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Full Length Research Paper

Culturing of immature inflorescences and *Agrobacterium*-mediated transformation of foxtail millet (*Setaria italica*)

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In previous reports, we developed an *Agrobacterium*-mediated transformation system for foxtail millet. Here, we report optimization of the system through improvement of the regeneration system efficiency and optimization of conditions for gene delivery. Immature inflorescences explants of foxtail millet cv. Jigu 11 varying in length (0.5 to 1.0, 1.1 to 1.5, 1.6 to 2.0 and >2.0 cm) were cultured on modified MS medium for callus induction and regeneration. The highest embryogenic callus-formation efficiency (90.72%) was achieved with 0.5 to 1.0 cm long inflorescences and 25 days old calli induced from 0.5 to 1.0 cm long immature inflorescences gave rise to the highest differentiation frequency (90.93%). In addition, factors affecting T-DNA delivery were examined by transient β-glucuronidase (GUS) expression. Calli induced from younger explants (0.5 to 1.0 cm immature inflorescences) were optimal. *Agrobacterium tumefaciens* strain LBA4404 performed significantly better than EHA105. Co-cultivation at 22°C with 0.15 g/l dithiothreitol (DTT) in the infection solution and co-cultivation medium led to higher GUS transient expression efficiency than with other treatments. Using this optimized procedure, the lysine-rich protein encoding gene *SBgLR* from potato was transformed into foxtail millet cv. Jigu 11 with 5.5% transformation efficiency. The procedure described here will be useful for genetic improvement of foxtail millet.

Key words: Foxtail millet, regeneration, *Agrobacterium*-mediated transformation, temperature, dithiothreitol (DTT).

INTRODUCTION

Foxtail millet (*Setaria italica* (L.) Beauv.), which belongs to the Poaceae family, is native to China and has a long history in cultivation of about 7000 years. As a traditional crop for food, feed and forage, it is mainly cultivated in Asian countries, especially in northern China. The crop is also well known for its natural drought resistance, so it is generally grown in arid, hilly areas or marginal soil. In addition, foxtail millet has been recommended for consumption by diabetics. Therefore, genetic improvement of this crop is of great significance to people in many developing countries where it is widely cultivated. Successful genetic engineering of plants relies on the availability of efficient and reproducible *in vitro* plant regeneration and transformation systems. Ban et al. (1971) first reported the tissue culture of foxtail millet using anthers as explants. Subsequently, *in vitro* regeneration using immature inflorescences (Xu et al., 1984; Yang and Xu, 1985; Vishnoi and Kothari, 1996), mature seeds (Rao et al., 1988; Reddy and Vaidyarath, 1990), shoot apices (Osuna-Avila et al., 1995) and immature

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Abbreviations: AS, Acetosyringone; **DTT,** dithiothreitol; **GUS,** β-glucuronidase; **MS medium,** Murashige and Skoog; **PVP,** polyvinylpyrrolidone; **SE,** standard error of the mean; **X-Gluc,** 5-bromo-4-chloro-3-indolylglucuronide.

glumes (Reddy and Vaidyarath, 1988; 1990) as explants was achieved. In addition, the tissue culture and regeneration system for *Setaria lutescens* and *Setaria glauca*, wild relatives of foxtail millet, were established by Xu et al. (1983) and Diao et al. (1997), respectively. Immature inflorescences are considered to be an important source of totipotent cultures in many monocots and dicots (Vasil, 1982; Eapen and Georges, 1997). Callus cultures have been initiated from immature inflorescences of many cereals, such as tritordeum (Barcelo et al., 1989), *Sorghum bicolor* (Brettel et al., 1980), *Triticum aestivum* (Eapen and Rao, 1985), *Pennisetum purpureum* (Wang and Vasil, 1982), *Panicum miliaceum* and *Panicum miliare* (Rangan and Vasil, 1983) and *S. italica* (Xu et al., 1984; Yang and Xu, 1985; Vishnoi and Kothari, 1996). However, research on millet biotechnology, especially for foxtail millet, has lagged behind compared with other gramineous crops such as maize, rice and barley.

Kothari et al. (2005) reviewed the application of biotechnology for improvement of millet crops. However, research on gene transfer to foxtail millet is very limited. Diao et al. (1999) studied factors affecting particle bombardment transformation of foxtail millet calli derived from immature inflorescences. Production of transgenic plants by particle bombardment of foxtail millet pollen and inflorescence is reported, but the transformation efficiency was low (Dong and Duan, 1999, 2000). *Agrobacterium*-mediated transformation has been proven to be more advantageous than the biolistic gun and is a better alternative for the delivery of transgenes. This system can produce a higher proportion of stable, low-copy number transgenic events with fewer DNA rearrangements (Ishida et al., 1996; Zhao et al., 1998; Dai et al., 2001; Travella et al., 2005), transfer larger DNA segments into recipient cells (Hamilton et al., 1996) and transgene expression is more stable over generations than with the direct gene transfer method (Shou et al., 2004) and is highly efficient (Ishida et al., 1996; Zhao et al., 1998).

Many factors influencing *Agrobacterium*-mediated transformation of monocotyledonous plants have been investigated and elucidated. The influence of temperature on *Agrobacterium*-mediated T-DNA transfer was studied in the 1940s (Braun, 1947, 1958) and its effect on genetic transformation during co-culture was first reported in soybean (Kudirka et al., 1986). Fullner and Nester (1996) concluded that 19°C was the optimal temperature for transfer via bacterial conjugation. However, the optimal temperature for transformation should be evaluated for each specific explant and *Agrobacterium* strain involved (Salas et al., 2001). Several antioxidants also improve T-DNA delivery in some plant species. Perl et al. (1996) reported that dithiothreitol (DTT) in combination with polyvinylpolypyrrolidone (PVP) promoted T-DNA delivery in grape (*Vitis vinifera* L.). There are also reports that DTT coupled with L-cysteine in soybean can improve T-DNA transfer (Olhoft and Somers, 2001; Paz et al., 2004; Olhoft et al., 2001, 2003) or maize (Vega et al., 2007).

Previously, *Agrobacterium*-mediated transformation was developed for foxtail millet (Liu et al., 2005, 2007) and the pollen-specific gene *Si401* was transformed utilizing this system (Qin et al., 2008). In this study, reproducible and effective protocol for callus induction was reported and plantlet regeneration from immature inflorescences of foxtail millet in more detail. To optimize the transformation protocol and enhance the transformation efficiency, several important factors that had not been studied in our previous work was investigated, including the *Agrobacterium* strain, callus age, temperature and DTT, analyzed by GUS transient expression. In addition, the *SBgLR* gene which encodes a lysine-rich protein from potato (*Solanum tuberosum* L. cv. Desiree) (Lang et al., 2004) was successfully transformed into foxtail millet cv. Jigu 11 using this optimized transformation system.

MATERIALS AND METHODS

Plant materials

Foxtail millet cv. Jigu 11, which is a high-quality and high-yield variety commonly cultivated in China, was grown in the greenhouse of China Agricultural University.

Media

The types and composition of media used for millet culture and transformation at different stages are listed in Table 1. The basal salt medium and sucrose were autoclaved and additives including vitamins, plant growth regulators, antibiotics, the antioxidant DTT and acetosyringone (AS) were filter-sterilized into the medium. The co-cultivation medium was overlaid with a piece of sterile filter paper.

Agrobacterium strains and vector

For transient transformation, *Agrobacterium* strain EHA105 (Hood et al., 1993) and a hypervirulent derivative of LBA4404 (Komari et al., 1996) containing the construct pCAMBIA2300-GUS, respectively, were used in this experiment. The 7 kb T-DNA region of this construct is shown in Figure 1a. The binary vector pCAMBIA2300-GUS was constructed by inserting the 35S::GUS::Tnos fragment from pBI121 (Chen et al., 2003) into pCAMBIA2300 (Hajdukiewicz et al., 1994) at the *Hin*d III and *Eco*R I sites. The *Cauliflower mosaic virus* (CaMV) 35S promoter was replaced by the maize Ubiquitin promoter from pCAMBIA1301-UbiN (kindly provided by Prof. Dawei Li, China Agricultural University) at the *Hin*d III and *Bam*H I sites.

For stable transformation, the vector pSB-SBgLR harbored by a hypervirulent derivative of LBA4404 (Komari et al., 1996) was used. pSB-SBgLR was constructed from the super-binary vector pSB130 (kindly provided by Prof. Samuel Sun, Chinese University of Hong Kong) by inserting the seed-specific promoter F128 (unpublished data) and *SBgLR* gene (Lang et al., 2004) into the multiple cloning site. The vector pSB-SBgLR carried two separate T-DNAs. One T-DNA harbored the hygromycin resistance gene *Hpt*II (selectable marker) under the control of the CaMV 35S promoter and the other possessed the target gene *SBgLR* under the control of the seed-specific promoter F128. The 4.5 kb T-DNA region of this

Table 1. Medium formulation for tissue culture and transformation.

Developmental stage	Medium	Medium composition
For tissue culture	Callus induction medium	MS + 2 mg/l 2,4-D + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 5 mg/l $AgNO_3$ + 3 % (w/v) Sucrose + 0.3 % (w/v) Phytagel, pH 5.8
	Regeneration medium	MS + 2 mg/l 6-BA + 0.5 mg/l NAA + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 3 % (w/v) Sucrose + 0.3 % (w/v) Phytagel, pH 5.8
	Rooting medium	1/2MS + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 3 % (w/v) Sucrose + 0.3 % (w/v) Phytagel, pH 5.8
For transformation	Infection medium	MS + 2 mg/l 2,4-D + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 100 µmol/l AS + 3 % (w/v) Sucrose + 1 % (w/v) Glucose, pH 5.2 (+ 0.15 g/L DTT)
	Co-cultivation medium	MS+2 mg/l 2,4-D + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 100 µmol/l AS + 3 % (w/v) Sucrose + 1 % (w/v) Glucose + 0.3 % (w/v) Phytagel, pH 5.8 (+ 0.15 g/L DTT)
	Resting medium	MS + 2 mg/l 2,4-D + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 5 mg/l $AgNO_3$ + 3 % (w/v) Sucrose + 0.3 % (w/v) Phytagel + 500 mg/l Cb, pH 5.8
	Selection medium	MS + 2 mg/l 2,4-D + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 5 mg/l $AgNO_3$ + 3 % (w/v) Sucrose + 0.3 % (w/v) Phytagel + 500 mg/l Cb + 5(or 8) mg/l Hyg, pH 5.8
	Regeneration medium	MS + 2 mg/l 6-BA + 0.5 mg/l NAA + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 3 % (w/v) Sucrose + 0.3 % (w/v) Phytagel + 500 mg/l Cb + 5 mg/l Hyg, pH 5.8
	Rooting medium	1/2MS + 1 mg/l L-Proline + 800 mg/l Casein acids Hydrolysate + 3 % (w/v) Sucrose + 0.3 % (w/v) Phytagel + 500 mg/l Cb + 5 mg/l Hyg, pH 5.8

Salt and vitamin compositions were the same as described for MS medium (Murashige and Skoog, 1962). 2.4-D, 2,4-dichlorophenoxyacetic acid; 6-BA, 6-benzylaminopurine; NAA, α-napthaleneacetic acid; Cb, carbenicillin; Hyg, hygromycin.

recombinant construct is shown in Figure 1b.

Callus culture and plant regeneration

Immature inflorescences of 0.5 to 1, 1.1 to 1.5, 1.6 to 2 and >2 cm in length were used as explants for initiating callus cultures. The inflorescences were surface-sterilized by wiping with cotton soaked in 75% ethanol when still enclosed by the flag and boot-leaf sheaths, then were cut transversely into about 0.4 to 0.5 cm long segments and plated on callus induction medium in a 10 cm diameter glass dish. Subculturing was carried out every two weeks. Cultures for callus induction as well as the subcultures were maintained in darkness at 26 ± 1°C. For regeneration, calli were transferred to the medium and maintained under 16 h light/ 8 h dark photoperiod with 1600 lux at 26 ± 1°C. Regenerated plantlets were further rooted on the medium in bottles. Thereafter, rooted plants were grown to maturity in the greenhouse and allowed to set seeds.

Transformation protocol

For transient transformation, *Agrobacterium tumefaciens* strains LBA4404 and EHA105 harboring the construct pCAMBIA2300-GUS, respectively, were streaked out from a -80°C glycerol stock onto a YEP medium plate (5 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, 15 g/l agar, pH 7.0) containing 100 mg/l streptomycin sulfate and 50 mg/l kanamycin. The plates were incubated at 28°C for 2 days until single colonies developed. Bacterial cells (OD_{600} = 0.8 to 1.0) cultured from a single colony were collected by centrifugation

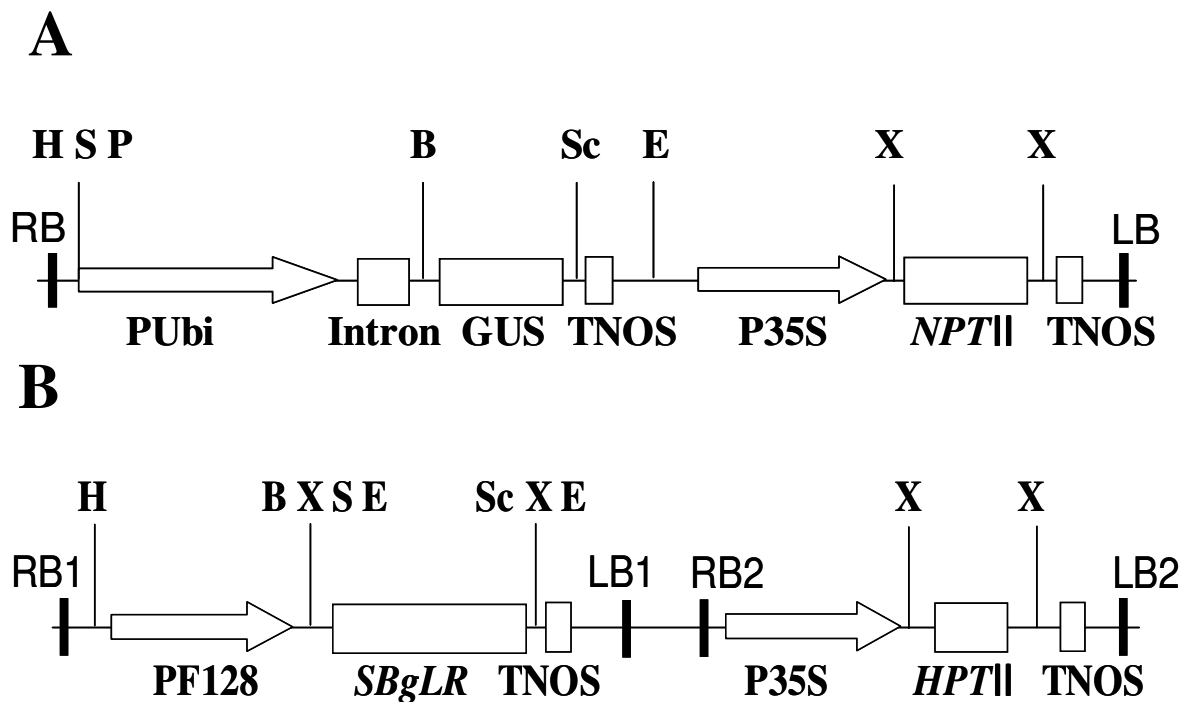

Figure 1. Schematic diagrams of the binary vectors used in this study (not drawn to scale). (A) pCAMBIA2300-GUS; (B) pSB-SBgLR. Only the T-DNA region is shown for each vector. LB and RB, left border and right border, respectively; PUbi, maize ubiquitin promoter; *SBgLR*, *SBgLR* gene; GUS, β-glucuronidase gene; P35S, CaMV 35S promoter; PF128, seed-specific promoter F128 from foxtail millet; *NPT* II, neomycin phosphotransferase II gene; *HPT* II, hygromycin phosphotransferase II; TNOS, nopaline synthase gene terminator; H, *Hin*d III; S, *Sph* I; P, *Pst* I; B, *Bam*H I; Sc, *Sac* I; E, *Eco*R I; X, *Xho* I.

at 3000 r/min for 10 min and suspended in infection medium supplemented with 100 µM AS in a 50 ml tube with OD_{600} = 0.8. The culture tube was shaken horizontally on a platform at 75 r/min at room temperature (26°C) for 4 to 5 h before being used for infection (Frame et al., 2002). For stable transformation, *A. tumefaciens* strain LBA4404 harbouring the vector pSB-SBgLR was used. The bacteria solution for infection was prepared as earlier.

The infection procedure was followed by the most advanced public protocol for maize embryo (Frame et al., 2002) with some modifications which made it suitable for foxtail millet callus as follows. Calli were dissected to 30 ml infection medium containing 100 µM AS in a 100 ml conical flask (100 calli per conical flask) and washed twice with this medium. The final wash solution was removed and 50 ml *A. tumefaciens* suspension was added to calli. Infection was carried out by shaking at low speed (100 r/min) for 30 min at room temperature (26°C). After infection, the *Agrobacterium* suspension was discarded. The calli were transferred to sterile filter paper and dried for 10 min on a clean bench before transferal to co-cultivation medium.

To test the effect of *A. tumefaciens* strain and callus age, the co-cultivation was conducted at 26°C for 3 days. To test the impact of temperature on transient transformation, the calli were co-cultivated with *A. tumefaciens* at 22 or 26°C. To test the effect of DTT on gene transformation, we conducted a side-by-side comparison between the absence and presence of 0.15 g/l (1 mM) DTT in the infection and co-cultivation medium and the co-cultivation was performed at 22°C. In the experiments in which temperature or DTT were compared, calli were washed and infected in the same conical flask before being distributed. In all the earlier mentioned experiments, the co-cultivation medium plates were wrapped with parafilm and placed in darkness for 3 days.

After co-cultivation, the explants were washed thoroughly with sterile distilled water containing 500 mg/l carbenicillin three times, then blotted on sterile Whatman filter paper and transferred to the resting medium containing 500 mg/l carbenicillin to suppress *A. tumefaciens* growth. Dishes were sealed and incubated in the dark for 7 days at 26°C. After resting, the explants, responsive or not, were transferred to selection medium (30 per plate) containing 5 mg/l hygromycin for two weeks, then subcultured on selection medium containing 8 mg/l hygromycin for further two weeks. The resistant calli were visible and the efficiency (%) was calculated by the number of hygromycin-resistant calli events per 100 explants infected. After that, calli were transferred to the regeneration medium and maintained under a 16 h light/8 h dark photoperiod with 1600 lux at 26 ± 1°C. Regenerated plantlets were further rooted on the rooting medium in bottles. Thereafter, rooted plants were grown to maturity in the greenhouse and allowed to set seeds.

Histochemical GUS assays

Transient GUS assay was carried out after resting for 7 days. The embryogenic calli were incubated overnight at 37°C in a solution containing 50 mM sodium phosphate buffer, pH 7.0, 0.1% (v/v) Triton-X 100, 10 mM EDTA and 1 mM 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) (Jefferson, 1987). The level of transient GUS expression was assessed by estimating the proportion of the area with blue foci visible on each callus. The calli were categorized as follows: high expression (>25% area of the callus stained blue), low expression (<25% area of the callus stained blue) and no

expression (no area of the callus stained blue). The number of calli in each group compared with the total number of calli was assessed to determine the percentage of each expression group. The experiment was carried out in triplicate.

Polymerase chain reaction (PCR) analysis

All of the putative transgenic (hygromycin resistant) and control non-transformed plants were analyzed by PCR for the presence of *SBgLR*. Genomic DNA was extracted from fresh leaves using the CTAB method (Murray and Thompson, 1980). The forward primer P_5F (30 nt; 5'-ATGGATCCATGAAGAATTGGAGGAACTTGC-3') and reverse primer P_1R (27 nt; 5'-ATAAGCTTGGAAATTCT CAACCTTGAA-3') were used. The PCR reactions were carried out in a total volume of 50 µl containing 100 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTPs, 2 U *Taq* DNA polymerase and 1 µM of each primer. Amplification conditions were 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 1 min and one cycle of 5 min at 72°C. The reactions were carried out in a Biometra Thermo cycler. The PCR products were visualized by running the completed reaction mixture on a 0.8% agarose gel containing ethidium bromide. To demonstrate positive PCR signals were not a result of residual *A. tumefaciens* in the leaves, the control PCR reaction using *vir*G specific primers (5'-CGATGTCGCTATGCGGCATCT-3' and 5'-CAATGAGAAGTTGCTCGCGCG-3') was conducted.

Western blot analysis of transgenic foxtail millet seeds

Total protein was extracted from mature seeds of T_0 transgenic foxtail millet plants in protein extract buffer (5 mM potassium phosphate, pH 6.0; 2.25% (w/v) sucrose, 0.15% (v/v) β-mercaptoethanol). Protein (50 µg) was separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose filter by electroblotting in the presence of transfer buffer (48 mM Tris base, 39 mM glycine, pH 9.2) for 90 min at 1 mA/cm^2 of gel area. The filter was blocked with 5% (w/v) non-fat dry milk for 1 h, then incubated with the primary polyclonal antibody anti-SBgLR at 1:1000 dilution in Tris-buffered saline plus Tween (TBST; 20 mM Tris-HCl, pH 7.5, 0.14 M NaCl, 0.1% (v/v) Tween 20) for 1 h. Finally, the filter was incubated with goat anti-rabbit alkaline phosphatase-conjugated IgG (Promega) as a secondary antibody at 1:3000 dilution in TBST for 1 h. Protein bands were visualized in alkaline phosphatase substrate buffer (Promega).

RESULTS

Callus induction from immature inflorescences in different size

Previous studies showed that calli can be produced from *S. italica* immature inflorescences (Xu et al., 1983; Yang and Xu, 1985; Vishnoi and Kothari, 1996). To test the relationship between the callus production rate and the developmental stage of inflorescences, as indicated by the length of immature inflorescences, four inflorescence length classes (0.5 to 1.0, 1.1 to 1.5, 1.6 to 2.0 and >2.0 cm) were distinguished. Then they were cut transversely into 0.4 to 0.5 cm long segments and plated on callus induction medium (Figure 2a). Most explants in the 0.5 to 1.0 and 1.1 to 1.5 cm classes formed callus at the cut ends within one week (Figure 2b), while others initiated callus later (data not shown). After one-month culture, two types of callus were observed: one was white and watery (non-embryogenic callus); the other was compact, nodular, light yellow and had embryoids (embyogenic callus) (Figure 2c) (Vishnoi and Kothari, 1996). The embryogenic calli production efficiency from the different inflorescence sizes is summarized in Table 2. Calli were obtained at the highest frequency (90.72%) from 0.5 to 1.0 cm immature inflorescences. The efficiency declined dramatically with increasing immature inflorescence size, namely 59.90, 40.78 and 19.80% for 1.1 to 1.5, 1.6 to 2.0 and >2.0 cm inflorescences, respectively. The results indicate that 0.5 to 1.0 cm immature inflorescences are the best explants for callus induction.

Regeneration of different-aged calli from different inflorescence sizes

To assess the regeneration potential, calli induced from 0.5 to 1.0 and 1.1 to 1.5 cm immature inflorescences were transferred to regeneration medium after culture on callus induction medium for 25, 50 and 100 days, respectively. Differentiation was defined as the development of green spots distributed discretely throughout the callus within two weeks of transfer (Figure 2d) and the differentiation rate (percentage of differentiated calli among the total number of embryogenic calli) was calculated. As shown in Table 3, the calli induced from 0.5 to 1.0 cm immature inflorescences and cultured for 25 days showed the highest differentiation frequency (90.93%). The differentiation frequency decreased with duration of induction culture, which was 75.33% for 50 days culture. In contrast, the differentiation efficiencies of calli induced from 1.1 to 1.5 cm immature inflorescences were 56.20 and 36.04% for 25 and 50 days culture, respectively. The 100 days old calli induced from both inflorescence sizes became brown, watery and failed to differentiate. Shoots developed from the green spots after a further one-month culture on regeneration medium (Figure 2e) and the number of shoots per callus varied from one to ten. Shortly after, the shoots (about 1 cm long) were excised and transferred to rooting medium for two weeks until roots developed (Figure 2f).

Regenerated plantlets with fully grown shoots and roots were gradually acclimatized in the greenhouse and the survival rate was more than 90%. These results demonstrate that 25 and 50 days old calli induced from 0.5 to 1.0 cm immature inflorescences are optimal for regeneration.

Influence of bacterial strain and callus age on gene transfer

In a previous study, *A. tumefaciens* strain LBA4404 was used for transformation. Here, the efficiencies of strains

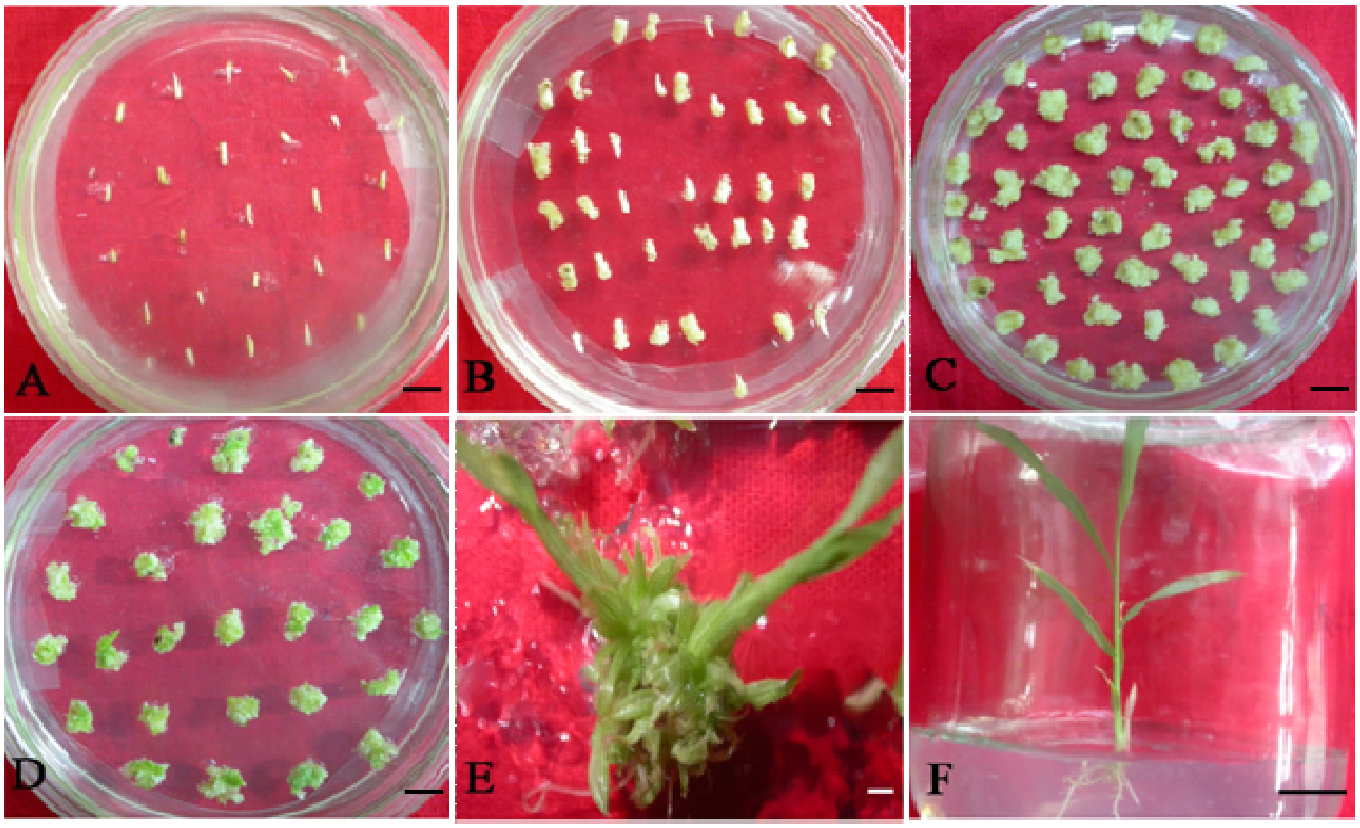

Figure 2. Somatic embryogenesis and regeneration of plantlets from calli from 0.5 to 1.0 cm long immature inflorescences. (A) Inflorescences (cut into 0.4 to 0.5 cm lengths) inoculated on callus induction medium; (B) calli formed from the cut ends after 1 week; (C) embryogenic calli formed on subculture medium after 1 month; (D) green spots differentiated on regeneration medium; (E) plantlets grown on regeneration medium; (F) plantlets with healthy roots on rooting medium. White (e) and black (a to d and f) scale bars represent 0.1 and 1 cm, respectively.

LBA4404 and EHA105 were compared, harboring the binary vector pCAMBIA2300-GUS, respectively, to transform foxtail millet calli. The bacterial culture at OD_{600} = 0.8 and the 25 days old calli induced from 0.5 to 1.0 cm inflorescences were used. No significant differences in the transient GUS expression efficiency after seven days resting culture were observed between the two strains and the percentage of resistant calli showed no obvious variation after one-month culture (data not shown) but the calli transformed by LBA4404 (44.63%) differentiated at a significantly higher rate (about 1.7 fold) than those by EHA105 (26.67%) after one-month culture on regeneration medium (Figure 3). Thus, the strain LBA4404 was used in further experiments.

The mentioned results also indicate that the differentiation efficiency decreases with duration of callus culture. Hence, experiments were carried out to determine if the callus age influenced gene transfer. Comparison of the transformation efficiency of the 25 and 50 days old calli induced from 0.5 to 1.0 cm inflorescences indicated the 25 days old calli showed much higher resistance and differentiation rate than the 50 days old calli (Figure 4), even though no obvious differences in GUS transient efficiency were observed (data not shown). Based on all of the earlier mentioned results, the following experiments were conducted with 25 days old calli induced from 0.5 to 1.0 cm inflorescences transformed by LBA4404 harboring pCAMBIA2300-GUS.

Impact of temperature and DTT on transformation efficiency

Experiments to examine the influence of co-cultivation temperature and DTT on foxtail millet transformation were performed. GUS histochemical analysis was carried out on a subset of calli co-cultivated at 22 and 26°C with or without DTT after 7 days resting. The GUS stained calli were categorized into three groups: high expression, low expression and no expression. Both of the frequencies of calli with high and low GUS expression were significantly higher when co-cultivated at 26°C (23.7 and 45.2%, respectively) than at 22°C (13.1 and 31.6%, respectively). When in addition with DTT, the percentage of calli showing high GUS expression increased distinctly from 23.7% (without DTT) to 32.1% (with DTT) with co-

Table 2. Callus induction efficiency of immature inflorescence at different length.

Inflorescence length (cm)	Number of calli induced	Number of segments inoculated	Callus induction efficiency (%)[a]
0.5 – 1.0	169 148 139	187 175 143	90.72 ± 6.32
1.1 – 1.5	86 48 89	137 105 125	59.90 ± 12.98
1.6 – 2.0	19 34 26	46 96 57	40.78 ± 5.12
> 2.0	8 13 26	86 93 72	19.80 ± 14.32

Callus induction efficiency (%) was calculated as the number of embryogenic calli per 100 total segments inoculated.
[a] Each value represents mean ± standard error (SE) of three replicates

Table 3. Differentiation efficiency of calli of different ages induced from different-sized inflorescences.

Inflorescence length (cm)	Calli age (days)	Differentiation efficiency (%)[a]
0.5 - 1.0	25	90.93 ± 7.33
	50	75.33 ± 3.79
	100	0
1.1 - 1.5	25	56.20 ± 13.19
	50	36.04 ± 2.40
	100	0

Differentiation efficiency (%) was calculated as the number of calli giving rise to green spots per 100 calli tested. [a] Each value represents mean ± SE of three replicates.

cultivation at 26°C. More interestingly, a much greater increase in the frequency of high GUS expression was observed when co-cultivated at 22°C, from 13.1% (without DTT) to 47.2% (with DTT). However, the frequency of low GUS expression declined in addition with DTT at both co-cultivation temperatures (Figure 5).

Production of transgenic foxtail millet

Taking into account the optimal conditions expected to produce the highest frequency of transgenic plants, 2000 embryogenic calli (Figure 6a) (about one month old) induced from 0.5 to 1.0 cm immature inflorescences of foxtail millet cv. Jigu 11 were transformed using LBA4404 harboring the vector pSB-SBgLR. After one month on selection medium, most transformed calli were brown or black; a few calli grew actively and these were found to be transformed calli (Figure 6b). The transformed embryogenic calli cultured on regeneration medium bore green regions (Figure 6c), but not all of these developed shoots. Plantlets with a height of 3 to 5 cm (Figure 6d) were transferred to rooting medium to develop roots for 2 weeks and about 1 to 3 roots were produced per plantlet. The hardened plants were transferred to the greenhouse (Figure 6e). All regenerated plants produced seeds (Figure 6f). Genomic DNA samples from soil-adapted plants obtained by hygromycin-resistant cultures were tested for integration of the *SBgLR* gene by PCR analysis. The plants produced from one resistant callus were calculated as one line. The expected 280 bp fragment was present in six lines of the 108 plants tested (Figure 7a). The transformation efficiency (number of molecular-confirmed lines among the total number of

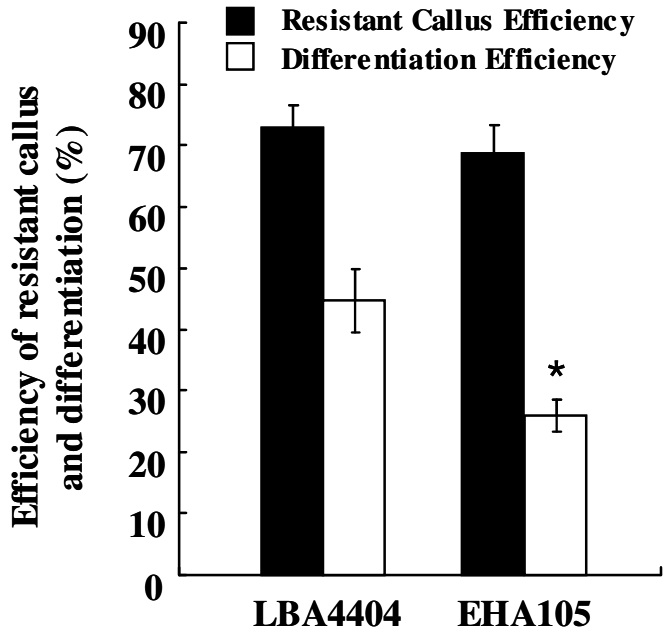

Figure 3. Effect of two *Agrobacterium* strains, LBA4404 and EHA105, on transformation efficiency. Data represents mean ± SE of three replicates with each treatment comprising between 50 and 60 immature inflorescence explants. Differentiation efficiency for EHA105 differed significantly from LBA4404 using Student's *t*-test, as indicated by an asterisk * ($0.01 < P < 0.05$).

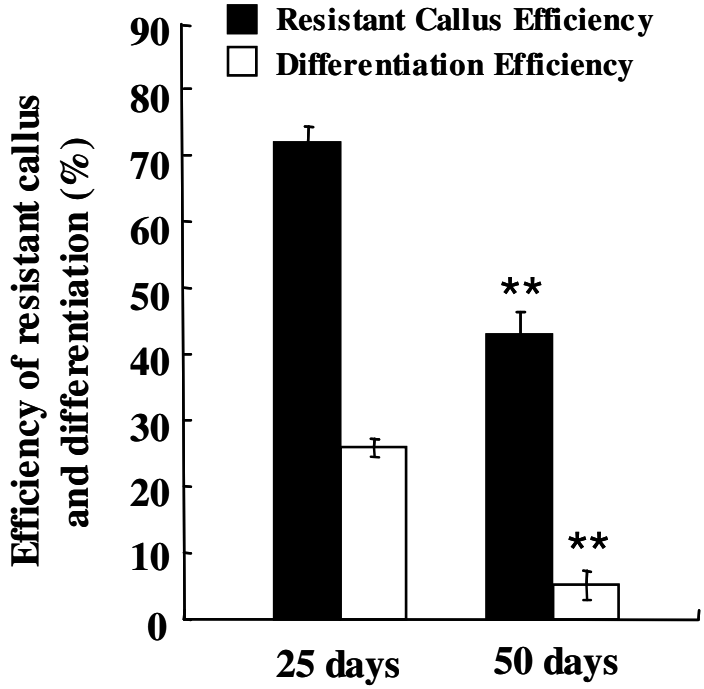

Figure 4. Effect of callus age on transformation. Data represents mean ± SE of three replicates with each treatment comprising between 48 and 66 immature inflorescence explants. Both resistant callus efficiency and differentiation efficiency for 50 days calli differed significantly from 25 days calli using Student's *t*-test, as indicated by two asterisks ** ($P < 0.01$).

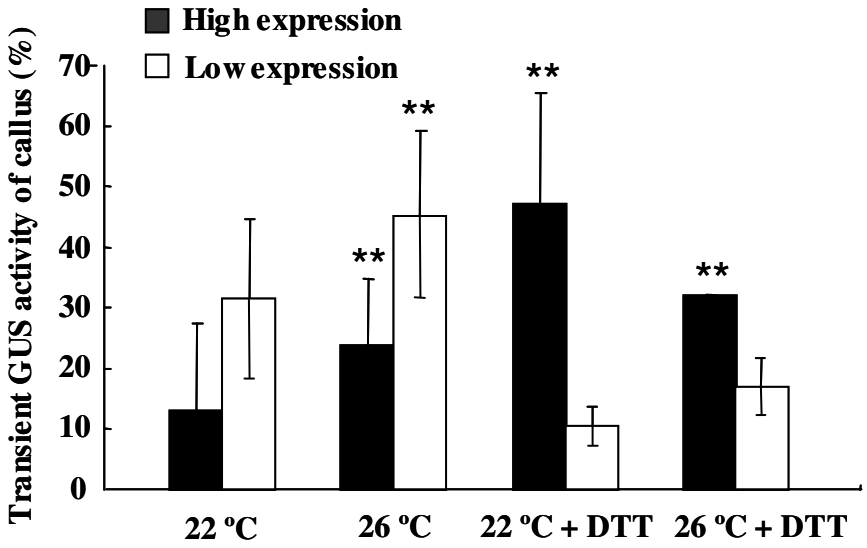

Figure 5. Impact of temperature and addition of DTT to the co-cultivation medium on gene transfer. Results were evaluated based on transient GUS expression assays of infected calli immediately after the 7 days resting stage. The GUS-stained calli were classified as showing either high expression or low expression. Data were from three independent experiments. Each error bar represents the standard error of the mean for each treatment. Compared with 22°C, the frequencies of high and low GUS expression is significantly different at 26°C. Compared with 26°C, the frequency of high GUS expression is significantly different at 26°C + DTT. Compared with 26°C + DTT, the frequency of GUS high expression is significantly different at 22°C + DTT. Significance was analyzed with Student's *t*-test and is indicated by two asterisks ** ($P < 0.01$).

regenerated plants) was 5.5%. Lack of amplification with the *vir*G gene primers demonstrated the absence of residual *Agrobacterium* in the leaves (data not shown).

The presence of the SBgLR protein in the seeds of the six transgenic lines was analyzed by Western blotting with rabbit polyclonal antiserum raised against the SBgLR protein. The SBgLR protein was present in the seeds of three out of the six transgenic lines analyzed (Figure 7b). There is a discrepancy between the deduced molecular mass of the SBgLR protein (23 kD) and its apparent molecular mass of 46 kD after SDS-PAGE, owing to the high content of glutamic acid and lysine or a S-S bond. A similar discrepancy was observed in previous reports on the SBgLR homologous protein, SB401 protein, in transgenic maize (Yu et al., 2004).

DISCUSSION

One of the most critical factors in the success of our transformation protocol is the efficient formation and maintenance of good-quality callus. The immature inflorescence regeneration system has several advantages. For example, donor plants are grown for a shorter period of time and therefore, are less likely to suffer from insect. More importantly, the explant isolation procedure needs less time and tissue sterilization is also simple because the immature inflorescences are enclosed by the flag and boot-leaf sheaths and surface sterilization with 75% ethanol is sufficient. However, the results indicate that the callus status is poor when the donor plants are planted too densely (data not shown). In addition, Maheshwari et al. (1995) suggested that immature inflorescence explants can produce a greater number of morphogenic cultures than other explants when cultured *in vitro* because they contain suppressed meristematic regions. Moreover, it is particularly suitable for genetic transformation using immature inflorescence as explants, since they contain highly meristematic tissue and some cells at developmental stages which were especially suitable for transformation (Barcelo et al., 1994).

According to Vishnoi and Kothari (1996), younger expants (0.5 cm) yielded a higher embryogenic calli formation and regeneration efficiency than larger explants (1.0 and 2.0 cm) irrespective of the growth hormones present in the culture medium. Similar observations were reported for *P. purpureum* (Wang and Vasil, 1982), *Echinochloa crusgalli* (Wang and Yan, 1984) and *Pennisetum americanum* (Eapen and Georges, 1992). In this study, the same trend was observed in that the youngest explants (0.5 to 1.0 cm) showed higher embryogenic calli production and plantlet regeneration efficiency than older explants (1.1 to 1.5 and 1.6 to 2.0 cm), even though the class interval of explant length was

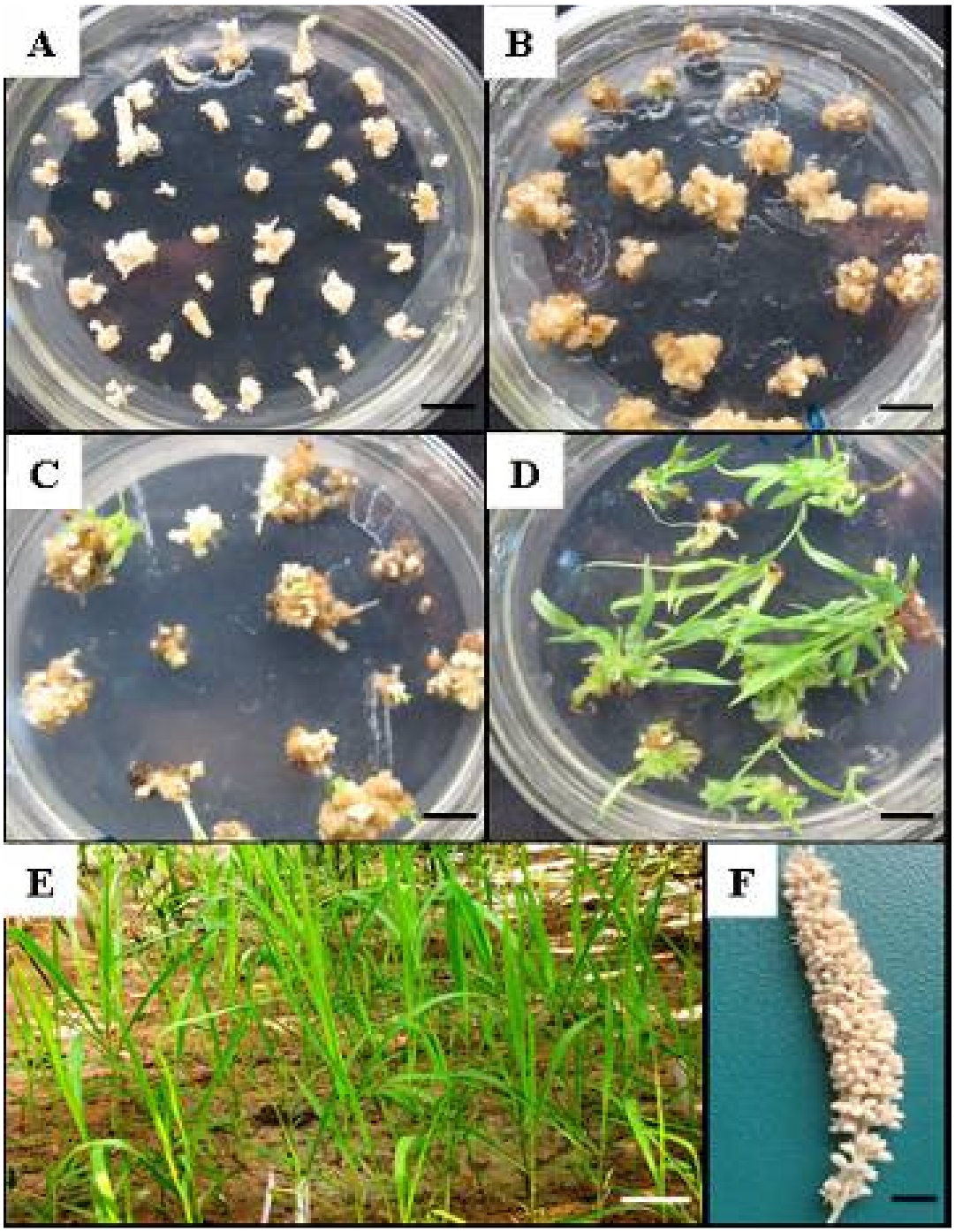

Figure 6. Stages of *Agrobacterium*-mediated transformation of the *SBgLR* gene into foxtail millet. (A) Calli induced from 0.5 to 1.0 cm long inflorescences (cut into 0.4 to 0.5 cm lengths) on the callus induction medium; (B) resistant calli cultured on selective medium for one month; (C) bud induction on regeneration medium for one week; (D) shoots developed for one month on regeneration medium; (E) regenerated plants after one-month growth in the greenhouse; (F) mature seeds from T_0 transgenic plants. White (e) and black (a to d and f) scale bars represent 10 and 1 cm, respectively.

slightly different.Calli regeneration capacity declined with prolonged duration of culture, which is common in many plant species (Barba and Nickell, 1969; Lustinec and Horak, 1970). The loss of totipotency after long-term subculture might be due to the disappearance of a specific factor contained in the primary explants or to polyploidization (Gautheret, 1966; Sheridan, 1975). In this study, a similar tendency was observed and 25 days

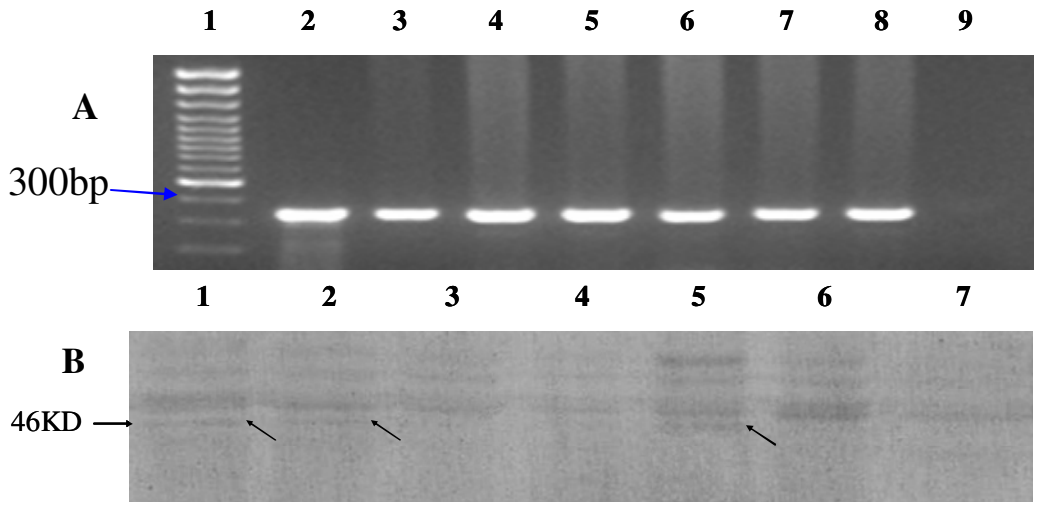

Figure 7. PCR and western blot analyses of *SBgLR* transgenic foxtail millet plants. (A) PCR analysis of genomic DNA to detect the presence of the *SBgLR* gene. Lane 1, molecular weight marker; lane 2, positive control; lanes 3 to 8, plants showing amplification of the predicted 280 bp *SBgLR*-specific sequence; lane 9, non-transformed plants; (b) western blot analysis of SBgLR protein expression in transgenic foxtail millet, probed with SBgLR antibody at 1:1000. Lanes 1 to 6, 50 µg protein from T_0 transgenic foxtail millet mature seeds; lane 7, 50 µg protein from non-transformed foxtail millet mature seeds.

old calli induced from young explants (0.5 to 1.0 cm) showed the highest differentiation frequency (90.72%), which is slightly lower (93%) than that reported previously for foxtail millet cv. Arjuna (Vishnoi and Kothari, 1996). The transformation test also showed that calli subcultured for a short period (25 days) were superior for transformation.

Agrobacterium-mediated genetic transformation has become the first choice for basic plant research and many agronomically and horticulturally important species are routinely transformed using this method (Stafford, 2000; Gelvin, 2003). Although *in vitro* culture techniques for foxtail millet were developed quite early, only four reports are available so far on transformation of foxtail millet mediated by *Agrobacterium* (Wang et al., 2003; Qin et al., 2008; Liu et al., 2005; 2007). Many factors influence transformation efficiency, such as *Agrobacterium* strains, vectors, explants, plant genotypes, inoculation and co-culture conditions and selective markers. The *Agrobacterium* strain is an important factor affecting the genetic transformation and the optimal strain varies depending on the plant species or cultivar. The results of this study indicate that LBA4404 is superior to EHA105 for transformation of calli derived from immature inflorescences of foxtail millet cv. Jigu 11. The medium and temperature for co-cultivation are also important factors for transformation efficiency. Several antioxidants, for example, DTT, improve T-DNA delivery in some plant species. For instance, low-salt media and addition of DTT along with L-cysteine to the co-cultivation medium improved transformation efficiency from 5.5 to 12% in maize Hi-II transformation (Vega et al., 2007). The inclusion of L-cysteine, DTT and sodium thiosulphate in the co-cultivation medium enhanced the transformation frequency by as much as 16.4% in soybean cotyledonary-node cells (Olhoft et al., 2003). The effect of temperature during co-cultivation on T-DNA delivery was first reported in dicot species (Dillen et al., 1997). In addition, co-cultivation at low temperature enhances the *Agrobacterium*-mediated transformation efficiency in *Phaseolus acutifolius* and *Nicotiana tabacum* (Dillen et al., 1997). Regarding foxtail millet transformation, co-cultivation was carried out at 22, 23 to 25 or 28°C in previous investigations (Wang et al., 2003; Liu et al., 2005; 2007). In this work, the effect of co-culture temperature on transformation efficiency was analyzed combined with or without DTT. The results show that 26°C is superior to 22°C without DTT, while the high GUS transient expression efficiency at 22°C is dramatically higher than at 26°C when coupled with DTT, but the mechanism remains to be explored. These results indicate that optimizing for only one parameter did not necessarily result in a high level of transformation overall, which is similar to a report on maize (Zhao et al., 2001). Therefore, balancing a set of factors was critical for developing a high-throughput transformation system.

To sum up, we built on our previous studies to optimize foxtail millet regeneration and transformation conditions. These include: (1) 0.5 to 1.0 cm long immature inflorescences cultured for 25 days which are optimal to induce embryogenic calli and transformation; (2) the *Agrobacterium* strain LBA4404 (OD_{600} between 0.8) is used and the inoculation time is 30 min; (3) co-cultivation was conducted at 22°C for 3 days with addition of 100 µM

AS and 0.15 g/l DTT to the co-cultivation medium. We successfully transformed the high-lysine protein-encoding gene (*SBgLR*) to foxtail millet using this optimized protocol with 5.5% transformation efficiency (number of transgenic lines per 100 regenerated plants). Western blot analysis revealed that the *SBgLR* gene was expressed successfully in the seeds of three out of the six transgenic lines.

There are some methods to define the transformation efficiency, for example, by the number of the independent transgenic plants per 100 embryos infected (Ishida et al., 1996) or the number of bialaphos-resistant events recovered per 100 embryos infected (Frame et al., 2002) in maize transformation, the mean number of kanamycin-resistant calli per cotyledon disk (Sunikumar and Rathore, 2001) or the proportion of kanamycin-resistant calli among the total number of calli (Wu et al., 2005) in cotton transformation, the number of PPT-resistant calli versus the number of initial explants in rice transformation (Enriquez-Obregon et al., 1999). Besides, the transformation efficiency is also evaluated by the number of transgenic plants per 100 produced antibiotic resistant plants in maize transformation (Ishida et al., 2004).

Here, the proportion of the transgenic lines among the plants produced was used as the transformation efficiency in order to compare with our earlier report (Liu et al., 2005), in which the same measure method was used. The transformation efficiency of 5.5% in this present work is a little lower than our previous study (6.6%) due to the reasons as follows. First, the vector pSB-SBgLR carries two separate T-DNAs. The target gene *SBgLR* and the selectable marker gene *Hpt* II were cotransformed into foxtail millet mediated by *Agrobacterium*. The T-DNAs introduced by this method behaved as independent Mendelian loci. Hence, some of the transformants carried the target gene and the selectable marker gene simultaneously, whereas others carried the selectable marker gene only or target gene only. Transformants with both genes are useful because transgenic plants free from selection marker can be obtained by segregation among the progeny (Komari et al., 1996). However, transformants with target gene only can not be obtained during transformation due to the absence of the selectable marker gene. If all the types of trans-formants are classified as transgenic lines, the transfor-mation frequency is much higher. Second, the materials used for stable transformation in this study were grown in the greenhouse due to the climate limitation in winter. The status of the calli induced from these materials is not as good as those derived from field-grown materials in summer. Field-grown materials in our previous work were used. According to Zhao et al. (2000), the source of the plant material has a large impact on transformation efficiency, with field-grown materials producing a significantly higher efficiency than greenhouse-grown materials in sorghum and maize transformation. Hence, the transformation efficiency was speculated which could be higher if field-grown materials were used as our earlier report.

Conclusions

In conclusion, efficient and reproducible foxtail millet regeneration and transformation procedures were established in this study. The protocol will enable the genetic improvement of foxtail millet with agronomically useful genes without a seasonal limitation. More importantly, transgenic foxtail millet plants free from selection markers can be obtained by this optimized *Agrobacterium*-mediated transformation procedure and segregation of the progeny.

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