

Factors influencing *Agrobacterium tumefaciens*-mediated genetic transformation of *Eleusine coracana* (L.) Gaertn

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Received: 28 May 2010 / Accepted: 1 September 2010 / Published online: 18 September 2010
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Abstract *Agrobacterium*-mediated transformation protocol has been developed for *Eleusine coracana* (var. PR-202) by varying several factors which influence T-DNA delivery. Green nodular regenerative calli with meristematic nodules of seed origin were used as the target tissue for *Agrobacterium tumefaciens*-mediated gene transfer. The highest frequency of transformation (44.4%) was observed when callus was infected, co-cultivated and incubated at 22°C. Incorporation of higher level of CuSO₄ in the regeneration medium had significantly positive effect on the recovery of transformed plants. PCR analysis of T₀ and T₁ generation plants with *nptII*-specific primers revealed the amplification of *nptII* gene. Southern blot analysis of six regenerated plants confirmed selectable marker gene integration in three plants. This is a first report on *Agrobacterium*-mediated genetic transformation of finger millet and will pave the way for further studies in this and other millet crops.

Keywords Finger millet · Plant regeneration · Transgenic plants · GUS · Ragi · *npt II*

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Abbreviations

AS	Acetosyringone
CaMV	Cauliflower mosaic virus
2,4-D	2,4-dichlorophenoxyacetic acid
GUS	β -glucuronidase
Kan	Kanamycin
LB	Luria-Bertani (medium)
<i>nptII</i>	Neomycin phosphotransferase
YEM	Yeast extract manitol (medium)

Introduction

Finger millet (*Eleusine coracana* (L.) Gaertn., fam. Gramineae) is cultivated as a staple food crop in parts of Africa and India. The nutritional quality of finger millet is superior to rice and is on a par with that of wheat (Latha et al. 2005). Finger millet has outstanding attributes to survive in poor soils under harsh and severe drought conditions. Its seed can be stored safely for several years without any insect damage. This crop is a traditional component of farmers' risk avoidance strategies in drought-prone areas (Latha et al. 2005).

An efficient regeneration system is a prerequisite for genetic transformation of crops. Immature embryos are the choicest starting material for cereal tissue culture and genetic transformation (Vasil 2007). However, availability of the immature embryos is restricted to the growing season. Therefore, mature embryos have also been used in cereals (Bi et al. 2007; Chen et al. 2006; He and Jia 2008). There are several reports of in vitro studies of finger millet (reviewed by Kothari et al. 2005; Ceasar and Ignacimuthu 2009), but there are no reports of *Agrobacterium*-mediated genetic transformation, although it has been transformed using particle gun (Latha et al. 2005). Gene transfer using a biolistic

method has also been reported in *Eleusine coracana* and *Echinochloa crusgalli* (Gupta et al. 2001), and in *Paspalum notatum* (Smith et al. 2002; Grando et al. 2002; Gondo et al. 2003, 2005; Altpeter and James 2005; James et al. 2008). *Agrobacterium tumefaciens*-mediated genetic transformation has been used as method of choice for several cereals (Smith and Hood 1995; Uze et al. 2000; Amoah et al. 2001; Haliloglu and Baenziger 2003; Wu et al. 2003; Cheng et al. 2004; Mitic et al. 2004). In *Eleusine*, *pin* gene was introduced via particle bombardment where fungicidal PIN protein was used to exert antimicrobial activity, analogous to that of Ceatropin A. Significant level of resistance was shown against leaf blast fungus (*Magnaporthe* spp.) in transgenic plants at the seedling stage (Latha et al. 2005). However, this millet crop still lacked any *Agrobacterium*-mediated protocol for gene transfer (Ceasar and Ignacimuthu 2009). Considering this, we standardized an *Agrobacterium*-mediated transformation protocol for *Eleusine coracana* by optimizing various physical and chemical parameters.

Materials and methods

Establishment of in vitro cultures

Seeds of *Eleusine coracana* PR-202 were procured from the University of Agricultural Science, GKVK campus, Bangalore. They were initially washed with 0.5% Tween-20 and left in 70% ethanol for 5 min. They were finally surface-sterilized with 2.5% sodium hypochlorite solution for 10 min followed by thorough washing with autoclaved distilled water. Seeds were placed on callus induction medium, i.e. MS medium (Murashige and Skoog's 1962) supplemented with 2.32 μM Kinetin and 9.04 μM 2,4-D. Green nodular callus was obtained after 6 weeks of culture and further maintained on modified maintenance medium containing 0.90 μM 2,4-D by repeated subculture in every 3 weeks (Kumar et al. 2001). It was decided to use nodular callus from 2nd and 3rd passage for transformation as it offers availability of experimental material throughout the year without somaclonal variation. MS medium was used throughout the study. The co-cultivated callus was transferred to control regeneration medium with normal levels of CuSO_4 (0.1 μM) and 2.88 μM GA_3 and modified regeneration medium having 10 times CuSO_4 (1 μM) (Kothari-Chajer et al. 2008).

Evaluation of factors influencing transformation

Agrobacterium strains and binary vector

The *Agrobacterium tumefaciens* strain LBA 4404 with plasmid pBI-121 and EHA-105 with plasmids pCNL-56

and p35S-GUS-INT possessing *nptII* gene as selectable marker conferring resistance against kanamycin under 35S CaMV promoter were used in the study to select the most suitable strain for transformation.

Preparation of explants for *Agrobacterium* infection

Small pieces (5–7 mm) of green nodular calli were pre-cultured on MS + 0.90 μM 2,4-D in Petri dish for 4–5 days.

Effect of bacterial density and infection time

Selected *Agrobacterium* strain EHA-105 possessing plasmid pCNL-56 was grown to different OD_{600} (0.5, 0.8, 1.0, 1.5 and 2.0). Pre-cultured calli were infected with *Agrobacterium* suspension of varying OD_{600} for 5, 25 and 45 min.

Effect of acetosyringone

Different concentrations of AS (100, 200, and 300 μM) were added to the infection medium. The infected calli were transferred on co-cultivation medium containing different concentrations of AS (0, 50, 100, and 150 μM).

Use of surfactants

Tween-20 or Pluronic acid F-68 (0.01, 0.05 and 0.1%) was added to the infection medium.

Effect of pH

Five different pH levels (5.0, 5.4, 5.6, 5.8 and 6.0) were tried to induce virulence in order to see the effect on the efficacy of transformation by the *Agrobacterium* strain. Infected calli were transferred on co-cultivation medium having the most recommended pH (5.8) for in vitro studies in plants.

Pre-treatment of target tissue with antinecrotic solution

Before *Agrobacterium* infection, the green calli pieces of 5–7 mm were soaked in liquid MS medium fortified with various concentrations of AgNO_3 (Ag) (5.88–29.41 μM), ascorbic acid (AA) (28.40–142.04 μM) and cysteine (Cys) (661.15–826.44 μM) for 1, 4 and 7 h. The effect of 27 combinations of these antinecrotic solutions was seen on the survival of callus after *Agrobacterium* infection.

Effect of purine and pyrimidine inhibitors

Callus pieces were soaked in purine and pyrimidine synthesis inhibitor azaserine and purine synthesis inhibitor

mizoribine (0.01, 0.03 and 0.05 mg/l) for 16 h before infection.

Effect of temperature

Green calli pieces were infected and incubated for 15 days in varying temperatures, i.e. 22, 24, 26 and 28°C, and then finally shifted to a standard in vitro incubation temperature (26 ± 1°C).

Determination of regeneration potential

The regeneration potential of callus after co-cultivation with *Agrobacterium* was evaluated in two different media. The media used in this study included control regeneration medium consisting of MS salts and vitamins supplemented with 2.88 μM GA₃ and modified regeneration medium consisting of MS salts and vitamins supplemented with 10 times CuSO₄. Each medium was supplemented with 30 g/l sucrose and 8 g/l agar, pH was adjusted to 5.8 before autoclaving at 121°C for 15 min. Antibiotics were filter-sterilized and added to media before use.

Genetic transformation, selection and regeneration of transformants

Agrobacterium cultures were initiated from glycerol stock and grown overnight in liquid Luria–Bertani (LB) medium (Maniatis et al. 1982)/yeast extract mannitol (YEM) medium (Vincent 1970) containing kanamycin (50 mg/l) and rifampicin (20 mg/l) at 28°C, on continuous shaking (200g) and were used at appropriate OD₆₀₀ (0.8). Bacterial cells were centrifuged at 4,000g for 5 min and resuspended in liquid MS to maintain desired OD (0.8). Calli pieces pre-cultured on 0.90 μM 2,4-D were added to the infection medium (liquid MS + 200 μM AS + factor modified) and incubated for 25 min on mild shaking (50g). Calli were transferred to co-cultivation medium at 26 ± 1°C in the dark for 3–4 days. Infected calli were washed with wash medium (liquid MS + 350 mg/l Cefotaxime) for 2–3 min and initially incubated on modified maintenance medium (MS + 0.90 μM 2,4-D + 10 times CuSO₄) without antibiotics for 10–15 days, and the final selection of resistant calli was made by exposing these to increasing concentration of kanamycin. It was shifted to modified maintenance medium with 10 mg/l of Kan in the 2nd round of selection where concentration of antibiotic was increased to 25 mg/l and final selection was made at 50 mg/l (each selection cycle consisted of 15 days). Green callus was transferred to control regeneration medium and modified regeneration medium. The total number of plantlets was counted after 8–10 weeks of culture. Cultures were

incubated at 26 ± 1°C under fluorescent light (75 μmol s⁻¹ m⁻²) and 16/8 h photoperiod. In vitro-regenerated putative transformants were successfully transferred in controlled field conditions and seeds collected at maturity.

Analysis of transgene integration

GUS assay

Agrobacterium-infected callus was tested for GUS expression after 7 days of co-cultivation (Jefferson 1987). Regenerated putative transformants and their parts like leaves and roots were checked for GUS expression by placing them in eppendorf tubes containing GUS-buffer (1 mM 5-Bromo, 4-Chloro, 3 Indolyl-D-glucuronide (X-Gluc), 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100). They were incubated overnight at 37°C, washed once with sterile distilled water and finally dipped in 70% ethanol overnight to extract any chlorophyll that may be present in the tissue. Explants were examined under a dissecting microscope and scored for blue coloration. Leaves, roots and entire shoots from control and co-cultivated callus were checked for GUS expression.

PCR analysis

DNA from putative transformants and control shoots of *Eleusine coracna* PR-202 was isolated from 100 mg of in vitro grown leaves using Qiagen mini DNA kit (Genetix). Desalted *nptII* gene specific primers of OD 2.0: forward (5'-CAA TCG GCT GCT CTG ATG CCG CGG-3') and reverse (5'-AGG CGA TAG AAG GCG ATG CGC TGC-3') were obtained from IDT, Coralville. Genomic DNA (approx. 40 ng) along with 1.5 mM MgCl₂ and 50 ng of *nptII* forward and reverse primers in 25 μl volume was subjected to PCR amplification (Thermocycler, Corbet). The PCR program had an initial denaturation for 3 min at 95°C, denaturation for 1 min at 94°C, annealing at 64°C for 30 s, primer extension at 72°C for 1 min for 30 cycles followed by a final extension of 7 min. The DNA of *nptII* positive plants was also checked for the presence of *Agrobacterium* contamination. The DNA was subjected with *vir C* gene specific forward (5'-ATC ATT TGT AGC GAC T-3') and reverse (5'-AGC TCA AAC CTG CTT C-3') primers under the following PCR conditions; denaturation at 94°C for 45 s, annealing at 50°C for 30 s, extension at 72°C for 45 s and final extension for 5 min. The amplified products were separated on 1.2% agarose (Himedia, India) gel electrophoresis and photographed using Gel Documentation System (Bio-Rad, Germany).

Southern blot hybridization

Approximately 8–10 μg genomic DNA of *nptII* positive T_0 transformants was digested with BamH1 at 37°C. It was electrophoresed on 0.8% agarose gel and blotted on immobilon-NY+membrane (Millipore, India; cat. no. 7104 633) as per Sambrook and Russel (2001). PCR amplified 700 bp *nptII* gene fragment (approx. $-2 \mu\text{g}$) labeled with digoxigenin dUTP and blotted membrane was hybridized using the standard protocol provided in DIG High prime DNA labeling and detection starter kit I (Roche; cat. no. 11 745 832 910) for color detection with NBT/BCIP.

Experimental design and statistical analysis

Each experiment was set in triplicate with at least 50 explants. The transformation frequency was calculated over the total number of plantlets showing *nptII* gene amplification out of the total number of plantlets evaluated for PCR analysis. Analysis of variance (ANOVA) was used to test the statistical significance of difference among

means was carried out using Duncan's (1995) multiple range test at a significance of $P = 0.05$.

Results

Agrobacterium strains and binary vector

In a preliminary experiment both super virulent *Agrobacterium* strains (LBA 4404 and EHA-105) with different vectors were grown to standard OD₆₀₀ 1.0 with 100 μM AS. Calli were infected for 25 min with the bacterial strains. The *Agrobacterium* strain EHA-105 possessing pCNL-56 was selected on the basis of maximum GUS response (59%) as well as maximum survival of the co-cultivated callus along with 3.5% regeneration response (Fig. 1a). The *Agrobacterium* strain EHA-105 possessing pCNL-56 was used to standardize essential basic conditions like bacterial density, concentration of AS during infection, co-cultivation, and infection time were optimized using varying ranges of basic factors.

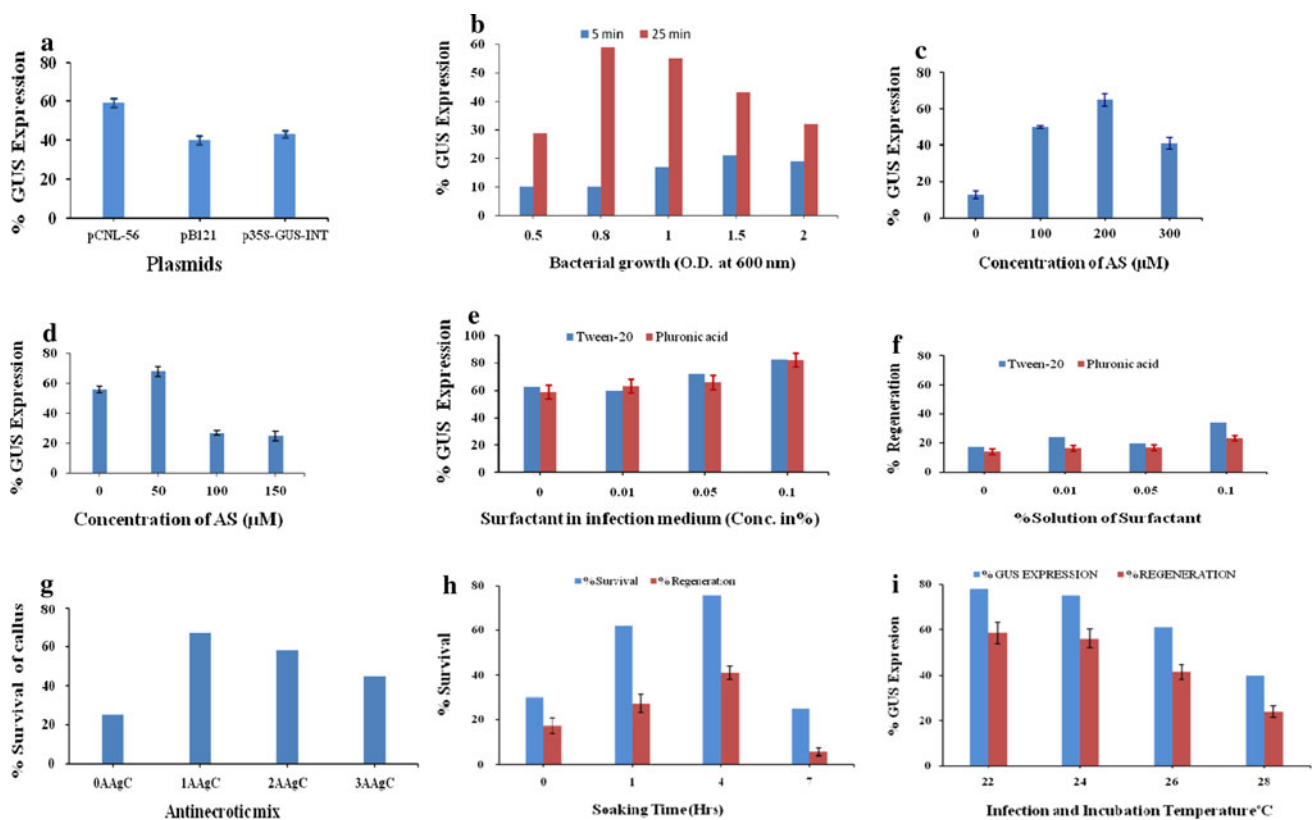


Fig. 1 a–i Factors influencing GUS expression and transgenic plant regeneration in *Eleusine*. **a** GUS expression using different plasmids, **b** effect of infection time and bacterial density on GUS expression, **c** effect of AS on GUS expression used in infection medium, **d** effect of AS on GUS expression used in co-cultivation medium, **e** effect of

surfactants on GUS expression, **f** effect of surfactants on transgenic plant regeneration, **g** effect of antinecrotic mixtures on callus survival, **h** effect of varying soaking time in antinecrotic mixture on transgenic plant regeneration, **i** effect of different temperatures on GUS expression and transgenic plant regeneration

Effect of pre-conditioning of explants for *Agrobacterium* infection

Incubation of explants on MS with 0.90 μM 2,4-D had a positive effect on subsequent transformation rates. Pre-conditioning of explants results in increasing morphogenic competency.

Effect of bacterial density and infection time

Bacterial cell density as measured by the optical density (OD) of bacterial suspension is directly related to their cell mass or cell number (Dutt and Grosser 2009). No significant GUS expression was noticed when infection was allowed for 5 min. Overgrowth of *Agrobacterium* was the real problem, when infection time increased to 45 min (data not shown). Out of these 15 combinations tried in *Eleusine coracana*, GUS expression was significantly high (58%) at 0.8 OD₆₀₀ when infected for 25 min (Fig. 1b). Therefore, optimized OD (0.8) was used with 25 min of infection time in further experiments.

Effect of acetosyringone

Maximum (65%) GUS expression was recorded in the presence of 200 μM AS (Fig. 1c) along with 7.1% frequency of transformation (Table 1). The presence of AS in co-cultivation medium kept GUS expression stable without affecting the growth of infected callus. An amount of 50 μM of AS in co-culture medium proved to be more effective for stimulating the virulence system of bacterium and hence generating maximum GUS response as well as GUS positive calli but did not affect the frequency of transformation. Presence of AS for more than 3 days caused browning of callus tissue. Omission of AS from infection and co-cultivation medium brings down the level of transient GUS expression (Fig. 1d). Presence of higher concentrations of AS (100, 150 μM) turned medium yellow and also affected the growth of calli adversely. Thus, 200 μM AS was used during infection medium in all experiments.

Effect of surfactants

High concentrations of Tween-20 (0.1%) expressed 82.7% of GUS response with assurance of recovery of putative plantlets (Fig. 1e) with 27.5% frequency of transformation (Table 1). The intensity of blue color and number of explants with blue color increased when higher concentrations of surfactants were used. Approximately 34% calli regenerated on modified regeneration medium along with

Table 1 Summary of transformation experiments with *Eleusine coracana* (finger millet)

Factors		No. of plantlets evaluated for <i>npII</i> gene amplification	No. of positive plantlets	Transformation frequency (%)
Bacterial density (OD ₆₀₀)	0.5	32	1	3.1 b
	0.8	28	2	7.0 a
	1	32	2	6.2 a
	1.5	15	0	0 c
	2	15	0	0 c
AS (μM) in infection medium	Control	0	0	0 c
	100	39	1	2.5 b
	200	42	4	7.1 a
	300	0	0	0 c
Tween-20 (%) in infection medium	Control	25	2	8 c
	0.01	35	6	17.1 b
	0.05	35	6	17.1 b
	0.1	40	11	27.5 a
Pluronic acid F-68 (%) in infection medium	Control	25	2	7.1 c
	0.01	18	2	11.1 b
	0.05	15	0	0 d
	0.1	18	3	16.6 a
Treatment with antinecrotic mix (1 h)	Control	28	2	7.1 d
	1AgAACys	35	7	20.0 a
	2AgAACys	24	3	12.5 b
	3AgAACys	25	2	8 c
Antinecrotic mix for varying time (h)	Control	25	2	8.0 c
	1AgAACys	45	6	13.3 b
	4	49	13	26.5 a
	7	12	0	0 d
Infection and incubation temperature ($^{\circ}\text{C}$)	22	72	32	44.4 a
	24	66	23	34.8 b
	26	25	2	8.8 c
	(Control)	28	15	0

Control-OD₆₀₀ = 0.8; Infection for 25 min at 26 $^{\circ}\text{C}$ + 200 μM (AS)
Means in a column followed by different letters are significantly different from each other at $P = 0.05$

1AgAACys (29.41 μM + 14.20 μM + 82.64 μM)

2AgAACys (147.05 μM + 5.68 μM + 330.57 μM)

3AgAACys (147.05 μM + 5.68 μM + 82.64 μM)

6.6 well-developed plantlets per explant. Although the GUS response with 0.1% pluronic acid (82.3%) was equal to 0.1% Tween-20, the number of plantlet induction and frequency of transformation were less. Regeneration response with pluronic acid also enhanced from 14.3 to 23.3% in comparison to control (Fig. 1f).

Effect of pH

pH 5.6 and 5.8 appeared to facilitate transformation, as 55 and 52% GUS expression was observed at these pH, respectively. Infected calli were transferred on co-cultivation medium having the most recommended pH (5.8) for in vitro studies in plants. Variation in pH did not suit the callus growth, and solidification of the medium was also a problem at modified pH values. The calli infected at lower pH did not show any encouraging response in terms of recovery of plantlets (data not shown).

Effect of pre-treatment of target tissue with Antinecrotic solution

Out of the 27 combinations tried, 3, i.e. (1) AgAA-Cys (29.41 μ M + 14.20 μ M + 82.64 μ M), (2) AgAACys (147.05 μ M + 5.68 μ M + 330.57 μ M), and (3) AgAACys (147.05 μ M + 5.68 μ M + 82.64 μ M) showed marked influence on the survival of calli after *Agrobacterium* infection. The maximum survival (67%) was noticed in combination (1) (Fig. 1g). GUS expression was almost similar (58–62%) in the best three treatments. The best combination (1) resulting in highest surviving calli was selected to observe further significant difference in response of calli soaked for 1, 4 and 7 h before infection. Survival of calli reached maximum (77.5%) in 4 h treatment and number of recovered putative transformants were 4 times better than control. Maximum plantlets (9.3) were obtained with 41% regeneration response and 26.5% frequency of transformation (Fig. 1h). This treatment helped in keeping infected calli green for longer duration and reduced the regeneration time by 4–5 days (Fig. 2a).

Effect of purine and pyrimidine inhibitors

Treatment with azaserine and mizoribine adversely affected the post-infection handling. Most parts of the treated callus turned brown. GUS expression could not be assessed accurately due to the browning. The explants did not survive more than 2 weeks.

Effect of temperature

Perceptible differences in callus growth and regeneration response were observed on varying temperature conditions. Calli which were infected and incubated at 22°C and 24°C showed prolific growth and there was a 6–7 times increase in the amount of callus compared with control (Fig. 1i, 2b). The regeneration potential of the callus also increased to 58.3% (Fig. 1i) and recovery of plantlets was also higher. Fifteen plantlets per callus were obtained with 86.3%

frequency of transformation at 22°C. The temperature increased beyond 24°C reduced the number of plantlets as well as frequency of transformation was also decreased and reached zero at 28°C.

Effect of medium on regeneration

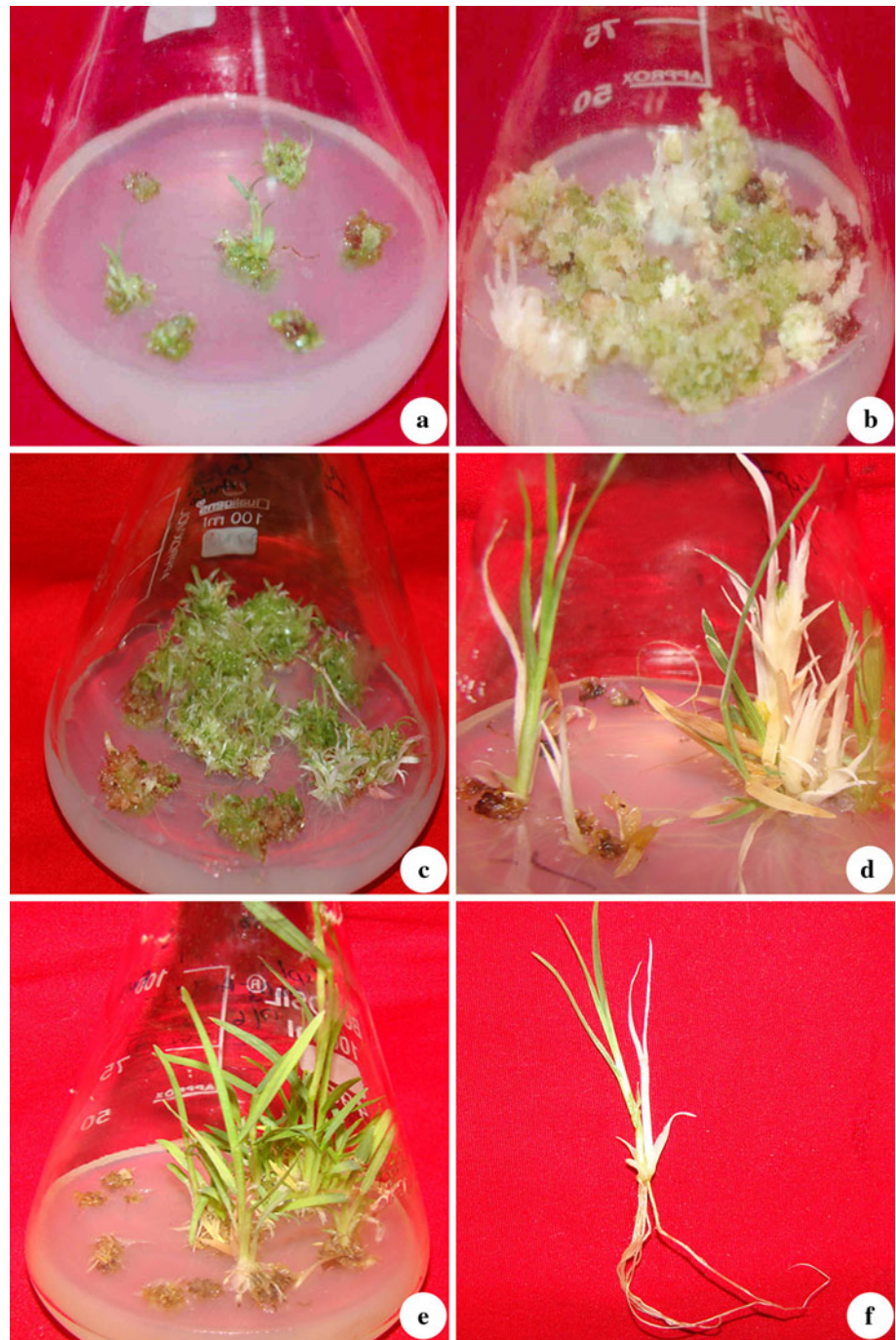
Control regeneration medium induced less regeneration response. The regeneration response was also delayed to 20–25 days with 1.2% regenerating calli while improvement in number of shoot bud induction as well as plantlet development was observed on modified regeneration medium which was clearly visible in all experiments. Both white and green (Fig. 2d) or only white or only green (Fig. 2e) well-developed plantlets were regenerated from the same callus, and their number continuously increased along with the growth of regenerable callus. The number of plantlets varied from 4 to 20 per callus piece along with increase in percentage of responding calli on modified regeneration medium after 8 weeks of culture in different experiments. Most plantlets were normal in appearance and morphology. Roots were small in length and brown in color and appeared weak, but this did not effect the successful establishment of in vitro plantlets in the field.

Molecular analysis

The control callus did not show any blue coloration (Fig. 3a) while calli pieces infected with *Agrobacterium* displayed varying GUS response (Fig. 3b). Blue coloration was observed in putative transformed T_0 plantlets (Fig. 3c) and parts of germinated seedlings of T_1 generation. DNA of T_0 putative transformed shoots from each treatment was subjected to standard conditions for PCR analysis with *nptII* gene-specific primers (Table 1). Putative transformants showed the expected 700-bp band size of the amplified product (Fig. 4a). The wild-type control plant did not show any amplification. Vector pCNL-56 was taken as positive control. The highest frequency of transformation (44.4%) was observed when callus was infected, co-cultivated and incubated at 22°C. The positive sample numbers were checked for *Agrobacterium* contamination and their DNA was amplified with *vir C* (729 bp) gene-specific primers. None of them showed amplification of *vir C* gene (Fig. 6). Seed samples were selected randomly from T_0 *nptII*-positive plants and germinated on MS medium supplemented with 50 mg/l of kanamycin. DNA isolated from seedlings of each T_1 generation was subjected to PCR amplification with *nptII* specific primers (Fig. 4b). DNA of *nptII* positive T_0 transformants were used for Southern blot analysis with PCR amplified *nptII* fragment as a probe. The results indicated three out of six selected plants have T-DNA integration in their genome and the transformants

Fig. 2 Callus proliferation and transgenic plant regeneration in *Eleusine* infected with EHA-105 possessing pCNL-56.

a Differentiation of shoot buds from callus treated with antinecrotic mix for 4 h and cultured on modified regeneration medium in 3 weeks, **b** Callus growth on modified maintenance medium in 5 weeks following *Agrobacterium* infection and incubation at 22°C, **c** shoot bud induction on modified regeneration medium in 3 weeks following infection with *Agrobacterium* and incubation at 22°C, **d,e** regeneration of green and albino plants on modified regeneration medium supplemented with kanamycin (50 mg/l) after 8 weeks of culture, **f** rooted putative transgenic plant

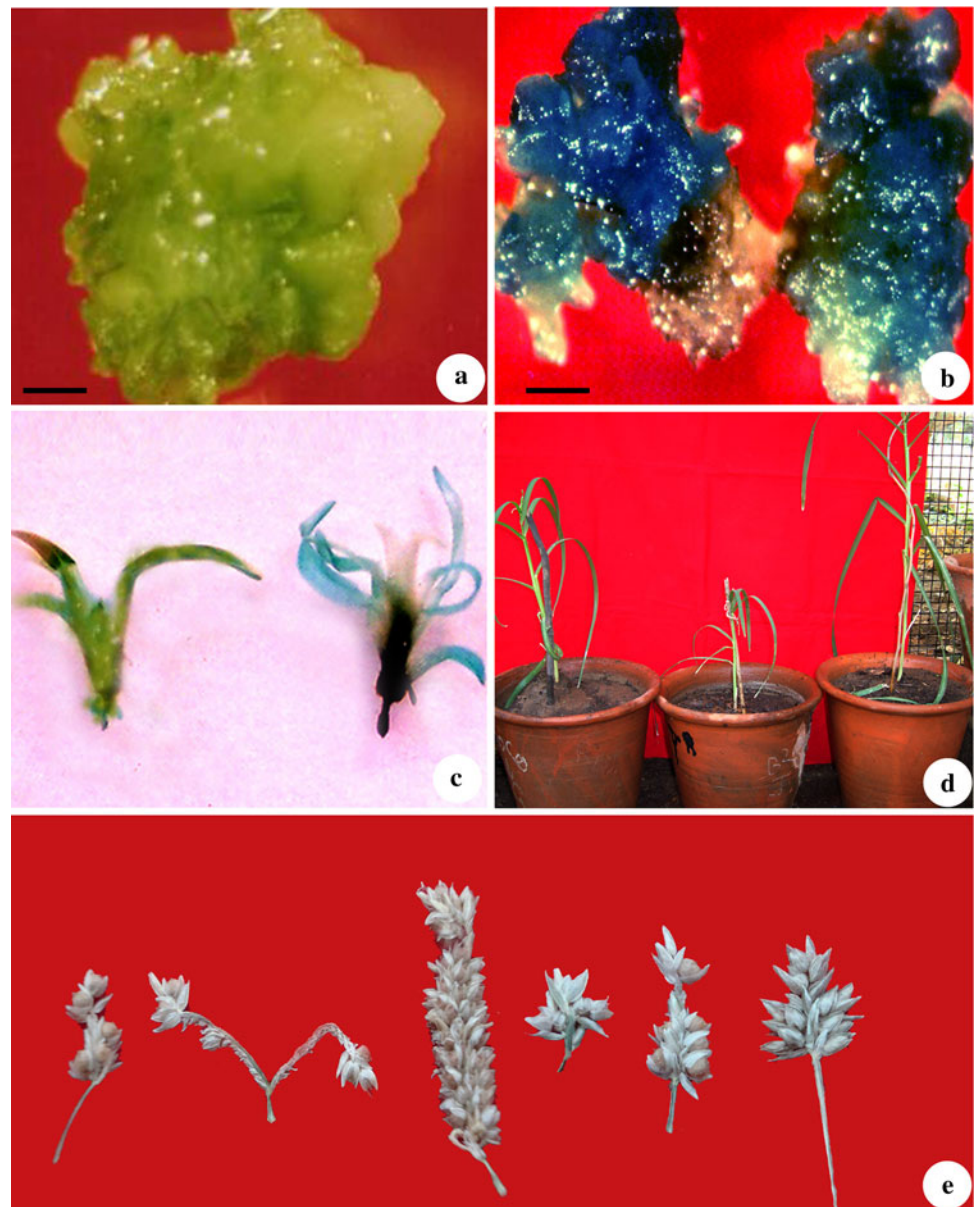


contain one insertion site of transgene (Fig. 5). No hybridization was detected in the non-transformed plants.

One hundred putative transformants were transferred in field conditions out of which 60 survived. Of these 31 plants showed slow growth, height of the plants was less and size of the inflorescence was also slightly smaller than normal plants (Fig. 3d). In the remaining 29 plants, variation in plant height was noticed from 12 to 15 cm.

Sometimes, the length of the plants did not go beyond 5–6 cm and bore inflorescences within 10–20 days of field transfer. Inflorescence appeared on many plants with 1, 2 and 3 fingers of which part of the finger or the entire finger was sterile (Fig. 3e). Seeds obtained from all the field-transferred plants were dark brown with a little red tint and the size of the seeds was equivalent to normal seeds.

Fig. 3 GUS Expression in *Eleusine* cultures following *Agrobacterium* infection. **a** Control uninfected callus (*bar* 1 mm), **b** GUS expression in infected callus (*bar* 1 mm), **c** GUS expression in T_0 plants (control plant on *left*), **d** field transferred T_0 putative transformants after 1 month, **f** inflorescences of T_0 plants at maturity



Discussion

The transformation can be effective only if we have a robust tissue culture protocol. In our study, callus derived from seeds proved to be the material of choice because of the high frequency of regeneration (Kumar et al. 2001). The transfer of T-DNA from bacteria to plant is a highly specific process and multiple factors are known to modify *Agrobacterium*-mediated T-DNA transfer and gene expression.

In various *Agrobacterium*-mediated transformation studies, vectors and *Agrobacterium* strains show variation

in T-DNA delivery. Available evidence suggests that T-DNA processing may be affected by the size and/or organization of the T-DNA region, leading to the production of intact double-stranded or single-stranded forms of T-DNA (Steck 1997), and this in turn has some effect on T-DNA mobility and delivery into plant tissue. What is exactly responsible for this variation is not clear but the results point towards the need to screen a number of vectors in order to obtain one that will be optimal for a given transformation system. In our experiment, vector pCNL-56 with genome size of 15.9 kb was well suited to the *Eleusine* cultures used for the first time for *Agrobacterium* infection.

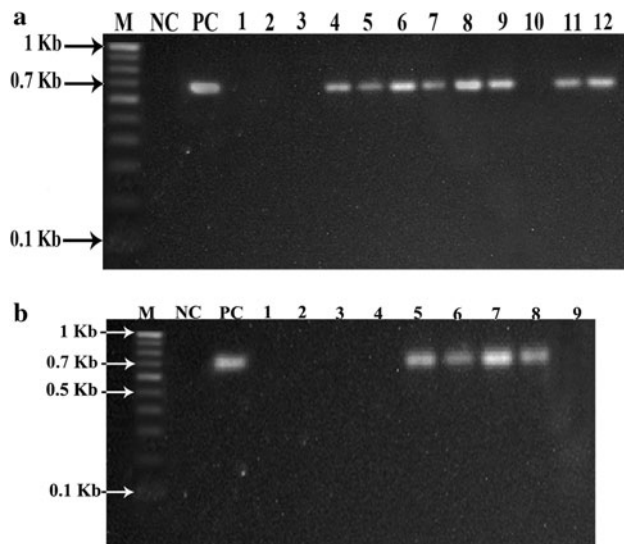


Fig. 4 PCR analysis of putative transformants. **a** Selectable marker gene (*nptII*) amplification in T_0 plants. *M* 100-bp ladder, *NC* negative control, *PC* positive control, lanes 1–12 DNA from putative transformants. **b** Selectable marker gene (*nptII*) amplification in T_1 generation plants. *M* 100-bp ladder, *NC* negative control, *PC* positive control, lanes 1–9 DNA from putative transformants

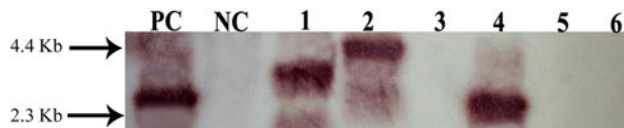


Fig. 5 Southern blot hybridization of putative transformants of *Eleusine*. *PC* Positive control, *NC* negative control (non transgenic), lanes 1–6 DNA from transformants



Fig. 6 DNA amplification for the presence of *vir C* gene in transformants. *M* 100-bp ladder, *PC* pCNL-56 plasmid DNA, lanes 1–12 DNA from different transformants

Selection of appropriate OD is an important factor of concern. A density of more than 1.0 was not manageable and overgrowth of *Agrobacterium* affected growth of calli adversely. Similarly, increase of infection time to more than 25 min caused browning of the target tissue and did not allow it to flourish. Our observations are in agreement with the previous studies (De Clercq et al. 2002; Amoah et al. 2001; Kumria et al. 2001). The recalcitrance of monocots to *Agrobacterium* transformation is due to a deficiency of phenolic compounds. Therefore, the range of acetosyringone concentration (0–300 μ M) was evaluated and maximum GUS expression was recorded at 200 μ M AS with 2.5% frequency of transformation. Our observations are in agreement with reports on wheat (Amoah et al.

2001), while in many other experiments variation in concentration of AS was confirmed as per the need of individual plant species (Hiei et al. 1994, 1997). It has clearly been indicated in different studies that low concentrations of AS (below 100 μ M) could not increase bacterial virulence, while high concentrations of AS as high as 500 μ M were found lethal to bacteria and explant growth (De Clercq et al. 2002).

In addition, an acidic pH is required for optimal expression of the virulence genes (Li et al. 2002). The Agropine strain in our experiments did not show any differential activity at various pH levels. The range of pH was varied from 5.0 to 6.0. Maximum GUS expression was shown at pH 5.6 (55%) but expression was not significantly different at 5.8 (52%). No encouraging results were obtained by varying pH in infection and co-cultivation medium. These results are in agreement with the work carried out in maize, where modification in sugar and pH was not needed (Rashid et al. 1996). Contrary to our results, maximum GUS expression was reported at pH 6.0 in wheat (Ali et al. 2007).

In another set of experiments, inclusion of surfactants such as Tween-20 and Pluronic acid F-68 in the infection medium had greatly affected T-DNA delivery. Addition of 0.1% Tween-20 to the infection medium enhanced the regeneration response to 34% with 27.5% frequency of transformation. The effect of silwett-77 has been evaluated on embryogenic callus of maize (Yang et al. 2006). Approximately 34.89% calli were GUS positive using 0.01% of silwett-77. Our results match with the observation made on wheat (Cheng et al. 1997; Wu et al. 2003) and maize (Yang et al. 2006), only the type of surfactant and its concentration were different. Surfactant enhances T-DNA delivery by aiding *A. tumefaciens* attachment or by elimination of certain substances that inhibit attachment. Other surfactants have also been shown to be beneficial during in plant transformation of *Arabidopsis thaliana* (Bechtold et al. 2000; Desfeux et al. 2000).

Treatment of explants in purine and pyrimidine inhibitor before infection was found to be favorable for development of transgenics in various monocots. Soaking of embryogenic callus for 16 h in 0.36 mM azaserine and 0.19 mM mizoribine showed good results when used for transformation studies in maize (Yang et al. 2006). In contrast, use of purine and pyrimidine inhibitor with *Eleusine* callus did not seem effective and calli turned completely brown within 2 weeks of culture.

The role of particular temperatures has been mentioned in delivery of T-DNA to plants irrespective of the type of helper plasmid used (Dillen et al. 1997). In monocots, the co-culture temperature for most of the crops ranged from 24 to 25°C and, in some cases, 28°C was used (Rashid et al. 1996; Arencibia et al. 1998; Hashizume et al. 1999).

In this experiment, a temperature of 22°C was observed to be optimum for T-DNA delivery as well as recovery of plantlets with 44.4% frequency of transformation. *Agrobacterium* infection and further incubation at 22°C for 15 days followed by final incubation at 26 ± 1°C had a profound effect on callus growth and recovery of putative transformants. The plantlets survived well in field conditions and reached maturity. Our study is in line with the results obtained by various workers (Kondo et al. 2000; Frame et al. 2002). Higher frequency of transformation was observed from maize immature embryo when co cultured at 20°C, followed by subculture at 28°C (Frame et al. 2002; Gordon-Kamm et al. 2002), while 23–25°C for wheat and 23°C in maize was found optimal for T-DNA delivery (Rout et al. 1996).

This is first time that we have used 10 times CuSO₄ compared with MS level in maintenance and regeneration medium for culturing *Eleusine* callus after *Agrobacterium* infection, where 5–6 times better growth of the callus was seen with good recovery of plantlets. Five times and 10 times CuSO₄ compared with MS level enhance regeneration response in *Paspalum* and *Eleusine*, respectively (Kothari-Chajer et al. 2008). In another experiment, three-fold concentrations of NH₄ NO₃ were found responsible for modifying the activity of host factors leading to a higher frequency of transgene integrations and possibly to the shift in the mechanism of transgene integrations (Boyko et al. 2009).

Necrosis and cell death of tissue after *Agrobacterium* infection were found to be major limiting factor as it severely reduces the transformation efficiency (Enriquez-Obregon et al. 1997; Dan 2008). Pre-treatment of callus with anti-necrotic mix considered to be imperative for reducing oxidative burst. Pretreatment of target tissue with mix of antioxidant compounds containing ascorbic acid (0.09 μM), cysteine (0.33 μM) and (0.01 μM) silver nitrate raised the frequency of transformation 10–35% in sugarcane (Enriquez-Obregon et al. 1998). A similar protocol was attempted in rice using seedling explants for improving transformation (Enriquez-Obregon et al. 1999). Use of antinecrotic compounds in various concentrations were found to be good for survival of the calli, which reached 77.5% in *Eleusine*. The regeneration potential of the calli rose from 16.5 to 41%, when treated with (1) AgAACys mix for 4 h. Inclusion of AgNO₃ in co-culture medium led to improved transient and stable transformation in maize (Armstrong and Rout 2001; Zhao et al. 2001).

In summary, we propose the following conditions: infection and incubation at 22°C, pretreatment of target tissue with antinecrotic mix, use of surfactant (Tween-20) during infection enhanced the survival of GUS positive calli. The presence of 10 times CuSO₄ compared with MS level in maintenance and regeneration medium was found

effective for enhancing the number of responding calli as well as for recovery of plantlets. This is the first time we have used modified medium during regeneration, but efforts are continuing to explore the role of CuSO₄ right from *Agrobacterium* infection to callus induction and regeneration. The proposed protocol is an important first step which offers the opportunity to transfer foreign genes for improved agronomic traits, such as fungal disease resistance in finger millet.

Acknowledgments We thank Department of Science Technology, New Delhi for providing postdoctoral fellowship to Dr. Manju Sharma, CSIR, New Delhi for providing Senior Research Fellowship to Aditi Kothari-Chajer and UGC, New Delhi for providing Senior Research Fellowship to Swati Jaggā-Chugh.

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