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Full Length Research Article

Factors Influencing *Agrobacterium*-Mediated Transformation of Shoot Apex-Derived Calli Explants of Sorghum

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Sorghum bicolor is an agronomical cereal crop providing food security to millions in tropics especially Africa, Asia and Central America. Generation of transgenic sorghum for insect resistance through *Agrobacterium* is a challenging task as there are several factors that determine the efficiency of successful transformation. The objective of the study was to evaluate the effect of critical factors such as the use of shoot apex explants for generating embryogenic calli as target tissue for transformation, the effect of combination of the transgene Bt cry2A with two different promoters –d35S and ST-LS1 respectively in pCAMBIA 1305.1 construct, the duration of co-cultivation and the concentration of acetosyringone during co-cultivation on transformation. The results showed that the transformation frequency was influenced by the above factors and the gus expression studies revealed that cry 2A gene driven by d35S promoter in pCAMBIA 1305.1, 3 days of co-cultivation of calli explants with *Agrobacterium* strain LBA4404 and a concentration of 200 µM acetosyringone in the co-cultivation medium was found to be optimal in increasing the transformation frequency.

Key words: *Agrobacterium*, acetosyringone, Co-cultivation, Cry 2A gene, Gus assay, *Sorghum bicolor*, Transformation.

INTRODUCTION

Sorghum bicolor is an economically important cereal crop noted for its ability to thrive in water scarcity areas particularly in arid and semi-arid tropics. Besides its use as food and forage, its potential application in the production of bioethanol has elevated its significance as a model crop for agronomical research. Sorghum crop improvement through *in vitro* regeneration system and genetic transformation is retarded by its recalcitrant nature that is due to the release of phenolics, lack of regeneration during long term *in vitro* cultures and the genotype-dependent response (Visarada and Sai, 2007). Due to these constraints, sorghum is categorized as one of the difficult plant species for manipulation in tissue culture and transformation (Zhu *et al*., 1998). Tissue culture protocols with improved *in vitro* regeneration potential are an essential prerequisite for successful genetic transformation. *In vitro* culturing of sorghum is greatly influenced by genotype, explant, media composition and plant growth regulators.

However, tissue culture and plant regeneration in sorghum have been considerably successful with certain explants such as mature and immature embryos, immature inflorescences and shoot tip explants (Amali *et al*., 2014). Transformation studies in sorghum have shown immature embryos, immature inflorescences and shoot meristem explants to be most amenable to gene transfer protocols (Casas *et al*., 1997; Girijashankar *et al*., 2005; Howe *et al*., 2006). Ever since the first successful *Agrobacterium*-mediated sorghum transformation reported by Zhao *et al*. (2000) with 2.1% transformation frequency, various modified transformation protocols have been continually studied in sorghum to enhance transformation efficiencies. *Agrobacterium*- mediated gene transfer offers many unique advantages in plant transformation such as precise transfer and integration of DNA sequences with defined ends, linked transfer of genes of interest along with the transformation marker, higher frequency of stable transformation with many single copy insertions, low incidence of transgene silencing and the ability to transfer long stretches of T-DNA (>150kb) (Veluthambi *et al*., 2003). The critical factors governing the successful transformation event include the right choice of *Agrobacterium* strain, size of the *Agrobacterium* inoculum, promoter/transgene combinations, concentration of acetosyringone in the co-cultivation medium, number of days of co-cultivation and source of the explants. An optimal combination of these factors would facilitate the development of a reliable and robust transformation technology that would surpass the genotype- dependent response in sorghum. Earlier, we have reported the *Agrobacterium*-mediated transformation of sorghum with Bt cry 1C gene to develop resistance to *Chilo partellus* (Ignacimuthu *et al*., 2014). The present study was carried out to investigate the effect of promoter/transgene combinations, duration of co-cultivation and the concentration of acetosyringone in the co-cultivation medium on transformation frequency through gus expression analysis.

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MATERIALS AND METHODS

Explant and Embryogenic callus induction

Sorghum seeds were obtained from International Crops Research Institute for the Semi-arid Tropics (ICRISAT), Andhra Pradesh, India. Seeds were washed in 100 ml of distilled water containing 50 µl of Tween -20 for 5-7 minutes. Then the seeds were surface sterilized using 70% ethanol for 1 minute and subsequently with 0.1% mercuric chloride for 5 minutes. Then they were rinsed vigorously in sterile distilled water several times to remove the surface sterilants. Then the seeds were dried in sterile filter papers and inoculated in Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium for 5 days under dark incubation. The shoot apex explants of 5-days old

were excised using a sterile scalpel and were cultured in callus induction medium (CIM) that contained MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L kinetin and 500 mg/L casein hydrolysate. The cultures were incubated for about 4 weeks at 25 ±2°C in dark for initiating the embryogenic calli that were to be used for transformation.

Agrobacterium and transformation vector

The Agrobacterium tumefaciens strain LBA4404 was used for this study. The binary vector pCAMBIA 1305.1, containing cry 2A gene driven by two different promoters -d35S promoter (source= CaMV) and ST-LS1 promoter (source= potato) respectively, in separate constructs were used for transformation. The medium used for introducing the transformation vector pCAMBIA 1305.1 with two different promoter-transgene combinations into *Agrobacterium* LBA4404 was AB medium (Chilton *et al*., 1974). The competent *Agrobacterium* cells for transformation were prepared by using 0.2M Tris HCl at pH 8.0 (Holsters *et al*., 1978) and the cry 2A gene constructs -d35S promoter driving cry2A/pCAMBIA 1305.1 (cry 2A) or ST-LS1 promoter driving cry2A/pCAMBIA 1305.1 (cry 2AST) were transformed into *Agrobacterium* using liquid nitrogen for freezing the mixture followed by heat shock at 37˚C for 5 minutes. The transformants were selected by using rifamycin (10 mg/L) and kanamycin (50 mg/L) antibiotics in the AB medium after incubation for 3-4 days at 28˚C in an orbital shaker with 180 rpm. The transformed positive colonies were confirmed by PCR using cry 2A gene specific-primers.

Agroinfection and Co-cultivation

Agrobacterium cultures harboring cry 2A in pCAMBIA 1305.1 were grown overnight in MS broth with respective antibiotics at 28˚C. The bacterial suspension (OD at A_{600} = 0.6-1.0) was concentrated by centrifugation at 3000 rpm at 20˚C for 10 min. The bacterial pellet was resuspended in 50 ml of fresh MS broth containing acetosyringone. For co-cultivation, the shoot apex –derived calli explants were incubated in an *Agrobacterium* suspension in the above medium for 30 min with occasional shaking. Following infection, the calli were blotted onto sterile Whatman No.1 filter papers to remove moisture and excess bacteria and then transferred to co-cultivation medium that contained acetosyringone incorporated in callus induction medium. The effect of duration of co-cultivation on transformation was studied by evaluating the co-cultivation period of 1-5 days using transient gus expression activity. Further, to determine the optimal concentration of acetosyringone for efficient transformation, acetosyringone was included in the co-cultivation medium at a concentration range of 100-500 µM and the transformation efficiency was studied using gus assay.

Histochemical assay for the GUS gene

Transient gus expression was used as a measure to study the frequency of transformation after co-cultivation. The expression of β-D-Glucuronidase (GUS) gene in co-cultivated calli was assayed with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as the substrate (Jefferson *et al*., 1987). The putative transgenic calli were incubated in sodium phosphate buffer (50 mM $NaPO_4$, pH 6.8) that contained 1% Triton -X- 100 at 37°C for 1 h and were later incubated overnight in a solution containing 1.0 mM X-Gluc, 10 mM ETDA, 100 Mm NaH_2PO_4, 0.1x Triton-X-100 and 50% methanol (pH 5.8). They were then washed twice in 99% methanol for two hours to remove chlorophyll pigment. The number of blue spots obtained was counted to be gus positive.

RESULTS AND DISCUSSION

The frequency of embryogenic calli and somatic embryo formation was higher with 95.5% as reported in our previous study (Amali *et al*., 2014), when shoot apex explants were cultured on callus induction medium for about 7 weeks in dark with subculture at regular intervals. Explants derived from meristematic tissues such as shoot apices at the early developmental stage respond better in tissue culture conditions and are most desirable for genetic transformation to reduce somaclonal variations (Girijashankar and Swathisree, 2009). The transformed colonies of *Agrobacterium tumefaciens* harboring pCAMBIA 1305.1 with cry 2A gene under two different promoter constructs respectively were selected using rifamycin (10 mg/L) and kanamycin (50 mg/L) antibiotics in the AB medium and the total DNA extracted by using the method of Chen and Kuo (1993) was subjected to PCR analysis and the results confirmed the presence of the transgene cry 2A in *Agrobacterium tumefaciens* (Fig. 1a & b). Selection of a suitable promoter that could drive transgene expression to detectable and desired levels is an essential factor for genetic transformation. Among the two cry 2A gene constructs driven by promoters d35S and ST-LS1 in pCAMBIA 1305.1 respectively, the transformation frequency was higher with d35S promoter/cry 2A gene since more number of transformed colonies were obtained (Fig. 1a). Consequently, the bacterial colonies harboring cry 2A gene driven by d35S promoter in pCAMBIA 1305.1 were used for agroinfection and sorghum transformation. Promoters 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 (Able *et al*., 2001; Tadesse *et al*., 2003; Kumar *et al*., 2011).

Transient gus expression

The most widely used reporter system in sorghum transformation is the histochemical gus expression studies, starting from initial transformation attempts (Hagio *et al*., 1991) till the latest reports of Wang *et al*. (2007). X-gluc (5-bromo-4-chloro-3-indolyl-β-glucouronic acid) is a very effective chromogenic substrate for histochemical localization of β-glucuronidase activity. The interruption of gus reporter gene by a plant intron (catalase intron) prevents the expression of the reporter gene at bacterial level and allows the expression of this gene only in plant cells. The gus gene expression in calli was calculated based on the number of blue loci per callus explants after staining with X-gluc following co-cultivation (Fig. 2). The optimal parameters influencing transformation were selected and used in stable transformation experiments based on their highest percentage of expression observed in transient gus assays.

Effect of duration of co-cultivation

The co-cultivation period varied from 1 to 5 days and the gus gene expression varied greatly with respect to the co-cultivation period. It was observed that the gus expression was maximum with 3 days of co-cultivation and subsequently, it decreased with the increase in the number of days of co-cultivation (Table. 1). This was largely due to the overgrowth of bacteria that led to necrosis of embryogenic calli explants as a result of prolonged co-cultivation period. Earlier reports have also shown, a period of 3 days to be optimum for co-cultivation in most of the cereal transformations involving variable explants such as immature embryo, embryogenic calli and shoot apical meristems (Nguyen *et al*., 2007; Nimnara *et al*., 2007; Indra *et al*., 2010).

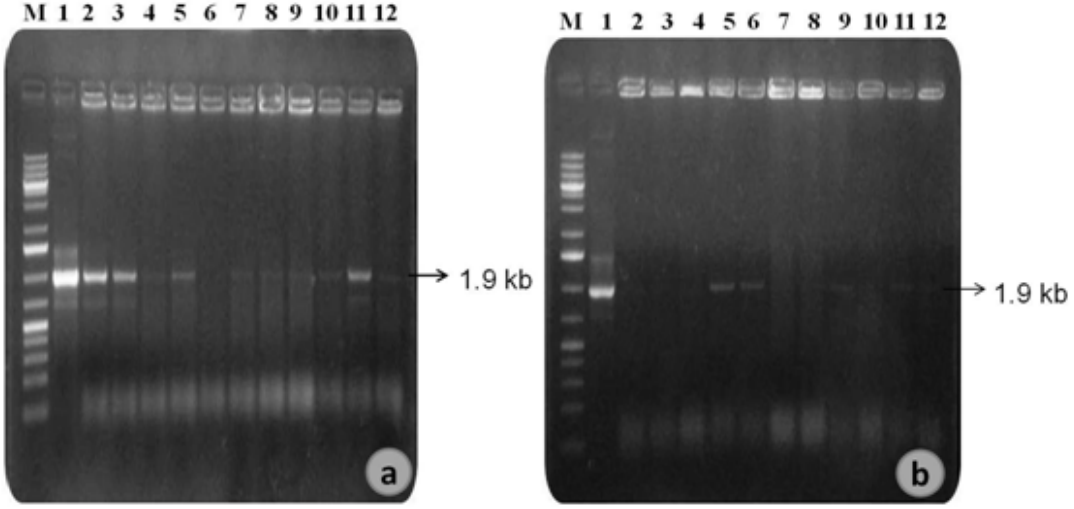

Fig. 1. PCR analysis for transformation of *Agrobacterium tumefaciens* LBA4404 with cry 2A gene in pCAMBIA 1305.1 vector
a) pCAMBIA1305.1 /cry 2A and b) pCAMBIA1305.1 /cry 2AST.
Lane M- Marker DNA, Lane 1- Plasmid DNA as positive control and Lane 2-12- DNA isolated from *Agrobacterium* colonies after transformation.

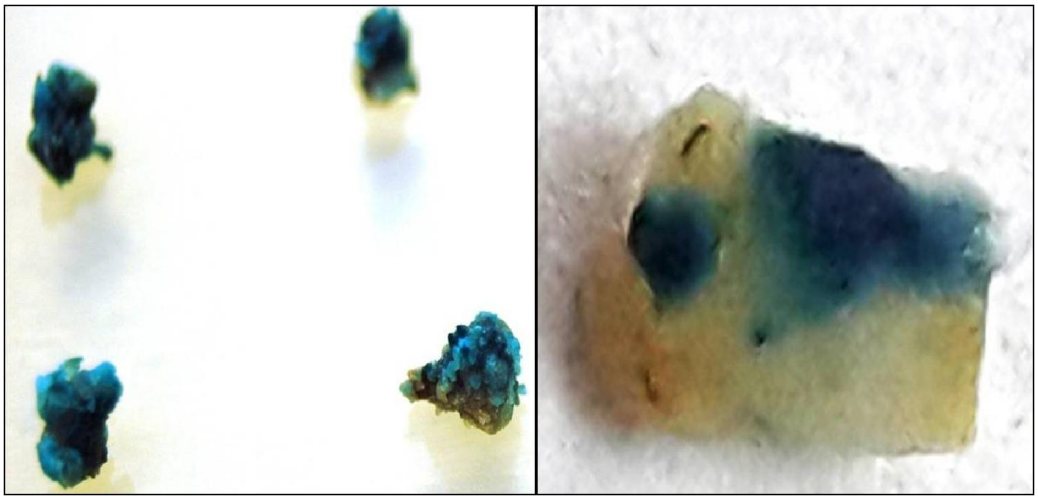

Fig. 2. The transient gus gene expression in sorghum calli after co-cultivation with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1305.1/cry 2A)

Table 1. Effect of duration of co-cultivation on transformation of sorghum using *Agrobacterium* strain LBA4404

Duration of co-cultivation period (days)	Number of explants under co-cultivation	Gus expression (%)	Mean number of GUS$^+$
1	20	0	0.0d
2	44	12.7	12.3±1.2b
3	50	28.6	27.2±0.8a
4	30	9.2	8.7±1.6c
5	28	0	0.0d

For each treatment three replicates were maintained. Values are expressed as (mean ± SD) based on ANOVA and compared using Fisher's LSD at *P*= 0.05%.

Effect of acetosyringone concentration

In our study, acetosyringone at a concentration of 200 µM was found to be optimal, as evidenced by the maximum number of gus positive loci (Table. 2). The inclusion of acetosyringone in the co-cultivation medium is crucial for efficient genetic transformation in monocots as it activates the *vir* genes in the T_i plasmid for the successful transfer of T-DNA from *Agrobacterium* to the plant genome (Arunkumar *et al*., 2010). However, the concentration of acetosyringone in the co-cultivation medium determines the relative extent of agroinfection. Similarly, a maximum number of 30 blue spots/ callus were reported when 200 µM acetosyringone was included in I_6 medium for sorghum transformation of rice chitinase gene using *Agrobacterium* strain EHA 105 (Indra *et al*., 2010).

Table 2. Effect of Acetosyringone concentration on transformation of sorghum using *Agrobacterium* strain LBA4404

Acetosyringone concentration (µM)	Number of explants under co-cultivation	Gus expression (%)	Mean number of GUS$^+$
0	35	0	0..0e
100	46	17.1	16.8±0.2b
200	55	24.8	25.3±1.6a
300	28	9.0	9.8±0.9c
400	39	1.6	1.2±0.6d
500	32	0	0.0e

For each treatment three replicates were maintained. Values are expressed as (mean ± SD) based on ANOVA and compared using Fisher's LSD at *P*= 0.05%.

Conclusion

Crop improvement technologies through genetic modification could be applied to overcome the biotic and abiotic stresses and this can greatly enhance the agricultural productivity along with the introduction of economically beneficial traits in sorghum. With the application of improved Agrobacterium-mediated transformation and

regeneration protocols, development of transgenic sorghum crops engineered for various biotic and abiotic stress tolerances and for quality enhancement can be routinized with higher adaptability, feasibility and recovery of large number of transgenic sorghum lines.

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