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Highly efficient sorghum transformation

Guoquan Liu · Ian D. Godwin

Abstract A highly efficient microprojectile transformation system for sorghum (*Sorghum bicolor* L.) has been developed by using immature embryos (IEs) of inbred line Tx430. Co-bombardment was performed with the neomycin phosphotransferase *II* (*nptII*) gene and the green fluorescent protein (*gfp*) gene, both under the control of the maize ubiquitin1 (*ubi*1) promoter. After optimization of both tissue culture media and parameters of microprojectile transformation, 25 independent transgenic events were obtained from 121 bombarded IEs. The average transformation frequency (the total number of independent transgenic events divided by the total number of bombarded IEs) was 20.7% in three independent experiments. Transgenic events were confirmed by both PCR screening and Southern hybridization of genomic DNA from primary transgenics (T_0). More than 90% of transformants were fertile and displayed normal morphology in a containment glasshouse. Co-transformation rate of the *nptII* and *gfp* genes was 72% in these experiments. The segregation of *nptII* and *gfp* in T_1 progenies was observed utilizing fluorescence microscopy and geneticin selection of seedlings indicating both were inherited in the T_1 generation. The transformation procedure, from initiating IEs to planting putative transgenic plantlets in the glasshouse, was completed within 11–16 weeks, and was approximately three-fold more efficient than the previously reported best sorghum transformation system.

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G. Liu · I. D. Godwin (✉)
School of Agriculture and Food Sciences,
The University of Queensland,
Brisbane, QLD 4072, Australia
e-mail: i.godwin@uq.edu.au

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench], the fifth most important cereal after wheat, rice, maize and barley, plays a unique role in food security and renewable energy (Belton and Taylor 2004). Sorghum is a drought-tolerant crop, which can grow in marginal areas not suitable for the other cereals. It is widely used for food especially in Africa and India, and for animal consumption (grain and stover) and as a biofuel energy source in Americas and Australia. Genetic engineering has the potential to have a great influence on improving sorghum yield and digestibility (Gurel et al. 2009). However, sorghum has been widely considered as a recalcitrant major crop in terms of tissue culture and genetic transformation (Grootboom et al. 2010). In fact, sorghum transformation has lagged far behind published efficiencies of rice (Hiei and Komari 2008), maize (Ishida et al. 2007) and barley (Ibrahim et al. 2010), even though it has been almost two decades since the first study on transgenic sorghum was published (Casas et al. 1993).

Microprojectile transformation and *Agrobacterium*-mediated transformation are two main approaches that have been utilized to obtain transgenic sorghum. The first report of successful transgenic sorghum was published by using particle bombardment in 1993 and a transformation efficiency of 0.286% was achieved (Casas et al. 1993). Seven years later, the first *Agrobacterium*-mediated transgenic sorghum was reported (Zhao et al. 2000) at 2.12% efficiency. Since then, several more reports have been published (Zhao and Tomes 2003; Williams et al. 2004; Gao et al. 2005b; Nguyen et al.

2007; Gurel et al. 2009; Able et al. 2001). Recently, up to 8.3% of stable *Agrobacterium*-mediated transformation efficiency has been reported (Gurel et al. 2009), although this was the best single experiment among a number using *Agrobacterium*-mediated transformation. However, there have been few significant advances in microprojectile transformation efficiency (Grootboom et al. 2010; Raghuwanshi and Birch 2010). Grootboom et al. obtained 27 transgenic plants from 3,499 immature embryos (IEs) of the cultivar P898012 utilizing *manA* gene, and the transformation efficiency at 0.77% was more than twofold higher than the first published attempt (0.286%) (Casas et al. 1993). Raghuwanshi and Birch obtained 16 transgenic plants of sweet sorghum from 17046 IEs bombarded. Even though the tissue culture system was optimized, the transformation efficiency of sweet sorghum (0.09%) was much lower than the first published attempt (0.286%) of grain sorghum (Casas et al. 1993). A highly efficient gene transfer system largely rests on an effective tissue culture system and an optimal DNA delivery system. Important progress has been made in both areas (Kaeppler and Pedersen 1997; Elkonin et al. 1995; Elkonin and Pakhomova 2000; Nirwan and Kothari 2003; Able et al. 1998; Karami et al. 2009; Carvalho et al. 2004; Hill-Ambroz and Weeks 2001; Jeoung et al. 2002; Able et al. 2001), but sorghum transformation efficiency remained significantly below 10% and was far behind the transformation efficiencies of rice 50–90% (Hiei and Komari 2008), maize 50% (Ishida et al. 2007) and barley 14.8% (Ibrahim et al. 2010).

An efficient genetic transformation system plays a key role in molecular breeding and understanding the genetic control of plant physiology and development. Sequencing of the sorghum genome was completed in 2009 (Paterson et al. 2009). Genome sequencing and gene discovery have gained momentum in the last decade. Yet, the absence of an efficient sorghum transformation protocol has hampered progress in sorghum genetic engineering and understanding of sorghum gene function.

The aim of the present study was to develop a highly efficient transformation system for sorghum. Focusing on an extensively used inbred sorghum line Tx430 (Gurel et al. 2009), we optimized callus induction medium (CIM), regeneration medium and rooting medium to reduce the production of phenolics from tissue and increase the callus induction rate and regeneration frequency. Other transformation variables were also optimized.

Materials and methods

Plasmids

Two plasmids, pUKN and pGEM-ubi-gfp (Dugdale et al. 1998), which contain *nptII* encoding NPT*II* and *gfp* encoding GFP, respectively, were kindly provided by Scott Hermann, Bureau of Sugar Experimental Stations (BSES) Limited. Both genes were driven by the maize ubiquitin1 (*ubi*1) promoter and terminated with A. *tumefaciens* nopaline synthase 3′ termination signal (*nos*) (Fig. 1a, b).

Plant materials and embryo isolation

Seeds of the inbred line Tx430 (Miller 1984) were planted in the temperature control glasshouse (18–28°C) and some were planted outside in the field during the summer. Plants were watered daily and fertilized with Osmocote® monthly. Panicles were covered with paper bags before flowering and immature seeds were harvested within 12–15 days after flowering. To sterilize immature seeds, they were soaked in 70% ethanol (v/v) while shaking at 200 rpm for 5 min. Seeds were rinsed once with sterilized water, before being soaked again in the solution containing commercial bleach (White King) 4% (v/v) sodium hypochlorite and 0.2% (v/v) surfactant Tween 20 while shaking at 200 rpm for 10 min. Finally, seeds were rinsed five times with sterilized water. IEs ranging from 1.0 to 2.0 mm in length were isolated onto 90 × 15 mm petri dishes containing CIM with their scutellums facing upward. Cultures were incubated in the dark in a tissue culture room, which was set up at 27 ± 1°C under fluorescent light of approximately 100 μmol s^{-1} m^{-2} (16 h/day).

Media for tissue culture

All media were based on MS medium (Murashige and Skoog 1962) containing 4.44 g/L MS powder with Gamborg vitamins (PhytoTechnology Laboratories), 30 g/L sucrose and 8 g/L agar. The pH of all media was adjusted to 5.7 before being autoclaved at 121°C for 15 min; 2,4-D was added to media before autoclaving. The other plant growth regulators and copper sulfate ($CuSO_4$) were sterilized with a 0.2-μm syringe filter and added after autoclaving.

The following media were used for microprojectile transformation experiments:

Callus induction medium (CIM): MS medium supplemented with 1 g/L L-proline, 1 g/L L-asparagine, 1 g/L potassium dihydrogenphosphate (KH_2PO_4), 0.16 mg/L $CuSO_4$ and 1 mg/L 2,4-D.

Regeneration medium: MS medium supplemented with 1 mg/L BAP, 1 mg/L IAA and 0.16 mg/L $CuSO_4$.

Rooting medium: MS medium supplemented with 1 mg/L NAA, 1 mg/L IAA, 1 mg/L IBA and 0.16 mg/L $CuSO_4$.

Selective regeneration medium: regeneration medium supplemented with 30 mg/L geneticin (G418) (disulfate salt solution, SIGMA).
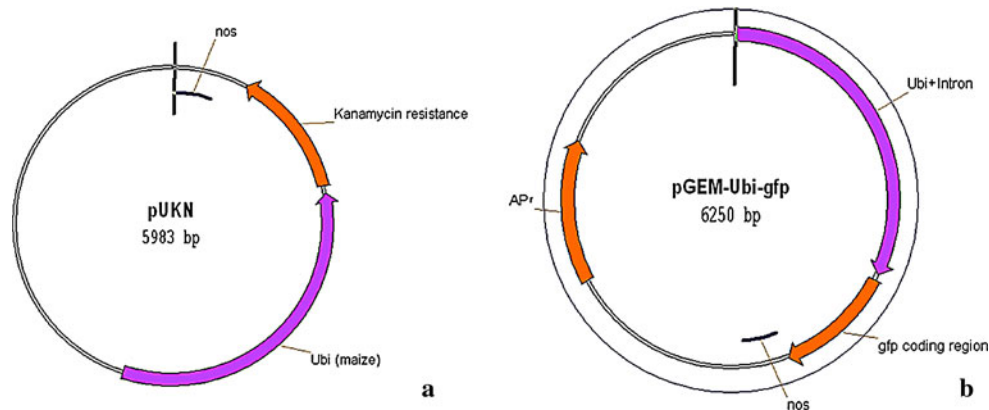
Fig. 1 Constructs, **a** the pUKN construct and **b** the pGEM-ubi-gfp construct

Selective rooting medium: rooting medium supplemented with 30 mg/L G418.

Osmotic medium: MS medium supplemented with 0.2 M D-sorbitol and 0.2 M D-mannitol.

Kill curve experimentation

A kill curve experiment was carried out utilizing IEs at 2 weeks old after initiation on CIM. IEs were subcultured onto selective regeneration media containing G418 at 0, 10, 20, 30 and 50 mg/L (5 IEs per plate, three plates for each G418 treatment) and placed under lights. IEs were subcultured fortnightly. Four weeks later, the numbers of IEs generating shoots and surviving (the total number of IEs minus the number of necrotic IEs) were recorded.

Microprojectile transformation

IEs at 9–11 days old after initiation were used for microprojectile transformation. Six IEs were placed at the center of a shallow petri dish 15 × 90 mm containing osmotic medium and stored for 2–3 h in the dark prior to bombardment (Fig. 6a). Operation of the particle inflow gun (PIG) was conducted as described by Vain et al. (1993) (Grootboom et al. 2010). For plasmids, DNA delivery occurred via 0.6 μm gold particles (0.42 mg per shot). The distance from the filter holder to the target cells was adjusted to 18.5 cm and the helium pressure was modified to 1,000 kPa. As much as 5 μg of each pUKN plasmid and pGEM-ubi-gfp plasmid were mixed and then equally loaded into the receptacle for six shots. A 500-μm nylon mesh screen was placed 8 cm above the target tissue and a vacuum of approximately −90 kPa was generated prior to shooting with a time duration of 0.05 s. Post-bombardment, IEs were kept on osmotic medium for 3–4 h before being transferred onto CIM.

Selection of putative transgenics

After IEs recovered on CIM for 3–4 days, they were transferred to selective regeneration medium and placed under lights in a tissue culture room. IEs were then subcultured fortnightly until putative shoots grew to 4–6 cm. Subsequently, shoots were then moved to selective rooting medium for 4 weeks without subculture. Once the plantlets had a well-developed root system (>10 roots of >1 cm length), the lids of petri dishes were opened for at least 3 days to acclimatize plantlets in tissue culture room. The plantlets were transferred into the containment glasshouse and planted in plastic pots (20 L) containing 15 L of potting mix (Green Fingers). Plants were watered once per day.

Reporter gene observation

The *gfp* reporter gene produces a protein which fluoresces in living cells when exposed to blue light at the wavelength of 395 nm (Ormo et al. 1996). The *gfp* gene expression in callus was monitored under the OLYMPUS BX60 fluorescence microscope 3 and 10 days after bombardment. Each callus piece (from one IE) was then categorized (based on the number of GFP foci) into the following groups: 0–20, 21–50, 51–100 and >100. The *gfp* gene expression in root tips of putative transgenic plantlets and T_1 seedlings was also monitored.

PCR analysis

Genomic DNA was extracted from young leaves of putative transgenic and non-transgenic line Tx430. To confirm the presence of *nptII* and *gfp*, fragments (722 bp of *nptII* and 732 bp of *gfp*) were amplified from genomic DNA using NPT*II*-specific primers: NPT*II*-1: 5′-GCTAT-GACTGGGCACAAC-3′; NPT*II*-2: 5′-GTCAAGAAG

GCGATAGAAGG-3′, and GFP-specific primers: GFP-1: 5′-CCGCGGTTACTTGTACAGCTCGTCC-3′; GFP-2: 5′-CCCGGGATGGTGAGCAAGGGCGAGG-3′ (INTE-GRATED DNA TECHNOLOGIES, www.idtdna.com). PCR analyses were performed in 25-μL reaction mixtures, each containing 50 ng of template DNA, 1× PCR buffer, 200 μM of each dNTP, 2.5 mM MgCl₂, 0.5 μM primer and 2 U Taq DNA polymerase (NEW ENGLAND BioLabs). The PCR program utilized for the *nptII* gene consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles, consisting of 94°C for 30 s, 60°C for 30 s, 72°C for 50 s and a final 7-min elongation step at 72°C. Similarly for the *gfp* gene PCR reactions, the annealing temperature was changed to 70°C for 30 s. PCR products were separated by gel electrophoresis in 1.0% agarose gels.

Southern hybridization analysis

Aliquots of ~10 μg total genomic DNA of each putative transgenic sample and non-transgenic Tx430 were digested with the restriction enzyme *Sac*I-HF™ (NEW ENGLAND BioLabs) at 37°C overnight. As the positive control, 100 pg of undigested pUKN plasmid was used. The digested DNA and plasmid were then separated on a 0.8% agarose gel for 16 h at 25 V. DNA samples were then transferred to a positively charged nylon membrane (Roche) via blot apparatus overnight. The following day, the membrane was hybridized using the digoxigenin (DIG)-labeled *nptII* probe at 42°C overnight (probe construction was carried out using *nptII* primers following the PCR conditions described previously). The next day, the membrane was washed, blocked and fluorescent signal detected according to manufacturer instruction (Roche).

Transgene inheritance assay

Six PCR-confirmed T₀ lines containing both *nptII* and *gfp* genes were selected for analysis of gene inheritance. Non-transgenic Tx430 seeds were used as a control. For each selected line, 25 seeds were placed onto a petri dish of 25 × 90 mm, lined with two filter papers (Whatman No. 1). Seeds were then partially covered by sterilized water and allowed to germinate in the dark at 27 ± 1°C for 3 days. The *gfp* gene expression in the T₁ seedlings was monitored utilizing the OLYMPUS BX60 fluorescent microscope. Seedlings were then soaked in sterilized water containing 30 mg/L G418. Plates were placed under light in the tissue culture room for 1 week. Observations of the health and vigor of seedlings for each treatment were recorded, with particular attention paid to detecting any signs of necrosis among the seedlings under G418 selection.

Results

Kill curve

IEs began to display G418 stress symptoms on selective regeneration media within 1 week. IEs on regeneration medium without G148 (G0) grew healthily and showed the beginnings of shoots within 2 weeks and generated vigorous shoots within 4 weeks. On G0 medium, all IEs survived and 14 of 15 IEs produced vigorous shoots (Table 1). In contrast, none of the IEs generated shoots on G30 or G50 media, and the majority were necrotic. After 4 weeks, the results demonstrated that G418 had a significant impact on callus growth and development in selective media with different G418 concentrations (G0, G10, G20, G30 and G50) (Table 1). Even though two and six IEs produced shoots on G20 and G10 media, respectively, the shoots were shorter and weaker than those on G0 media. The pattern in the kill curve experiment was observed as follows: the higher the G418 concentration in selective regeneration media, the lower the shoot regeneration frequency was evident and the more necrotic IEs were observed (Table 1). In this experiment, 30 mg/L G418 in medium was sufficient to prevent IEs from producing shoots and was therefore selected as the optimal concentration for Tx430 transformation.

GFP observations

Strong *gfp* gene expression was observed in all bombarded IEs with at least 20 GFP foci. More than 100 GFP foci were spotted in about 80% of IEs (Figs. 2, 3a). When examining root tips of putative transgenic plantlets, the *gfp* gene expression was shown to be systemically green under the fluorescent microscope (Fig. 3c). No GFP was found in root tips of non-transgenic plantlets and the root tips of these plantlets displayed yellow color under the fluorescent microscope (Fig. 3b).

Molecular analysis of putative transgenic (T₀) lines

PCR results confirmed the presence of the *NPTII* gene in all transgenic lines and no *nptII* escapes were observed

Table 1 G418 kill curve of Tx430 immature embryos (IEs) under lights in 4 weeks

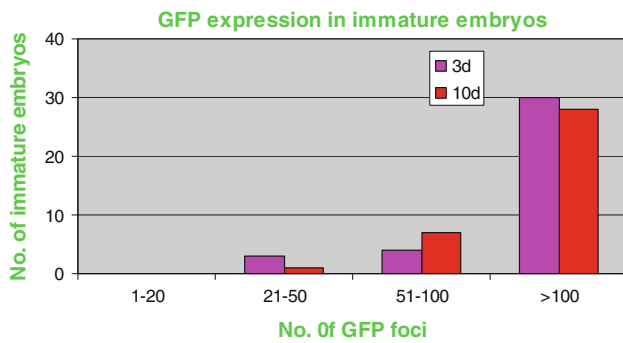| Selective media	G418 (mg/L)	IE with shoots	IE survival
G0	0	14	15
G10	10	6	11
G20	20	2	8
G30	30	0	3
G50	50	0	1

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Fig. 2 Transient *gfp* gene expression in bombarded Tx430 immature embryos

(Fig. 4a). The result of *gfp* PCR analysis showed that 18 of 25 plants were positive (Fig. 4b). Overall, the co-transformation frequency of the *gfp* and *nptII* genes was 72%. Southern hybridisation confirmed the integration of the *nptII* gene into the genome of all T_0 plants (Fig. 5). Single copy and multiple copies of *nptII* gene were found in various plant lines. Among the 25 transgenic lines, 24% had a single *nptII* gene copy, 28% had two to three copies and 48% had at least four copies.

Selection of putative transgenics

IEs recovered and grew quickly on CIM in the dark 3 days after bombardment. The growth of IEs was clearly constrained on selective regeneration medium by the application of G418. Two weeks after selection, most of the IEs displayed necrotic symptoms due to G418. However, a few IEs started to generate shoots. In 4–8 weeks, the transgenic sectors proliferated and produced healthy shoot selection medium (Fig. 6b). Vigorous roots of putative transgenic plantlets were generated on selective rooting medium (Fig. 6c). As much as 100% of all plantlets transferred to pots in the containment glasshouse environment survived (Fig. 6d). The transformation efficiency varied from 18.4 to 25% (mean efficiency of 20.7%) in the three independent experiments (Table 2).
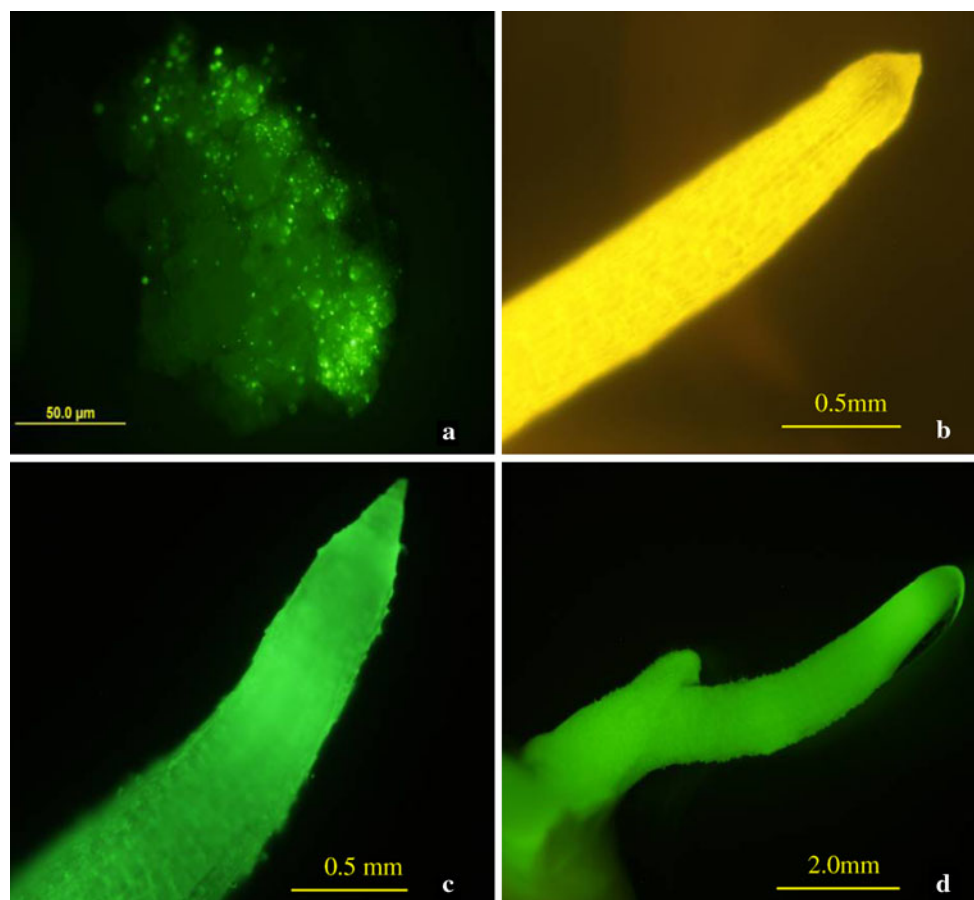

Fig. 3 GFP expression under the blue light at the wavelength of 395 nm of the OLYMPUS BX60 fluorescence microscope; **a** Tx430 immature embryo-derived callus 3 days after bombardment; **b** root tip of non-transgenic Tx430 plantlet; **c** root tip of transgenic Tx430 plantlet; **d** 3-day-old T_1 seedling
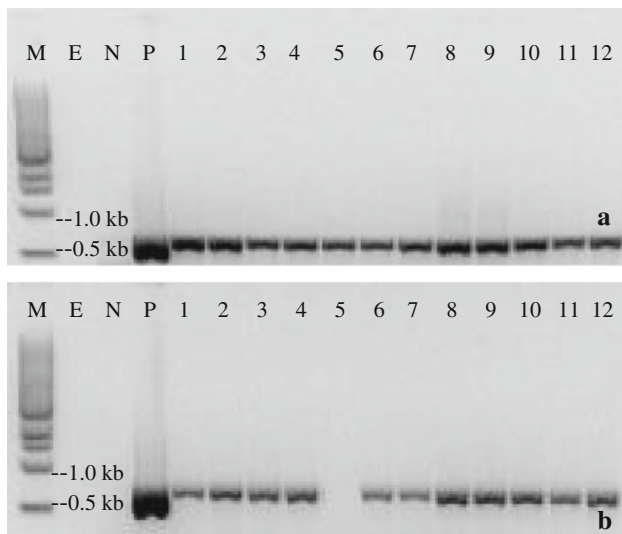
Fig. 4 PCR detection of transgenes in putative transgenic sorghum lines. **a** PCR for the *nptII* gene and **b** PCR for the *gfp* gene. Lines from *left* to *right*: *M* 1 kb DNA Ladder (NEW ENGLAND BioLabs). *E* empty (no template), *N* non-transgenic Tx430, *P* plasmid, *1–12* 12 samples of putative transgenic lines
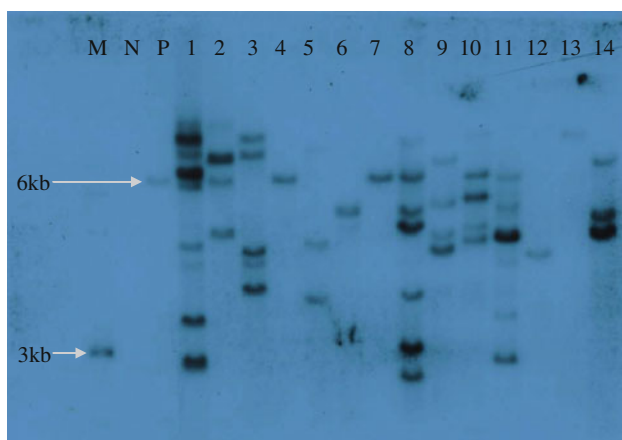

Fig. 5 Southern blot hybridization of 14 putative transgenic lines with *nptII* probe. Lines from *left* to *right*: *M* DNA ladder. *N* NC, *P* equivalent single copy control (pUKN plasmid), *1–14* individual transgenic lines

Transgene inheritance assay

The *gfp* gene expression in T_1 seedlings was observed under the fluorescence microscope in all tested lines (Fig. 3d). The seedlings with the *gfp* gene expression were uniformly fluorescent green. However, null segregants of the *gfp* gene was also observed and appeared yellow. Similarly, the expression of the *nptII* gene in T_1 seedlings was confirmed by their survival under G418 selection. T_1 null segregants of the *nptII* gene and non-transgenic Tx430 seedlings were necrotic in G418 solution after 1 week

(Fig. 7a). T_1 seedlings with the *nptII* gene expression were healthy and grew vigorously (Fig. 7b).

Discussion

Sorghum transformation has been widely considered as challenging since the first transgenic sorghum was reported in 1993 (Casas et al. 1993). Here, we report the highest recorded sorghum transformation efficiency to date of 20.7%. This result is more than 70 times higher than the first successful transformation rate of 0.286% reported via microprojectile transformation, and more than 25 times higher than the biolistic sorghum transformation efficiencies cited by Grootboom et al. (2010) (0.11–0.77%) and Raghuwanshi and Birch (2010) (0.09%). We have more than doubled the best published frequency of *Agrobacterium*-mediated transformation of up to 8.3% (Gurel et al. 2009). The enhancement of sorghum transformation efficiency can be largely attributed to three crucial factors: (1) tissue culture system; (2) DNA delivery system; and (3) selection strategy.

The tissue culture system plays a fundamental role in the success of sorghum microprojectile transformation and the *Agrobacterium*-mediated transformation system. There are many factors which directly influence the effectiveness of the tissue culture system, from the method of initiating explants to the procedure of planting putative transgenic plantlets in containment glasshouses. Genotype selection is of paramount importance to obtain an efficient tissue culture system. For example, P898012, C401 and Tx430 have been widely studied (Gao et al. 2005b; Sato et al. 2004). In our laboratory, we also routinely transform genotype 296B and SA281, although these do not allow for such a high efficiency as Tx430 (data not shown). Choosing the appropriate explant is also important to constantly produce reliable embryogenic callus. IEs have been shown to be the most successful and productive explants for sorghum tissue culture (Elkonin and Pakhomova 2000; Grootboom et al. 2010; Gurel et al. 2009). Optimization of the callus induction medium, regeneration medium and rooting medium is another approach to improve tissue culture performance. M11 medium, which contains MS medium supplemented with 1 g/L KH_2PO_4, has been shown to be more effective than MS medium for sorghum tissue culture (Sato et al. 2004; Elkonin and Pakhomova 2000). The addition of L-proline and L-asparagine has improved the production of sorghum embryogenic callus and reduced the incidence of toxic phenolic compounds in medium (Elkonin et al. 1995). Furthermore, the presence of high levels of copper in CIM and regeneration medium has been shown to enhance the callus induction rate and regeneration frequency (Nirwan and Kothari 2003). Based on reports in
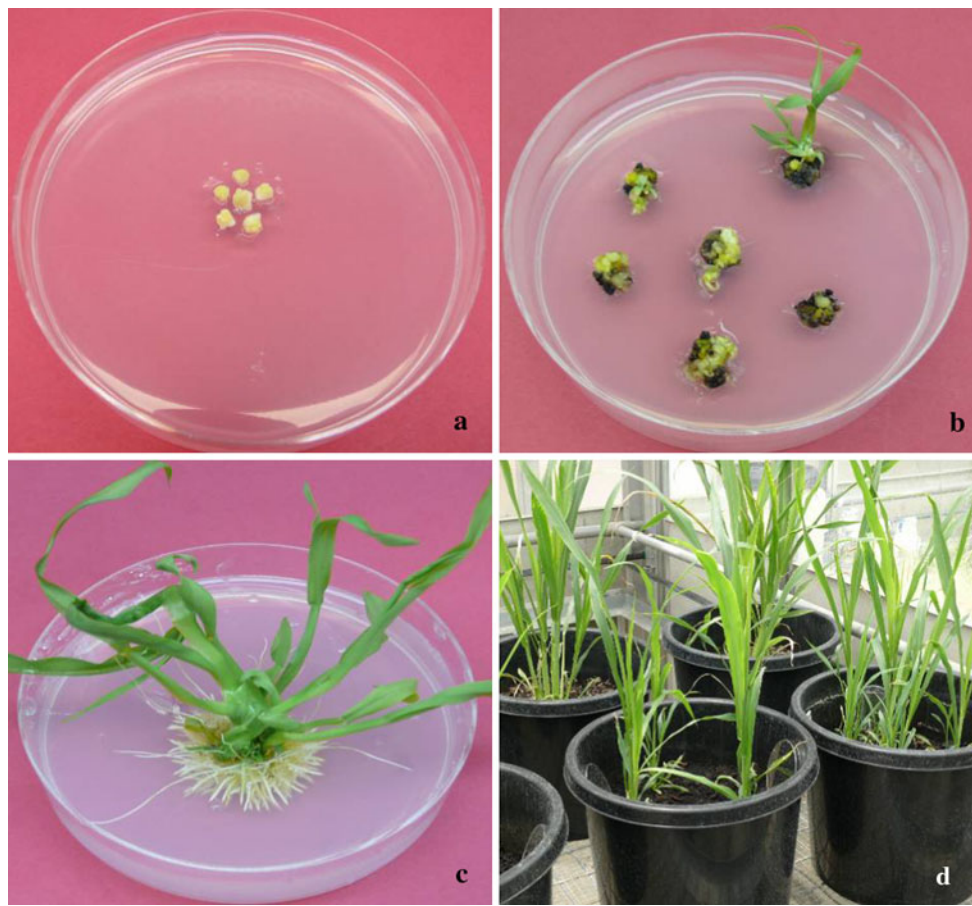
Fig. 6 The development of putative transgenic sorghum plants. **a** The initiation stage—immature embryo-derived calli ready for microprojectile transformation; **b** putative transgenic shoots on selective (30 mg/L G418) regeneration medium at 6 weeks; **c** putative transgenic plantlet on selective (30 mg/L G418) rooting medium at 4 weeks; **d** putative transgenic plants in containment glasshouse

literature, we optimized the usage of additional ingredients including $CuSO_4$, KH_2PO_4, L-proline, L-asparagine and plant growth regulators including 2,4-D, IAA, IBA, NAA and BAP in callus induction medium, regeneration medium and rooting medium. After optimization of the sorghum tissue culture system for inbred line Tx430, an efficient tissue culture system was established. More than 95% of IEs form callus, and of these more than 70% regenerate shoots with no further loss of in vitro plantlets during the transition from the tissue culture environment to containment glasshouse conditions.

Promoters and reporter genes play an important role in optimizing the DNA delivery system. There are many promoters which have been studied for plant transformation, but the strength and suitability of promoters are variable and result in different levels of gene expression in target tissue (Kumar et al. 2011; Tadesse et al. 2003; Able et al. 2001). It was reported that the number of GUS foci was significantly higher from *ubi*1 than from *actin*1 and CaMV 35S (Able et al. 2001). Similarly, this result was further refined utilizing GUS histochemical staining and

Table 2 Transformation efficiency of three independent experiments with immature embryo (IE)-derived calli of *Sorghum bicolor* Tx430

Experiment	Bombarded IE	Transgenic events	Transformation efficiency (%)
1	49	9	18.4
2	36	7	19.4
3	36	9	25.0
Total	121	25	20.7

enzymatic activity assay, showing the strength of promoters in descending order: $ubi1 > act1D > adh1 > $ CaMV 35S (Tadesse et al. 2003). Recently, CaMV 35S promoter activity was reported to be very low in small T_0 sorghum transgenic plants (Kumar et al. 2011). The *ubi*1 promoter has proven to be the most successful promoter in sorghum transformation in recent years (Grootboom et al. 2010; Raghuwanshi and Birch 2010; Gurel et al. 2009). GFP is a highly versatile reporter gene, because the *gfp* gene expression can be monitored any time in living cells under a fluorescence microscope in a non-destructive manner
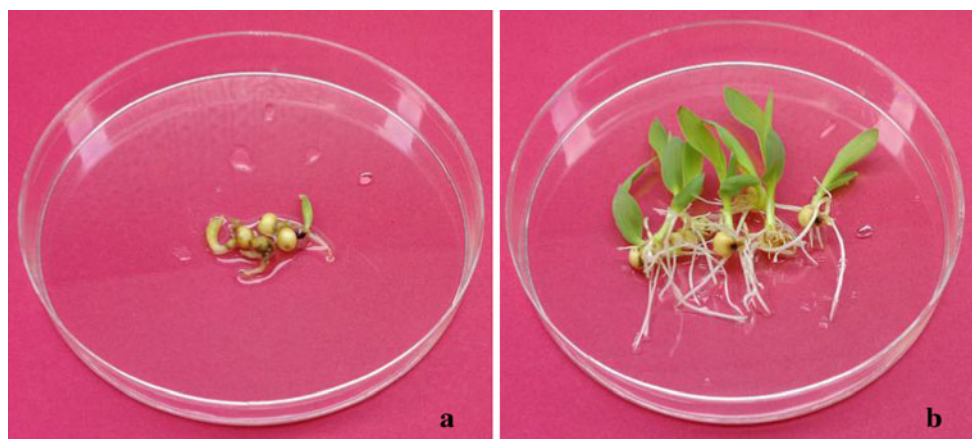
Fig. 7 G418 germination assay. **a** T_1 susceptible seedlings under 30 mg/L G418 selection after 1 week and **b** T_1 resistant seedlings under 30 mg/L G418 selection after 1 week

(Chiu et al. 1996). Hence, visual examination may be used to optimize helium pressure, shooting distance and other parameters very rapidly, and non-destructively by counting the GFP foci. The same tissues may then be used for regeneration of stable transformants, which is not possible with other maker genes requiring destructive or toxic enzyme assays. Visual marker genes like *gfp* can even be used for sorghum transformation without using antibiotics or herbicides as the selection agents (Gao et al. 2005a).

Transgenic sorghum plants have been successfully obtained from IEs with the aid of three selective markers (Kumar et al. 2011; Grootboom et al. 2010; Gurel et al. 2009). The herbicide-resistance *bar* gene was used in the first transgenic sorghum report (Casas et al. 1993). The phosphomannose isomerase (*pmi*) gene, which converts mannose-6-phosphate to fructose-6-phosphate (Joersbo et al. 1998), was proven to be an efficient selective marker for sorghum *Agrobacterium*-mediated transformation (Gurel et al. 2009), and the hygromycin phosphotransferase (*hpt*) gene, which gives the plant resistance to the antibiotic hygromycin, was reported to be a good selective marker for sorghum transformation (Kumar et al. 2011; Carvalho et al. 2004). The neomycin phosphotransferase (*nptII*) gene has been shown to be successful in sugarcane microprojectile transformation (Bower and Birch 1992). It was also shown to be an efficient selective marker for sorghum microprojectile transformation during the present study.

Genetic modification technologies play an important role in modern agriculture. An efficient genetic transformation system greatly enhances the ability of scientists to study gene function and improve economically important traits in crop plants. A high frequency of reliable sorghum transformation makes genetic engineering technology more practical and feasible, which will be critical in an era where the demand for food and biomaterials is ever expanding, and the amount of arable land is limited.

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