Agrobacterium-mediated transformation studies in sorghum using an improved *gfp* reporter gene[1]

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Abstract

Agrobacterium-mediated transformation of M 35-1, a *rabi* (postrainy season) sorghum cultivar, was envisaged with pKU352NA vector containing *SgfpS65T*, an improved *gfp* reporter gene and i*Ac* encoding transposase. Among various explants tried, immature inflorescence gave the highest callus induction (81.9%). pKU352NA-carrying *Agrobacterium* was induced by acetosyringone (200 µM) and used for Agroinfection followed by three-day co-cultivation of 15-day-old calli. Co-cultivated calli were washed with cefotaxime (200 mg L^{-1}) for 8 min and transferred to the regeneration medium with hygromycin (20 mg L^{-1}). In total, 25 putative transgenic plants were obtained, of which nine survived later. When eight plants were tested with i*Ac*-specific PCR, six yielded an expected 541bp amplicon indicating a transformation efficiency of 4.28%. *SgfpS65T* expression was evident in the leaves of all six i*Ac*-specific PCR positive plants.

Introduction

Sorghum (*Sorghum bicolor*) belongs to the family Gramineae and is the fifth most important cereal crop after wheat (*Triticum aestivum*), maize (*Zea mays*), rice (*Oryza sativa*) and barley (*Hordeum vulgare*). It is a dietary staple for over 500 million people in more than 30 countries. In India, sorghum is grown on 9.2 million ha (21% world sorghum area) and is the main source of calories among the resource-poor rural people. Sorghum is predominantly a self-pollinating crop with a chromosome number of 2n=20 and a total genome of 735Mb. Its value as a dietary staple for millions, adaptation to extreme environments and its placement within the grass family make it a valuable target for genome sequencing (Bedell et al. 2005). Large-scale shotgun sequencing of sorghum was initiated at the end of 2005 and completed on 25 January 2007 (http://www.phytozome.net/sorghum#A) (Paterson et al. 2009).

Gene discovery and function identification for the predicted genes using functional genomics provides the genomic resources for crop improvement. Though novel approaches such as site directed mutagenesis (Terada et al. 2002), specific gene expression knockouts by RNA interference or virus-induced gene silencing (Lu et al. 2003) and TILLING (Bhat et al. 2007) are used for determining gene function, the most direct way is to produce mutants by random insertion mutagenesis using a two component (i*Ac*/*Ds*) system (Hirochika et al. 2004). As a first step in this approach, an effort was made in this study to establish a protocol for *Agrobacterium*-mediated genetic transformation of sorghum using an i*Ac* vector which contained *SgfpS65T*, an improved *gfp* as the reporter gene.

Materials and methods

Agrobacterium strain LBA4404 harboring the binary vector pKU352NA [National Center for Biotechnology Information (NCBI), GenBank accession no. DQ225751] containing *SgfpS65T* reporter gene in fusion with ubiquitin promoter and *nos* terminator was provided by Dr NM Upadhyaya, CSIRO Plant Industry, Canberra, Australia. This vector carried Ω-i*Ac* gene encoding transposase, and *hpt* as the selectable marker (Upadhyaya et al. 2006).

Fresh seeds of M 35-1, a *rabi* (postrainy season) sorghum cultivar were obtained from Sorghum Scheme, All India Co-ordinated Research Project on Sorghum, University of Agricultural Sciences (UAS), Dharwad, Karnataka, India. Four different explants, viz, mature embryo-, young seedling-, in vitro grown leaf base- and

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immature inflorescence-derived calli were used for co-cultivation. Mature dry seeds soaked in distilled water for 32 h were surface sterilized with 70% alcohol for 1 min and finally in 0.05% mercuric chloride for 10 min followed by 3–4 rinses with sterile distilled water under aseptic conditions. The embryos from such seeds were excised for inoculation. The shoots of 6- to 7-day-old in vitro grown seedlings were excised, and were cut into 1 cm long pieces for inoculation. Leaf bases were collected from 12- to 15-day-old in vitro grown seedlings when they attained 3–5 leaf stage. The 3rd and 4th leaf bases were excised and cut into segments of 2–3 mm. Young inflorescences were collected from field-grown plants. After removing all the leaf sheaths except 2–3 inner ones, the enclosed inflorescences were surface sterilized by dipping them in absolute alcohol for 1 min. After air drying, the inflorescence was cut open aseptically, and chopped into 2–5 mm pieces. All these explants were inoculated onto MS medium (Murashige and Skoog 1962) supplemented with 2 mg L^{-1} 2,4-D. Calli obtained from different explants were subcultured on MS medium supplemented with 2 mg L^{-1} 2,4-D.

Transformation studies were done using immature inflorescence-derived calli. Embryogenic calli taken at 15 and 30 days after first subculture were immersed in suspension of *Agrobacterium* induced with 200 µM acetosyringone for Agroinfection (20 min) with gentle shaking in dark. Further, the calli were taken out of bacterial suspension (and the excess bacteria were dry blotted) and placed on the basal MS medium supplemented with 10 µM acetosyringone for co-cultivation (1, 2, 3 and 4 days). After co-cultivation, calli were washed (8, 10 and 15 min) with sterile distilled water and cefotaxime, blotted dry with sterile blotting paper and transferred to regeneration medium with hygromycin (0, 15, 20, 30 and 40 mg L^{-1}) as the selection agent. Subculturing was done after every 20 days on the same medium. These cultures were incubated in light (about 1000 lux) under a 10-h photoperiod at 25±2°C until green shoots appeared. Such shoots were aseptically shifted to rooting medium without hygromycin. Rooted plants were hardened and transferred to small cups containing sterile peat and sand in the ratio of 4:3.

DNA isolated from putative transgenic lines and non-transgenic (control) plants by following the method of Draper and Scott (1988) were tested for i*Ac*-specific PCR (polymerase chain reaction). Expression of *SgfpS65T* was checked in calli (after 5 and 12 days of co-cultivation) and the leaves of PCR positive transgenic plants by observing under stereomicroscope (Olympus, SZX-16) at National Centre for Biological Science (NCBS), GKVK campus, Bangalore, India. Images of *SgfpS65T* expressing tissues were captured.

Results and discussion

Maximum callus induction frequency was obtained with immature inflorescence (81.9%) followed by seedling tissue (61.0%) (Table 1). Mature embryo and leaf bases resulted in limited callus induction. Cereals, in general require 2,4-D for callus initiation (Mott and Cure 1978), and 2 mg L^{-1} has been found optimum (Bhat and Kuruvinashetti 1994). The callus produced from inflorescence was greenish, hard, compact and organized. Though immature inflorescence is most suitable to initiate cultures, the problem in procuring sufficient inflorescence in seasons other than *rabi*, demanded other explants to be tested.

Though callus induction from in vitro grown seedling was high (61.0%), it was unorganized, and could not be maintained further. Similarly, shoot explants produced embryogenic callus which was white and hard but regeneration was low. Similar observations have been made by Sai Kishore et al. (2006). The largely unorganized calli obtained from mature embryos produced only roots subsequently, similar to the observations of Vasil and Vasil (1984). Similarly, in vitro grown leaf bases were not suitable to initiate callus cultures, possibly due to the detrimental effect of phenolics (Mishra and Khurana 2003).

Maintenance of callus cultures for prolonged duration in a regenerable state is one of the most important requirements for using in vitro cultures for plant improvement. Generally, calli were maintained satisfactorily on MS medium with 2 mg L^{-1} of 2,4-D for 30–45 days, without appreciable changes in morphology. However, calli

Table 1. Callus induction response from different explants of sorghum cultivar M 35-1.

Explant	No. of explants inoculated	No. of explants producing callus	Response (%)
Mature embryo	200	47	23.5
Young seedling	200	122	61.0
In vitro grown leaf base	150	25	16.7
Immature inflorescence	320	262	81.9

derived from immature inflorescence produced fast growing, nodular, white, hard and embryogenic calli. Appearance of such embryogenic callus during subculturing might be due to the carryover of small undetected groups of embryogenic cells (Maddock 1985, Jabri et al. 1989). Considering the frequency and type of callus produced, immature inflorescence was used as the explant for generating callus for transformation.

The transfer of T-DNA and its integration into the plant genome is influenced by several *Agrobacterium*- and plant tissue-specific factors (Klee 2000). Young regenerable calli might offer the advantage of possessing actively dividing cells that are competent for Agroinfection, and target cells which are capable of regenerating into whole plant (totipotent). In the present study, 40% and 10% putative transformants were obtained with 15- and 30-day-old calli (after first subculture), respectively (Table 2). Though longer duration of co-cultivation is likely to be effective (Cervera et al. 1998), it is difficult to check the growth of *Agrobacterium*, which reduces regeneration frequency (Cervera et al. 1998). In the present study, calli co-cultivated for three days gave the highest putative transformants (30%) and none from calli co-cultivated for four days (Table 3), possibly due to over-infection and overgrowth of *Agrobacterium*.

Duration and number of washings given for the co-cultivated calli to get rid of *Agrobacterium* is a rate deciding step. Washing for eight minutes was found optimum to get maximum putative transformants (30%) (Table 4). Longer duration of washing resulted in browning of calli, which failed to regenerate. This could be due to excessive injury and loss of outermost (peripheral) cells that have greater chance to be transformed because of direct access to *Agrobacterium*. For the selection of transformants, hygromycin at 20 mg L^{-1} was considered optimum, because largely the transformed calli got selected and most of the shoots were green and normal (Table 5), except a few albino shoots.

Table 2. Influence of age of callus on recovery of putative transformants.

Age of callus (days after the first subculture)	No. of calli inoculated	No. of putative transformants	Putative transformants (%)
15	20	8	40
30	20	2	10

Table 3. Effect of duration of co-cultivation on recovery of putative transformants.

Duration of co-cultivation (days)	No. of calli inoculated on media containing hygromycin	No. of putative transformants	Putative transformants (%)
1	20	0	0
2	20	0	0
3	20	6	30
4	20	0	0

Table 4. Effect of duration of washing the callus on recovery of putative transformants.

Duration (min)	No. of calli tested	Re-growth of *Agrobacterium*	No. of putative transformants	Putative transformants (%)
15	20	No	0	0
10	20	No	2	10
8	20	No	6	30
5	20	Yes	0	0

Table 5. Sensitivity of calli to different levels of hygromycin during shoot regeneration.

Hygromycin concentration (mg L^{-1})	No. of calli inoculated	No. of putative transformants	Putative transformants (%)
0	20	11	55.0
15	20	8	53.3
20	20	6	30.0
30	20	0	0
40	20	0	0

In total, 25 plants regenerated on MS medium with 0.5 mg L^{-1} BAP in the presence of hygromycin selection (Fig. 1). Only 9 out of 25 plants could survive and establish in pots. And six of them yielded the expected 541bp amplicon (Fig. 2) with i*Ac*-specific PCR. Leaf samples from these i*Ac*-specific PCR positive plants showed strong expression of *SgfpS65T*, a visible reporter (Fig. 3), indicating a transformation efficiency of 4.28% (6 plants out of 140 explants co-cultivated), which is higher than success rates reported so far (Gao et al. 2005). These transgenic plants with i*Ac* are now being tested for copy number of T-DNA insertions before they are used for crossing with T-DNA/*Ds* lines for sorghum functional genomics.

Figure 1. Putative sorghum transformants in (left) test tube and (right) cup.

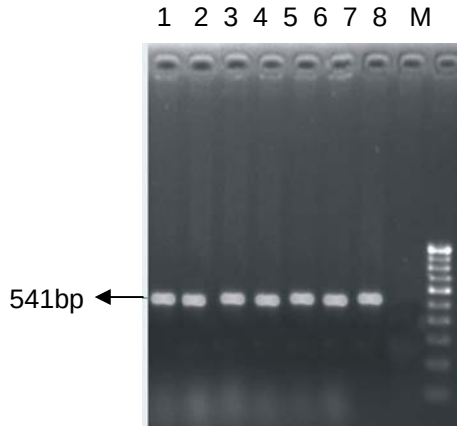

Figure 2. i*Ac*-specific PCR for putative transformants (Lanes 1–6: Sorghum transgenic plants; lane 7: positive control; lane 8: negative control; M: 100bp ladder DNA marker).

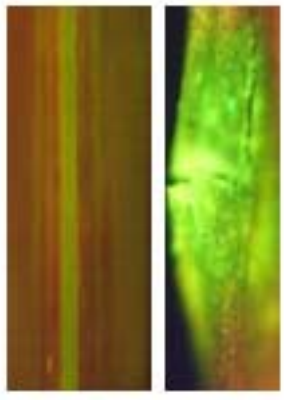

Figure 3. *SgfpS65T* expression in sorghum: (left) wild type and (right) transgenic plant.

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