencies of IEs expressing GFP (Fig. 2a). Frequencies of IEs surviving, producing callus and blackening steadily decreased from 0 to 5 days (Fig. 2b). Blackening rates were nearly fourfold lower at 5 days compared to 0 day,

Table 1 Effect of cold and centrifugation treatments on transient and stable transformation frequency of sorghum

Treatment number	Treatment type	Total # IEs used	# calli GFP^+	% GFP^{+} [A,B]	# calli surviving selection	% surviving selection[A,B]	# selected IEs with shoots	Transformation frequency[A,B,C]
1	0-day cold	120	27	22.5[a]	14	11.7[a]	4	3.3[a]
2	1-day cold	111	22	19.8[a]	15	13.5[a]	2	1.8[ab]
3	3-day cold	116	16	13.8[b]	7	6.0[b]	1	0.9[b]
4	5-day cold	94	6	6.4[c]	1	1.1[c]	0	0.0[c]
5	NH/NC	116	24	20.7[cde]	16	13.8[cd]	3	2.6[bcd]
6	C only	105	16	15.2[def]	4	3.8[f]	0	0.0[d]
7	37-3 min/ICE/NC	117	34	29.1[bc]	21	17.9[bc]	5	4.3[b]
8	37-3 min/ICE/C4C	107	13	12.1[efg]	5	4.7[f]	2	1.9[bcd]
9	40-3 min/ICE/NC	112	42	37.5[a]	26	23.2[a]	8	7.1[a]
10	40-3 min/ICE/C4C	108	26	24.1[bcd]	4	3.7[f]	2	1.9[bcd]
11	43-3 min/ICE/NC	105	41	39.0[a]	21	20.0[ab]	8	7.6[a]
12	43-3 min/ICE/C4C	99	16	16.2[def]	10	10.1[de]	3	3.0[bcd]
13	46-3 min/ICE/NC	103	33	32.0[ab]	16	15.5[c]	4	3.9[bc]
14	46-3 min/ICE/C4C	97	15	15.5[def]	9	9.3[e]	1	1.0[bcd]
15	43-5 min/ICE/NC	102	24	23.5[bcd]	4	3.9[f]	1	1.0 [cd]
16	43-5 min/ICE/C4C	112	10	8.9[fg]	2	1.8[f]	0	0.0[d]
17	43-10 min/ICE/NC	96	16	16.7[def]	2	2.1[f]	0	0.0[d]
18	43-10 min/ICE/C4C	108	12	11.1[fg]	1	0.9[f]	0	0.0[d]
19	43-30 min/ICE/NC	105	11	10.5[fg]	2	1.9[f]	0	0.0[d]
20	43-30 min/ICE/C4C	114	4	3.5[h]	0	0[f]	0	0.0[d]
21	NH/NC in water	113	10	8.8[e]	4	3.5[f]	0	0.0[d]
22	NH/NC medium	115	27	23.5[cd]	18	15.7[de]	3	2.6[c]
23	43-3 min/ICE/NC	117	40	34.2[bc]	22	18.8[cd]	9	7.7[a]
24	43-3 min/ICE/C4C	107	20	18.7[de]	15	14.0[de]	3	2.8[bc]
25	43-3 min/ICE/CRT	117	32	27.4[cd]	11	9.4[ef]	3	2.6[c]
26	43-3 min/25C/NC	108	53	49.1[a]	34	31.5[a]	9	8.3[a]
27	43-3 min/25C/C4C	109	38	34.9[bc]	26	23.9[bc]	4	3.7[b]
28	43-3 min/25C/CRT	112	44	39.3[b]	30	26.8[ab]	5	4.5[ab]
	Total	3,045	672		340		80	

NH no heat, *NC* no centrifugation, *ICE* IEs cooled on ice, *25C* IEs cooled at 25°C, *C4C* centrifuged at 4°C for 10 min at 16,000 × *g*, *CRT* centrifuged at room temperature for 10 min at 16,000 × *g*

[A] Three replicates of each experiment were averaged and used to determine frequency (%) of independent calli showing transient GFP expression, survival following mannose selection and the percent regenerable, which was used to calculate stable transformation frequency. P898012 immature embryos were inoculated with *A. tumefaciens* strain LBA4404 carrying pPZP201-PMI-GFP

[B] Means with the same letter within columns of each set of experiments (i.e., treatments 1-4, 5–20 and 21–28) are not significantly different at $P = 0.05$

[C] Transformation frequency = number of independent, regenerable, mannose-selected calli divided by total number of IEs treated × 100

8.2 versus 30.6%, respectively, similar to the fourfold loss in GFP expression in the same timeframe (20.9% at 0 day to 5.7% at 5 days; Fig. 2b). Rate of decrease in survival and callus production was not as sharp as the loss in GFP expression. Survival and callus formation decreased only ~25%, i.e., mean rates for the four treatments were 85.7 and 79.6 at 0 day versus 59.2 and 55.0 at 5 days. Regardless of sorghum genotype or *Agrobacterium* strain similar frequencies of survival, callusing and blackening were obtained (data not shown).

Pretreatment with heat and/or centrifugation

Given similarities in results of preliminary treatment experiments on frequencies of GFP-expressing calli for both sorghum genotypes (data not shown), we chose to focus on P898012 to perform in-depth examinations of the effects of heat and centrifugation treatments on frequencies of IEs expressing GFP. IEs of P898012 were heated at 37, 40, 43 and 46°C for 3 min, which was followed by no centrifugation or centrifugation at 4°C for 10 min at

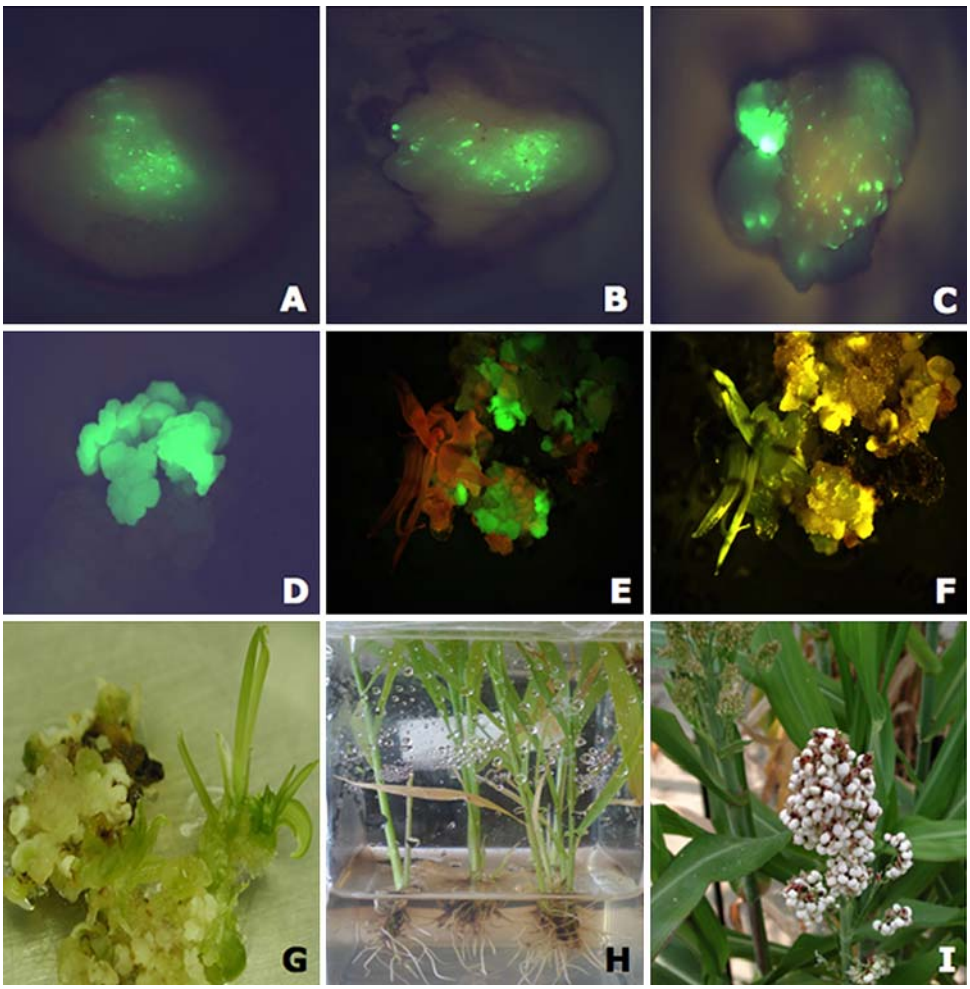

Fig. 1 GFP expression of P898012. IEs of P898012 at **a** 5 days **b** 10 days **c** 25 days and **d** 72 days following *Agrobacterium* infection with pGFP-PMI. Following mannose selection, early-stage developing shoots from putatively transformed callus were cultured on regeneration medium and photographed under **e** blue excitation and **f** white light. **g** Well-developed shoots after 3–4 weeks on regeneration medium. **h** Well-rooted regenerants after 4 weeks on rooting medium. **i** Transgenic plants from event 11E (Table 1) transferred to soil and setting seed in the greenhouse

16,000 × *g*. Preliminary results showed that 43°C heat treatment was the most effective at increasing GFP expression frequencies. Therefore, using a 43°C treatment, heating times were varied (3, 5, 10, 30 min) with or without centrifugation (Fig. 3a). Effects of these different treatments on the frequencies of IEs that survived, produced callus and experienced blackening were determined at 22-day post-inoculation (Fig. 3b).

Heat pretreatment of IEs alone at all temperatures for 3 min prior to *Agrobacterium* infection enhanced significantly the numbers of IEs showing GFP expression (Fig. 3a). There was a steady increase in frequency from 37 to 43°C with no centrifugation; the 43°C treatment yielded nearly twice as many GFP-expressing embryos compared to the no-heat/no-centrifugation control, 39.0 versus 20.7%, respectively. When heat treatment at 43°C was extended from 3 min to 5, 10 or 30 min with no centrifugation, there was a dramatic and steady reduction in the frequency of IEs expressing GFP. For example, treating for 5 min produced a mean of 23.5% GFP-expressing IEs, while treating for 30 min led to less than half that number (10.5%).

When heat and centrifugation treatments were combined, it was clear that centrifugation had a severe negative effect (Fig. 3a). The numbers of IEs expressing GFP at 10-day post-inoculation were significantly lower at all tested temperatures, e.g., at 43°C; 39.0% of IEs expressed GFP while only 16.2% expressed when IEs were centrifuged prior to inoculation. This trend was also observed when longer duration heating times at 43°C were used. Even in the absence of heating, centrifugation had a negative effect on the numbers of IEs expressing GFP. A mean GFP expression frequency of 15.2% was obtained versus 20.7% in the no-heat/no-centrifugation control (Fig. 3a). On average, centrifugation reduced GFP expression frequencies by 50%, i.e., 26.1 versus 13.3% (data not shown).

Centrifugation at 16,000 × *g* at 4°C also had a negative effect on IE survival and callus production (Fig. 3b). There was a significant decrease in the frequency of IEs surviving and producing callus when embryos were centrifuged, compared to no-heat/no-centrifugation control embryos: 72.2 versus 88.9% for survival rate and 61.0 versus 88.9% for callus production rate, respectively. However, when IEs were heat-treated at 37°C, and to a lesser extent at 40°C,

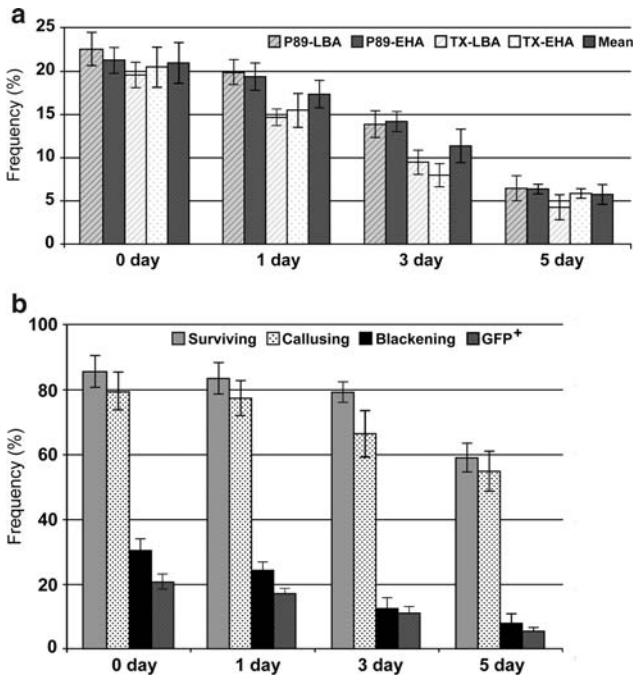

Fig. 2 **a** Effect of cold pretreatment on frequency of IEs expressing GFP. Immature embryos of P898012 and Tx430 were pretreated at 4°C for 0 day, 1 day, 3 days and 5 days and inoculated with *Agrobacterium* strain LBA4404 or EHA101, both with pPZP201-PMI-GFP. The frequency (%) of IEs expressing GFP at 10-day post-inoculation was determined. Data represent mean ± SD of three repetitions. Each repetition used 22–41 IEs; total number of IEs used was 1,027. **b** Effect of cold pretreatment on frequency of IEs surviving, producing callus, blackening and expressing GFP. Immature embryos of P898012 and Tx430 were pretreated at 4°C for 0 day, 1 day, 3 days and 5 days and inoculated with *Agrobacterium* strain LBA4404 or EHA101, both with pPZP201-PMI-GFP. Frequency (%) of IEs surviving, producing callus, blackening at 25 day post-inoculation, and transiently expressing GFP at 10 day post-inoculation (Fig. 2a) was determined. Values are means of four treatments (two sorghum varieties and two *Agrobacterium* strains from Fig. 2a). Data represent mean ± SD of three repetitions. Each repetition used 22–41 IEs; total number of IEs used was 1,027

there was little to no significant differences between centrifuged and non-centrifuged IEs. At 43°C, effects of centrifugation were significant; 85.0 versus 68.8% for survival rate and 85 versus 62.5% for callus production rate, non-centrifuged versus centrifuged, respectively. At the highest temperature, 46°C, heating alone was inhibitory to both embryo survival and callus production, irrespective of centrifugation. For longer heat durations at 43°C, effects of 5-min treatment were similar to those of 3 min but at 10 min, and more dramatically at 30 min, heat treatments in the absence of centrifugation reduced both survival and callus frequencies significantly. In all treatments, a clear majority of surviving IEs, 81.8–100%, were able to produce callus (Fig. 3b).

Centrifugation also increased the frequency of IEs that blackened at all temperatures and for all durations, even in the non-heat treated control. Frequencies of blackened embryos when heated at all temperatures and for all durations did not appear to be statistically different, in the absence of centrifugation. In all cases except for the 30-min heat treatment at 43°C, blackening increased significantly at a given temperature and for a given duration relative to the non-centrifuged counterpart. In other words, heat treatment did not have significant effects on the frequency of blackening relative to the no-heat/no-centrifugation control, but centrifugation did. Thus, there was a negative correlation between % of embryos expressing GFP (Fig. 3a) and blackening (Fig. 3b), i.e., the higher the blackening (which increased with centrifugation), the less the GFP expression at all temperatures. Heat and centrifugation increased the degree of de-differentiation (callusing) of the embryo, which was notable in the first 2 weeks of culture following bacterial infection. De-differentiation appeared greater when embryos were both heat-treated and centrifuged (data not shown). Comparisons of the effects of centrifugation at 16,000 × *g* at RT rather than at 4°C, both before and after heat treatment at 43°C and before and after bacterial inoculation, did not reveal significant differences in frequencies of IEs expressing GFP (data not shown).

Treatment of embryos pre- and post-heat treatment and during centrifugation

Effects of storing isolated IEs prior to inoculation in distilled water versus in liquid co-cultivation medium were compared. Storing IEs in water compared to culture medium dramatically decreased the frequency of GFP expression by nearly threefold (8.8 vs. 23.5%, respectively) (Fig. 4). No heat or centrifugation pretreatment was used.

Two additional parameters were tested to determine effects on the frequency of IEs expressing GFP: (1) treatment of IEs following heat treatment (no centrifugation) and (2), if centrifuged, the subsequent temperature at which centrifugation was done. Following heat treatment but before centrifugation, IEs were placed either on ice (0°C) or at 25°C for 2 min; centrifugation was at either 4°C or RT (Fig. 4). Centrifugation at both 4°C and RT decreased frequency of GFP expression. When IEs were cooled on ice following heat treatment and centrifuged at 4°C or RT, frequencies were 18.7 and 27.4%, respectively, versus 34.2% for heat treatment alone. When embryos were placed at 25°C following heat treatment and centrifuged at 4°C or RT, frequencies were 34.9 and 39.3%, respectively, versus 49.1% for heat treatment alone (Fig. 4; Table 1).

With regard to the % embryos expressing GFP, centrifugation of IEs at RT following heat treatment at 43°C and placing them on ice appeared to compensate in part for the negative effects of centrifuging IEs at 4°C, 27.4 versus

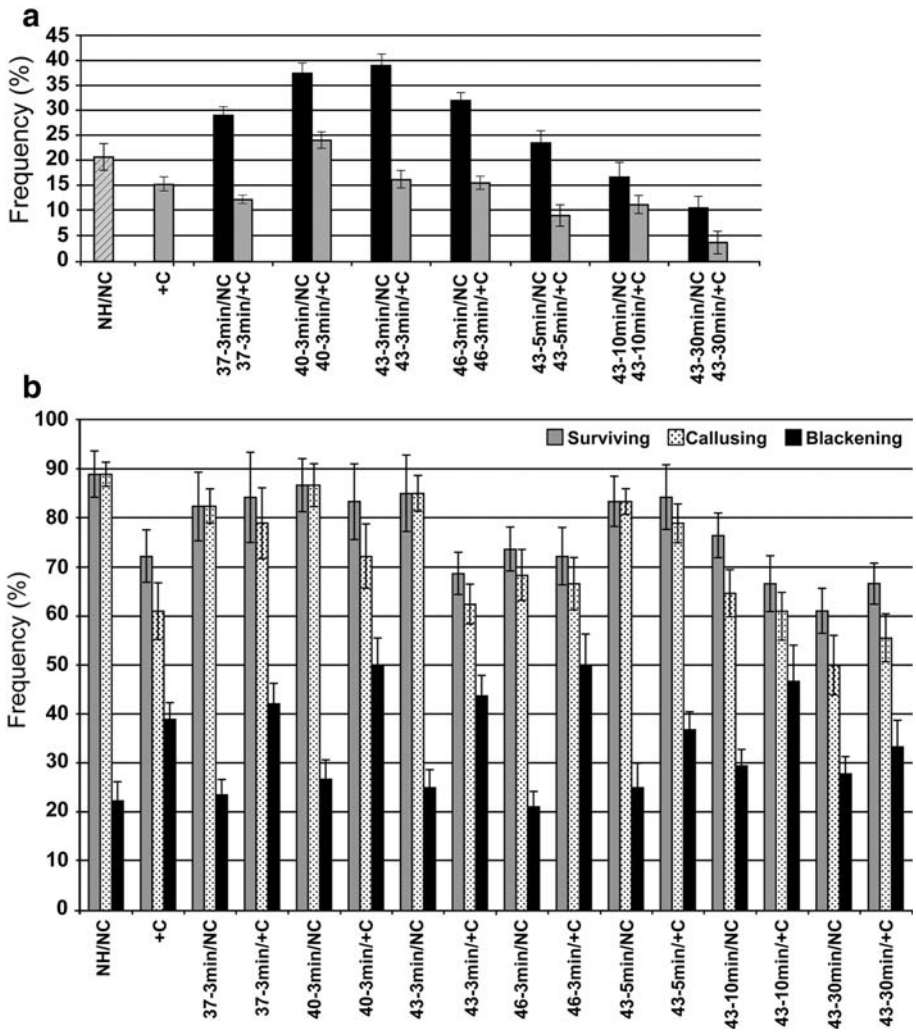

Fig. 3 **a** Effect on GFP expression of IEs following treatment of IEs at varying temperatures and durations of heat treatments with and without centrifugation. Immature embryos were exposed to 3-min heat treatments at varying temperatures and to heat treatment at 43°C for varying times either with (+C) or without (NC) a 10-min centrifugation step at 4°C at 16,000 × *g*. Percentage of P898012 IEs showing transient GFP expression at 10-day post-inoculation with LBA4404 harboring pGFP-PMI was determined. *NH* no heat, *NC* no centrifugation, *+C* centrifugation. Data represent mean ± SD of three repetitions. Each repetition used 29–43 IEs; total number of IEs used was 1,706. **b** Effect on survival, callusing and blackening of IEs following treatment of IEs at varying temperatures and duration of heat treatments with and without centrifugation. Immature embryos were exposed to 3-min heat treatments at varying temperatures or heat treatment at 43°C for varying times either with (+C) or without (NC) a 10-min centrifugation step at 4°C at 16,000 × *g*. Percentage of P898012 IEs surviving, producing callus and blackening at 22 day post-inoculation, which transiently expressed GFP at 10-day post-inoculation with LBA4404 harboring pGFP-PMI (Fig. 3a), was determined. *NH* no heat, *NC* no centrifugation, *+C* centrifugation. Data represent mean ± SD of three repetitions. Each repetition used 29–43 IEs; total number of IEs used was 1,706

18.7%, respectively (Fig. 4; Table 1). Much more striking was the compensation realized by placing IEs at 25°C rather than on ice following heat treatment and before centrifugation at 4°C, 34.9 versus 18.7%, respectively. Similar trends were observed when centrifugation was carried out at 25°C. Notably, if centrifugation was carried out at RT rather than at 4°C, significantly higher frequencies were realized, regardless of incubation conditions for IEs following heat treatment (Table 1; compare treatments 25 and 28 with 24 and 27). Placing IEs at 25°C prior to centrifugation produced significantly higher frequencies than cooling at 0°C regardless of centrifugation condition (Table 1; compare treatments 24 and 25 with 27 and 28). The highest frequency of IEs expressing GFP at 10-day post-inoculation, 49.1%, was achieved when IEs were heat-treated at 43°C for 3 min and placed at 25°C without centrifugation, a value significantly higher than the 34.2% achieved when IEs were cooled at 0°C (Table 1; treatments 23 and 26).

Using IEs heat-treated at 43°C for 3 min, effects of centrifuging IEs at RT at different speeds, 1,000, 8,000, 16,000 and 20,000 × *g* for 10 min, were tested. The %

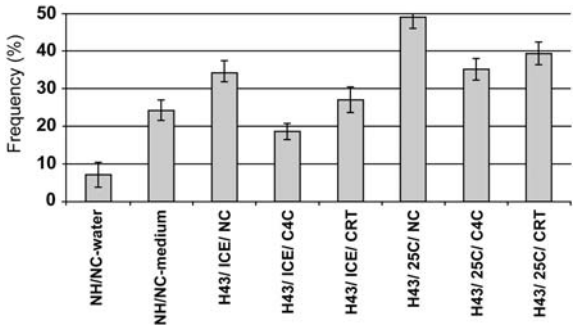

Fig. 4 Effect of two cooling regimes for IEs following heat pretreatment and of different centrifugation temperature. Immature embryos were exposed to heat treatments at 43°C for 3 min either with (+C) or without (NC) a 10-min centrifugation step at 16,000 × *g* at RT or 4°C. Following heat treatment, IEs were cooled either on ice (0°C) or at 25°C. Percentage of IEs showing transient GFP expression at 10-day post-inoculation with LBA4404 harboring pGFP-PMI was determined. NH/NC-water: no-heat/no-centrifugation, IEs stored in water before inoculation; NH/NC-medium: no-heat/no-centrifugation, IEs stored in culture medium before inoculation; H43/ICE/NC: IEs treated at 43°C for 3 min, cooled for 2 min on ice, no centrifugation; H43/ICE/C4C: as previous with centrifugation at 4°C; H43/ICE/CRT: as previous with centrifugation at RT; H43/25C/NC: IEs heated at 43°C for 3 min, cooled for 2 min at 25°C, no centrifugation; H43/25C/C4C: as previous with centrifugation at 4°C: H43/25C/CRT: as previous with centrifugation at RT. All centrifugations were for 10 min at 16,000 × *g*. Data represent mean ± SD of three repetitions. Each repetition used 36 to 40 IEs; total number of IEs used was 898

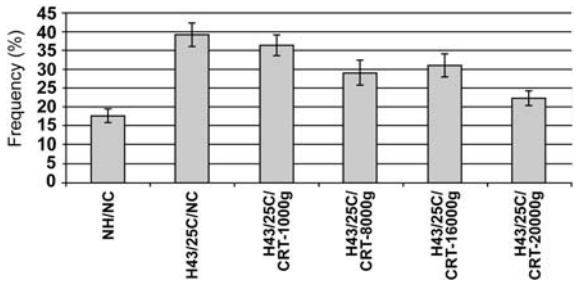

Fig. 5 Effects of centrifugation speed on frequency of IEs transiently expressing GFP. Immature embryos were heated at 43°C for 3 min prior to inoculation with LBA4404 harboring pGFP-PMI and centrifuged at varying speeds and observed for GFP expression 10 day post-inoculation. NH/NC: no heat and no centrifugation; H43/25/NC: IEs heated at 43°C for 3 min, cooled at 25°C for 2 min with no centrifugation: H43/25/CRT-1,000 × *g*: as previous but centrifuged at RT for 10 min at 1,000 × *g*; H43/25/CRT-8,000 × *g*: as previous but centrifuged at 8,000 × *g*; H43/25/CRT-16000: as previous but centrifuged at 16,000 × *g*; H43/25/CRT-20000: as previous but centrifuged at 20,000 × *g*. Data represent mean ± SD of three repetitions. Each repetition used 24–29 IE; total number of IEs used was 485

GFP expression decreased with increasing speeds; the 20,000 × *g* speed reduced GFP expression frequency significantly versus the no-centrifugation control, 22.3 versus 39.2%, respectively (Fig. 5). The difference in frequency between the no-centrifugation control (39.2%) and the lowest speed (1,000 × *g*) (36.4%), however, was not significant.

Mannose selection and plant regeneration

After 6 weeks on mannose selection medium, a varying percentage of calli from the different treatments survived (Table 1), proliferated (Fig. 1e) and produced shoots (Table 1; Fig. 1f, g). Other calli, presumably non-transgenic or expressing *pmi* at low levels, turned black and eventually died (data not shown). Frequency of IEs producing callus that survived mannose selection varied significantly across treatments from 1.1 to 13.5% in the first set of experiments (treatments 1–4), from 0 to 23.2% in the second set of experiments (treatments 5–20) and from 3.5 to 31.5% in the third set of experiments (treatments 21–28) (Table 1). The highest frequency (31.5%) was obtained when IEs were heated at 43°C for 3 min, cooled at 25°C without centrifugation. In general, frequencies of calli surviving selection were lowest when IEs were centrifuged. Following heat treatment at 43°C for 3 min, treating IEs at 25°C produced a significantly higher frequency of surviving calli (31.5%) than cooling IEs on ice (18.8%). Of the four temperatures tested in the second set of experiments (treatments 5–20; Table 1), heat treatment followed by cooling on ice and no centrifugation, heat-treating at 40 and 43°C yielded the highest percentage of tissues surviving selection, 23.2 and 20.0%, respectively.

A final hurdle to transformation success is the ability of callus to regenerate plantlets following selection. Upon transfer to regeneration medium, selected calli produced shoots at varying efficiencies (Table 1). Treatments producing the highest frequencies of IEs with transient GFP expression and calli surviving selection yielded the highest number of independently selected calli that regenerated shoots. For example, IEs heated at 40 and 43°C for 3 min and cooled on ice with no centrifugation yielded a 7.1 and 7.6% transformation frequency, respectively, defined as the number of independent, regenerable, mannose-selected calli divided by the total number of IEs treated (Table 1). The highest frequency, 8.3% was achieved when IEs were heat-treated at 43°C for 3 min and cooled at 25°C without centrifugation prior to *Agrobacterium* infection. Shoots from all tested events readily produced roots on rooting medium (Fig. 1h) and survived transfer to soil (Fig. 1i).

Analysis of putative transgenic (T_0) plants

Presence of the *pmi* transgene in genomic DNA of T_0 sorghum leaf tissue was confirmed by PCR (e.g., Fig. 6a). PCR results confirmed that tissues from all mannose-resistant plants tested contained *pmi* with no escapes. For

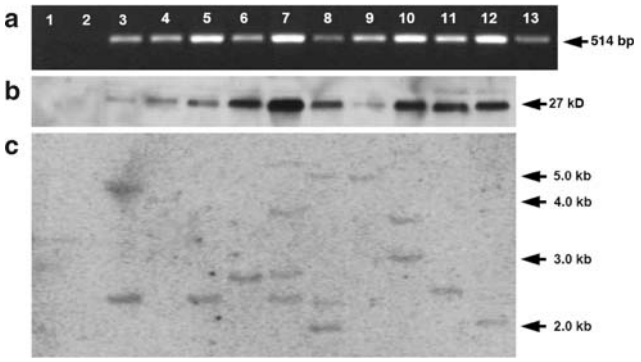

Fig. 6 Molecular analysis of ten putative lines by PCR, western and DNA hybridization blots. Of the 80 IEs that expressed GFP, survived growth on mannose and regenerated shoots, ten were selected at random and analyzed for the presence of **a** *pmi* by PCR amplification, **b** GFP by western blot, and **c** the *nos* terminator present in the pGFP-PMI construct by DNA hybridization blot. *Lane 1* water (negative control), *lane 2* non-transgenic shoot (negative control), *lanes 3–12* shoots from ten individual putative transgenic plants. For panel **a** *lane 13* is a positive control, i.e., LBA4404 harboring pGFP-PMI. Molecular weights in kb or kD are indicated on right

more complete analyses, 10 plants were chosen at random from the 80 independent events generated from the 28 different treatments (Table 1): two putatively independent events from treatment 1, two from treatment 5, one each from treatments 7 and 8, and two each from treatments 9 and 11. Western blot analyses for GFP expression were done on the ten selected plants (Fig. 6b); all ten expressed GFP although at differing levels.

Integration of the introduced genes into the genome and independence of the integration events was confirmed by DNA hybridization blot analysis on genomic DNA samples (Fig. 6c). In the latter case, genomic DNA was restricted with *Hin*dIII, which cuts pGFP-PMI at two locations (Gao et al. 2005b), one just inside the right border and the other between the *pmi* and *sgfp* cassettes. Restricting genomic DNA with this enzyme results in one fragment of 3,423 bp, which contains the *ubiquitin* promoter, *pmi* and the 35S 3′ terminator and this fragment does not hybridize with the *nos*-containing probe. Restricting at the internal *Hin*dIII site releases a fragment that includes the *ubiquitin* promoter, *sgfp* and the *nos* 3′ terminator, the left border and extends into the genome. Since the cut site in the genome varies from insertion site to insertion site, the size of the released fragment that binds to the *nos* probe varies from one independent event to another. Genomic DNA from each of ten putative transgenic lines transformed with pGFP-PMI yielded simple, yet different, hybridization patterns confirming their independence and six of the ten independent lines had single site insertions. PCR analyses of leaf tissues from T_1 plants using the *pmi* primers confirmed stable inheritance of the transgene.

Discussion

Given the pivotal role cereal crops play worldwide, efforts to improve them for human and animal feed and, more recently, for bioenergy production are important. Genetic engineering is one of the tools that can be used. While routine and efficient methods are available for *Agrobacterium*-mediated transformation of rice, maize and to a lesser extent wheat and barley (Shrawat and Lörz 2006), sorghum is the least successful of the major cereals in terms of in vitro culture and *Agrobacterium*-mediated transformation (Shrawat 2007).

In the present study, several treatments were used to improve *Agrobacterium*-mediated transformation efficiency of sorghum. Treatments included (1) using different temperatures and centrifugation conditions to pretreat IEs prior to *Agrobacterium* infection, (2) altering cooling temperatures following heat treatment of IEs, (3) varying temperatures during and after centrifugation, and (4) pretreating spikes in the cold prior to IE isolation. The effects of the different treatments on frequencies of transient and stable transformation were determined by monitoring GFP expression during callus formation and mannose selection and by conducting PCR, DNA hybridization and western analyses of regenerated shoots.

Culture, selection and regeneration

Optimizing the condition of the target tissue is important to transformation success. Nguyen et al. (2007) found that a 1-day, 4°C pretreatment of immature seeds significantly improved callus formation from IEs of an African red sorghum cultivar and reduced the need for frequent subculturing due to reduction of phenolics. In the present study, 1-day pretreatments at 4°C of two US sorghum lines, P898012 (Type II) and Tx430 (Type I), did not significantly increase frequency of IE survival or callus induction; in fact with Tx430 the frequency decreased significantly after 1 day of pretreatment (Fig. 2a). Prolonged (5 days) cold pretreatment of spikes prior to isolation of IEs decreased significantly frequencies of IEs from both genotypes that survived culturing, produced callus, blackened and expressed GFP (Fig. 2b). Cold pretreatment, however, did reduce phenolic production, most likely due to effects of low temperature on reducing key enzyme activities (polyphenol oxidases and peroxidases) that are involved in phenolic compound synthesis (Dicko et al. 2006).

To optimize transformation frequencies, the choice of selectable marker is also critical. Two bacterial genes, the herbicide-resistant gene, *bar* (Thompson et al. 1987), and the antibiotic resistance gene, hygromycin phosphotransferase, *hpt,* have been commonly used for sorghum transformation (Casas et al. 1993, 1997; Zhu et al. 1998; Zhao et al. 2000; Emani et al. 2002). While *bar* has been used to generate transgenic sorghum, its use can result in tissues that, although not transgenic, escape selection (Gao et al. 2005b). In addition, *bar*, which confers resistance to the herbicide, Liberty®, could be problematic if it were transmitted via pollen to wild relatives and/or compatible weedy species of sorghum, like johnsongrass or shattercane (e.g., Arriola and Ellstrand 1997; Ellstrand et al. 1999; Morrell et al. 2005). Resistance to the antibiotic hygromycin, also used for selection, is engineered using *hpt,* the product of which converts the antibiotic to a nontoxic form. Use of antibiotic resistance genes raised concerns that such genes might move into bacteria in the human gut, although there is no evidence this will occur under normal circumstances nor is this antibiotic used in clinical practice today (Lemaux 2008).

Nonetheless, the FDA recommends developers of transgenic crops avoid using antibiotic resistance genes if their presence could compromise clinical use of the antibiotic (Center for Food Safety and Applied Nutrition 1998). Such concerns led to development of a strategy using a gene encoding phosphomannose isomerase (PMI), which converts mannose-6-phosphate to fructose-6-phosphate (Joersbo et al. 1998). This approach is feasible because certain carbohydrate sources, like mannose, are not utilized efficiently by plant cells but can be metabolized if they express, for example, the product of the bacterial *man*A gene (Miles and Guest 1984), PMI. Non-transformed cells either grow slowly relative to transformed cells or fail to grow.

Using such positive selection approaches, non-transformed cells die, but the usual production of phenolic compounds observed with negative selection was not observed when using *pmi* and mannose selection (Wenck and Hansen 2004). Lack of phenolic production can lead to higher transformation efficiencies, since these compounds can negatively influence cell growth. In contrast to the earlier work, in our study with the P898012 variety both transformed and non-transformed cells produced phenolic compounds, due perhaps to the Type II nature of P898012 (Dykes and Rooney 2006). We reduced the negative effects of phenolics in this study by very frequent subculturing and use of a phenolic adsorbant, PVP, in all culture media.

To achieve optimal selection pressure with mannose, use of sucrose in combination with mannose has been suggested because sucrose significantly decreases the phytotoxic effects of mannose (Joersbo et al. 1998). In the present study, both sucrose and mannose concentrations were decreased during selection at the 2-week subculture intervals, starting with 3.0% sucrose/2.0% mannose in the first selection step and ending with 1.5% sucrose/1.0% mannose in the third selection step. Mannose alone was used during regeneration. In other studies, a combination of mannose and sucrose was used and in some reports, depending on the relative concentrations of sucrose used, the frequency of escapes seemed to increase (He et al. 2006; Jain et al. 2007). Therefore, in our study we followed a stringent selection strategy, using only 1% mannose during regeneration. Employing this approach we obtained a stable transformation frequency of up to 8.3% (Table 1), with no evidence of escapes. Consistent with our results, studies in onion reported a 23.4% stable transformation frequency with no escapes when mannose alone was used during regeneration (Aswath et al. 2006). Likewise, during regeneration of wheat, following bombardment with *pmi* and selection with mannose and sucrose, escapes were virtually eliminated when mannose alone was used during rooting (Wright et al. 2001). In the same study, bombarded maize tissues selected on mannose alone and regenerated on mannose plus sucrose led to no escapes. Thus, it is likely that selection and regeneration conditions need to be optimized for a given species. It is also important to consider that, for certain species, use of sucrose with mannose during regeneration increases shoot regeneration frequency (Ramesh et al. 2006; Li et al. 2007; Min et al. 2007); it might also increase escapes.

Heat, centrifugation and subsequent temperature treatments

Based on published results, it is apparent that treatment of monocot transformation target tissues with heat shock and/or centrifugation can increase transformation efficiency. Khanna et al. (2004) showed that heat shock treatment of banana suspension cells increased transformation efficiency. Studies by Hiei et al. (2006) showed that, as a general trend, heat treatment had greater effects than centrifugation on indica rice and maize; however, centrifugation effects were greater than heat for japonica rice. Despite these tendencies, some combination of both treatments was most effective in all varieties of rice and maize tested.

A seminal finding with regard to the effects of heat shock on cultured plant cells came with the observation that co-cultivation of *Agrobacterium* with plant cells often resulted in cell death in a process resembling apoptosis (Hansen 2000). Just as heat shock protects mammalian cells from cell cycle arrest and apoptosis (e.g., Iordanskjy et al. 2004), heat shock of plant cells was shown to

reverse the process of apoptosis, thus allowing survival of greater numbers of transformed cells following *Agrobacterium* infection (Hansen 1998). Effects of heat shock on banana suspension cells were thus speculated to be due to prevention of programmed cell death (Khanna et al. 2004).

While improvements in *Agrobacterium*-mediated transformation frequencies of banana embryogenic cell suspensions were observed when incubated at 45°C for 5 min, it was concluded that heat shock alone did not lead to significant improvements in reporter gene expression, but did double viability of post-infection cell suspensions, thus indirectly improving transformation efficiency. In contrast, Hiei et al. (2006) found that transgene expression did increase significantly in IEs of rice and maize after heat treatment, leading them to speculate that the manner in which heat improves transformation frequency may vary among plant species.

Centrifugation of target tissue of banana suspension cells and IEs of maize and rice were also found to have positive effects on transformation efficiencies; however, effects varied among the plant species. A fourfold increase in frequency was observed when banana suspension cells were centrifuged with *Agrobacterium*, suggested to be the result of increased binding of bacteria to plant cells (Khanna et al. 2004). Hiei et al. (2006) centrifuged target tissue before *Agrobacterium* infection, not during infection as with banana, and an increase in bacterial binding to plant cells was not observed. Callus growth promotion and decreased shoot elongation, however, were seen following centrifugation of rice IEs, suggesting that centrifugation slows normal differentiation and promotes de-differentiation, perhaps rendering them more amenable to transformation.

No positive effect on GUS expression frequency was observed in rice following centrifugation of plant cells before *Agrobacterium* infection; however, centrifugation of indica rice was found to have positive effects on transformation that were greater than heat treatment alone (Hiei et al. 2006). Conversely, heat treatment alone of japonica rice and maize was found to have a greater effect on transformation than centrifugation alone, but in all cases for these three cereal species centrifugation had positive impacts. The studies on banana, rice and maize demonstrated that optimal treatment conditions vary markedly depending on species and genotype.

In the present study in sorghum, a 3-min heat pretreatment alone (at 37, 40, 43 and 46°C) resulted in significant increases in the percent of IEs with GFP-expressing cells compared to untreated IEs, consistent with the findings for rice and maize (Hiei et al. 2006). Heat treatments of sorghum also translated into increased numbers of calli surviving selection and regenerating plantlets. Longer heat treatments (5, 10 and 30 min at 43°C) resulted in decreased numbers of GFP-expressing IEs and tissues surviving selection. That heat treatment in sorghum resulted in higher numbers of putative transgenics likely reflects the ability of short periods of heat shock to reverse cell death.

Negative effects of centrifugation on transformation frequencies of sorghum were observed in all treatments tested in this study. When centrifugation was used in the absence of heat treatment, 3.8% of GFP-expressing calli survived selection versus 13.8% with no centrifugation (Table 1, treatments 5 and 6). When centrifugation was combined with heat treatment, in all cases the numbers of IEs with cells expressing GFP and the numbers of calli surviving selection decreased. For example, with the most optimal heat treatment at 43°C (Table 1, treatment 26) 8.3% of independent tissues were found to give rise to regenerable tissues, while the addition of centrifugation either at 4°C or RT (Table 1, treatments 27 and 28) reduced frequencies to 3.7 and 4.5%, respectively. Consistent with data of Hiei et al. (2006), both heat and centrifugation led to an increase in de-differentiation of sorghum tissues from IEs during the first weeks of culture (data not shown). This suggests that effects of centrifugation are complex and their impact on transformation efficiency should be tested empirically on different plant species.

Hiei et al. (2006) observed that heat and centrifugation together enhanced both transient and stable transformation of rice and maize. During preliminary experimentation, they found that the order of heat and centrifugation did not affect transient frequency of GUS expression or frequencies of stable transformation. Ultimately they performed heat treatment first, followed by centrifugation. In our experiments we compared effects of centrifugation at 16,000 × *g* at RT both before and after heat treatment of IEs and of centrifugation with and without *Agrobacterium* infection. There was no clear pattern indicating that any order of treatment resulted in reproducible improvements in transformation efficiencies.

For japonica rice, effects of centrifugation were greater than that of heat, while for indica rice and the A188 maize variety heat had greater positive effects than centrifugation (Hiei et al. 2006), but a combination of treatments was most effective for all varieties tested. In contrast, for sorghum, heat treatment of IEs before infection with *A. tumefaciens* enhanced transformation efficiency significantly, but centrifugation clearly had negative effects when used alone or in combination with heat treatments (Table 1). Centrifugation also increased blackening and decreased the percent of IEs that survived and produced callus.

Temperatures at which IEs were cooled after heat shock and variations in temperatures at which IEs were

centrifuged were compared in sorghum; certain modifications had significant effects on both the number of IEs with GFP expression and the number of calli surviving selection. For example, when no centrifugation was used, 31.5% of IEs survived selection when IEs were cooled at 25°C following heat treatment at 43°C versus 18.8% when IEs were cooled on ice (Table 1, treatments 26 and 23, respectively). Temperatures at which centrifugation was performed had no significant effect on transformation frequency. When IEs were cooled at 25°C post-infection, centrifugation at RT versus at 4°C resulted in slightly higher frequencies of calli surviving selection, 26.8 versus 23.9%, respectively. Conversely, when IEs were cooled on ice, centrifugation at 4°C relative to RT resulted in higher numbers of calli surviving selection, 14.0 versus 9.4%, respectively. The difference perhaps reflects the effects of temperature on the changing fluidity of the membrane, which impacts susceptibility to infection.

Conclusions

In summary, several treatments to increase transformation frequency were investigated in this study. These included (1) pretreatment of IEs at 4°C, (2) heat and centrifugation treatment of IEs pre-inoculation, (3) temperature treatment following heat shock and centrifugation and (4) temperature during centrifugation. The optimal treatment, using freshly isolated IEs, was to heat shock IEs for 3 min at 43°C, followed by cooling at 25°C and no centrifugation (Table 1, treatment 26), which resulted in 49.1% of IEs having cells that expressed GFP, 31.5% of GFP-expressing calli surviving selection and 8.3% of selected calli being regenerable. Reproducibility of transformation procedures is important to its utility for future users. In this study, reproducibility was demonstrated by the fact that statistically significant differences in transformation frequencies compared to a control treatment were obtained over 5 months of testing using multiple related treatments: 7.1% (Table 1; treatment 9), 7.6% (Table 1; treatment 11) and 7.7% (Table 1; treatment 23). All tested calli surviving selection were shown to be PCR-positive for *pmi* and the ten lines chosen at random from the treatments were shown to contain independent insertions of 1–3 copies of the transgene, to express GFP and to set seed.

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