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Infection of Early and Young Callus Tissues of Indica Rice BPT 5204 Enhances Regeneration and Transformation Efficiency

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Abstract: A rapid and reproducible method to develop transgenic plants with enhanced transformation efficiency using *Agrobacterium* has been developed for the elite indica rice variety BPT 5204. Different rice calli aged from 3 to 30 d were co-cultivated with pre-incubated *Agrobacterium* suspension culture (LBA4404: pSB1, pCAMBIA1301) and incubated in dark for 3 d. Based on the transient *GUS* gene expression analysis, 6-day-old young calli showed high transformation frequency followed by 21-day-old ones. Thus, both 6- and 21-day-old calli were used for assessing the stable transformation efficiency. It was observed that the 6-day-old young transformed calli showed about 2-fold higher regeneration frequency when compared with 21-day-old calli. The transformation efficiency was enhanced for young calli to 5.9% compared with 0.8% of the 21-day-old calli. Molecular and genetic analysis of transgenic plants (T_0) revealed the presence of 1–2 copies of T-DNA integration in transformants and it follows Mendalian ratio in T_1 transgenic plants. From the present study, it was concluded that the development of transgenic rice plants in less duration with high regeneration and transformation efficiency was achieved in BPT 5204 by using 6-day-old young calli as explants.
Key words: *Agrobacterium*-mediated transformation method; rice callus; transformation; transgenic plant; indica rice

Rice (*Oryza sativa* L.) is one of the major food crops in the world, which is amenable to genetic transformation and has emerged as the model cereal for the study of genome organization, gene expression and function as well as the behavior of transgenes (Christou, 1994; Tyagi et al, 1999, 2004; Giri and Laxmi, 2000; Datta et al, 2002; Bajaj and Mohanty, 2005). Engineering of plants for traits like yield or resistance to various stress (biotic and abiotic) requires a thorough understanding of cellular and functional aspects of the host plants (Tyagi et al, 2004). Different transformation methods are available for introduction of novel genes into host genome. Among these methods, *Agrobacterium*-mediated transformation method is the most preferred one due to its precise transfer, low copy number and single integration of foreign DNA (Koncz et al, 1994; Hansen et al, 1997; Nagadhara et al, 2003).

Remarkable progress had been made in the development of efficient systems for *A. tumefaciens*-mediated transformation in rice. Raineri et al (1990) attempted to raise and regenerate transgenic calli after *Agrobacterium*-mediated transformation. Later, Chan et al (1992) showed regeneration of *Agrobacterium*-transformed calli from root explants and immature embryos. Several factors were found to have impact on the efficiency of *A. tumefaciens*-mediated transformation, including Ti plasmid type (Hiei et al, 1994; Cheng et al, 1998), bacterial strains with broad host range (Aldemita and Hodges, 1996; Dong et al, 1996; Hiei et al, 1997), culture conditions prior to and during inoculation (Aldemita and Hodges, 1996; Mohanty et al, 1999) and activation of T-DNA transfer process by exogenously added acetosyringone (Aldemita and Hodges, 1996; Rashid et al, 1996; Khanna and Raina, 1999). Hiei et al (1994) used mature embryos of rice as explants and successfully developed transgenic plants. Besides, other explants such as inflorescence (Dong et al, 2001) and green tissues induced from mature embryos (Cho et al, 2004) were also used as source materials for transformation. Among various explants used, scutellum-derived calli were the material of choice for efficient transformation in rice (Hiei et al, 1994; Kant et al, 2001). Mostly callus based transformation and selection has been the routine process which takes normally 3–4 months to get transgenic plants. A successful rice

transformation was reported with 5-day-old embryogenic calli from japonica rice and obtained transgenic plants within a month (Toki et al, 2006). Recently, transgenic plants were developed from leaf base derived calli in indica cultivar ADT43 with improved transformation efficiency (Karthikeyan et al, 2011a). Strong influence on genotypic background in indica rice was reported for lower regeneration and transformation frequency (Aldemita and Hodges, 1996; Mohanty et al, 1999), but the same was not apparent for japonica cultivars (Hiei et al, 1994). However, in indica variety, prolonged tissue culture results in browning of callus, which could be one of the reasons for low regeneration frequency (Zhao et al, 2009).

Many factors influenced the transformation frequency in indica rice varieties because of poor tissue culture response of either callus induction or regeneration (Ge et al, 2006). Explant source for rice transformation plays an important role in successful development of transgenic plants. Scutellum derived calli were mostly used as source for transformation in indica varieties (Kumar et al, 2005; Sripriya et al, 2011), and also there were other explant sources reported in rice transformation such as shoot apex (Park et al, 1996; Arockiasamy and Ignacimuthu, 2007; Baskaran and Dasgupta, 2012), *in-planta* (japonica) (Supartana et al, 2005), leaf base (Karthikeyan et al, 2011a) and immature embryos (Aldemita and Hodges, 1996; Hiei and Komari, 2008). Nevertheless, scutellum-derived rice callus is the most preferable source of explants (Sahoo et al, 2011; Endo et al, 2012).

In the present study, we selected an indica rice variety BPT 5204, which is popularly known as Samba Mahsuri, a widely cultivated variety in India for its good quality and marketability (Reddi et al, 1979). It has fine grain, high yielding and excellent cooking quality, therefore attracting rice farmers and consumers. Although it has good agronomic qualities, BPT 5204 is susceptible to biotic and abiotic stress (Directorate of Rice Research, 2006). Genetic engineering is an alternate method of choice for development of

rice variety against pest and disease and other abiotic stress. For trait enhancement of this particular variety or functional genomics studies through transgenic approach, establishment of larger number of transformed events is indispensable. Thus, we attempted to develop transgenic plants with improved transformation efficiency with seed derived young calli as explants.

MATERIALS AND METHODS

Plant materials and plasmid construct

Mature seeds of indica variety BPT 5204 maintained at the Directorate of Rice Research, India, were used. The binary plasmid pCAMBIA1301 (CAMBIA, Australia) was mobilized into *Agrobacterium* strain LBA4404 (pSB1) by triparental mating method (Lichtenstein and Draper, 1985). The binary plasmid pCAMBIA1301 has *gus* and *hpt* as reporter and plant selection marker genes, respectively, which are driven by CaMV 35S promoter and *npt* II gene outside the T-DNA region as bacterial selection marker (Fig. 1).

Agrobacterium-mediated rice transformation

Mature rice seeds were dehulled manually and sterilized with 70% ethanol for 2 min followed by 0.1% $HgCl_2$ for 4 min and then washing with autoclaved double distilled water for three times. After dried on sterile filter paper, the seeds were inoculated onto callus induction medium solidified with 0.3% phytagel [MS basal salt (Murashige and Skoog, 1962) supplemented with 2 mg/L 2,4-D, 0.5 mg/L kinetin, 500 mg/L *L*-proline, 500 mg/L casein enzymatic hydrolysate and 30 g/L maltose, pH 5.8], and were maintained in dark at (28 ± 2) °C for 6 to 30 d. The *Agrobacterium* strain LBA4404 (pSB1, pCAMBIA1301) was freshly streaked in YEB medium containing 10 mg/L rifampicin, 10 mg/L tetracycline and 50 mg/L kanamycin solidified with 1.5% agar for 3 d at 28 °C in dark. Single colonies of *Agrobacterium* culture were inoculated in AB minimal medium containing 50 mg/L
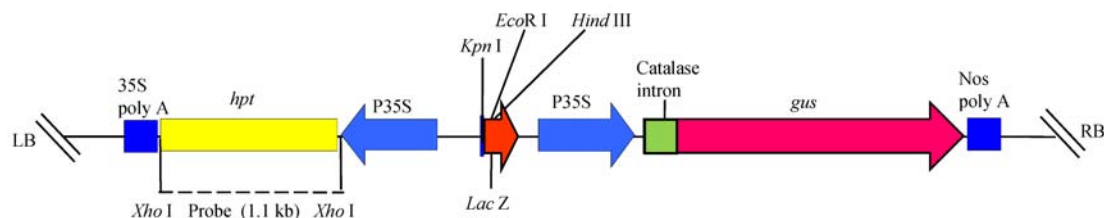

Fig. 1. Linear map of binary vector pCAMBIA1301 T-DNA region.
LB, Left border; RB, Right border; P35S, CaMV 35S promoter.
Dashed line represents *Xho* I digested 1.1 kb fragment of *hpt* gene used as the probe in Southern blot analysis.

kanamycin and 10 mg/L tetracycline and incubated in a shaker at 220 r/min for 16 h. A 50 mL culture of *Agrobacterium* (OD_{600} = 0.5) was checked with a ND-1000 spectrophotometer (NanoDrop Technologies, USA) and centrifuged at 3 000 r/min for 15 min at 25 °C. The bacterial pellet was suspended in 50 mL of MS-AS liquid medium (MS basal salt, 2 mg/L 2,4-D, 68.5 g/L sucrose, 36 g/L glucose, 500 mg/L casein hydrolysate, 100 mg/L myo-inositol, 876 mg/L *L*-glutamine, 266 mg/L *L*-aspartic acid, 174 mg/L *L*-arginine, pH 5.2) with 100 μmol/L acetosyringone (Sigma-Aldrich, USA) and incubated at 28 °C for 2 h. Embryogenic calli of 6- and 21-day-old were immersed in the pre-incubated *Agrobacterium* solution for 15 min and then blotted on a sterilized Whatman No. 1 filter paper to remove excess bacteria. The infected calli were transferred onto co-cultivation medium (MS basal salt, 2 mg/L 2,4-D, 68.5 g/L sucrose, 36 g/L glucose, 100 mg/L myoinositol, 300 mg/L casein hydrolysate, pH 5.2; solidified with 0.3% phytagel) with 200 μmol/L acetosyringone for 3 d at 28 °C in the dark. The co-cultivated calli were washed thoroughly with sterilized water and again washed with liquid callus induction medium containing 250 mg/L cefatoxime. The washed calli were blotted dry on a filter paper and cultured on selection medium [callus induction medium containing 50 mg/L *hygromycin* (Invitrogen, USA) and 250 mg/L cefatoxime] under dark at 28 °C for two weeks. The selection process was followed for three cycles of 14 d in each selection medium. Newly grown *hygromycin* resistant calli were transferred to the second and third selection media for two weeks each. After the third selection, the resistant calli were transferred to regeneration medium (RM) I [MS basal salt, 2 mg/L kinetin, 0.3 mg/L naphthyl acetic acid (NAA), 30 g/L sucrose and 30 g/L *D*-sorbitol and pH 5.8, 0.4% phytagel] with 25 mg/L *hygromycin*. The cultures were maintained in a 16-h-light/8-h-dark photoperiod. The plantlets arising from the calli were transferred to rooting medium solidified with 0.4% phytagel (1/2 MS basal salt, 15 g/L sucrose and pH 5.8) to induce roots. Fully rooted plants were transferred to hardening medium (Yoshida et al, 1976) for two weeks, then transferred to pots and maintained at transgenic biosafety glass house. The schematic transformation protocol is shown in Fig. 2.

Molecular analysis of transgenic plants

GUS histochemical staining assay

The histochemical assay of *GUS* gene expression was performed with leaf tissues and calli of putative transgenics according to the method of Jefferson et al (1987) using 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide X-Gluc (Biosynth AG, Staad, Switzerland) as a substrate. The histochemical solution contained 50 mmol/L phosphate buffer (pH 7.0), 1% Triton X-100, 1 mmol/L EDTA (pH 7.0), 1 mmol/L potassium ferricyanide, 1 mmol/L potassium ferrocyanide, and 1 mmol/L X-Gluc. The samples were incubated at 37 ℃
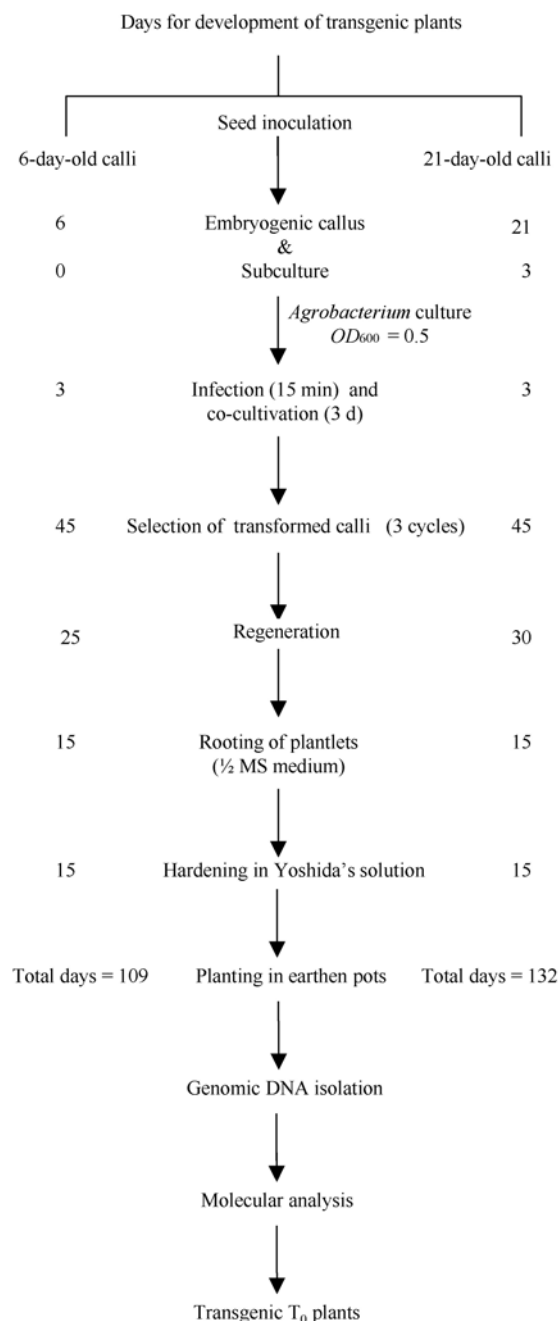

Fig. 2. A schematic protocol for development of transgenic rice from 6- and 21-day old calli of BPT 5204 and its time frame for development of transgenic rice plants.

for 24 h and the transient transformation frequency was calculated based on *GUS* expression ratio between the number of calli showing blue staining and the total number of calli kept for staining.

Genomic DNA isolation, PCR and Southern blot analysis

Leaves collected from 30-day-old plantlets of transformed and non-transformed control plants were used to isolate DNA by the modified CTAB method (Dellaporta et al, 1983). PCR was performed with 50 ng of template DNA, *GUS* gene primers (F: 5′-CATGAAGATGCGGACTTACG-3′ and R: 5′-ATCC ACGCCGTATTCGG-3′) using a Thermal Cycler (Bio-Rad, USA). The PCR reaction mixture (20 µL) was prepared with genomic DNA, 1 × *Taq* assay buffer (containing 1.5 mmol/L MgCl$_2$), 125 µmol/L dNTPs, 0.4 µmol/L each of forward and reverse primers and 1 U of *Taq* DNA polymerase (Bangalore Genei, India). The PCR profile was set at 95 ºC for 5 min + 35 cycles of (95 ºC for 30 s + 58 ºC for 30 s + 72 ºC for 1 min) + 72 ºC for 7 min.

Restriction digestion of plant genomic DNA, electrophoresis and Southern hybridization analysis followed Sambrook and Russell (2001). About 5 µg of genomic DNA from PCR positive plants and non-transformed control plants (NT) was digested with *Xho* I or *Hind* III. Restricted DNA samples were resolved on 0.8% agarose gel at a constant voltage of 20 V. The DNA was transferred onto positively charged nylon membrane (Hybond-N$^+$; GE Health Care Ltd., UK) by alkali transfer method. A 1.1 kb *Xho* I digested fragment of *hpt* gene was used as probe using ready-to-go-labeling kit (GE Health Care Ltd., UK). Probe was prepared as the manufacture's instructions. The blot was hybridized with the probe labeled with 50 µCi of α-^{32}P dCTP (BRIT, India). Pre-hybridization, hybridization and washing procedures were carried out at 65 ºC as described by Sambrook and Russell (2001).

Segregation analysis of transgenic plants

Seeds harvested from the primary transgenic plants (T$_0$) were used for raising T$_1$ progenies. Seeds of selected T$_1$ transgenic lines and non-transformed plants were surface sterilized with 70% ethanol and 0.1% HgCl$_2$ solution. The sterilized seeds were inoculated in hormone free ½ MS basal medium with 50 mg/L *hygromycin*. The plates were incubated under the light for 15–20 d. Number of germinating seedlings resistant/sensitive to *hygromycin* was scored for transgene inheritance pattern.

RESULTS

Callus induction and regeneration frequency of young callus

To increase transformation efficiency in popular indica rice variety BPT 5204, first we tested the callus induction and the regeneration frequency of different aged calli. Dehulled mature rice seeds were cultured on callus induction medium for 30 d. There was no callus proliferation on 1 and 2 d after inoculation (Fig. 3-A and -B). After 48 h incubation in the callus induction medium, the young callus from scutellum was initiated on the 3rd day onwards and the callus proliferation was prominent till three week incubation on the same medium (Fig. 3-C and -I). On the 3rd day, the callus was 2 mm in diameter while on the 21st day, it was 8 to 10 mm. The appearance of embryogenic calli (yellowish white, compact, globular structure) was clearly visible on the 6th day (Supplementary Fig. 1). From 10 d of incubation onwards, the callus size was gradually increased, moreover, changes in callus morphology were also observed. The embryogenic calli on the 6th day represented more compact and globular in nature, whereas prolonged incubation of up to three weeks showed less embryogenic portion and more watery translucent callus. Thus, 6-day-old young calli were selected for transformation (Supplementary Fig. 1-S). The callus frequency gradually increased when the age of the callus increased. The highest callus induction frequencies were recorded in 21- (85.8%) and 30-day-old calli (94.5%) whereas 43.0% of 6-day-old calli were induced (Table 1). However, 6-day-old calli showed more compact embryogenic calli than 2- or 3-week-old calli.

To check the regeneration potential, the different aged calli of BPT 5204 were placed on RM I. Previously, a different concentration of kinetin and NAA was studied with 21-day-old embryogenic callus, where kinetin (2 mg/L) and NAA (0.3 mg/L) contributed to higher plant regeneration. The calli at different ages were initially incubated under dark for 7 d and then transferred to light conditions till regeneration of plantlets. Regeneration frequency in terms of the ratio of shoot number to total callus number for regeneration was determined. Of all the six different aged calli, high regeneration frequency (61.3%) was observed in 6-day-old calli, while it was 48.7% in 21-day-old calli. It was observed that the regeneration frequency was gradually decreased as the age of the calli increased. Also, the regeneration frequency of 6- and 21-day-old
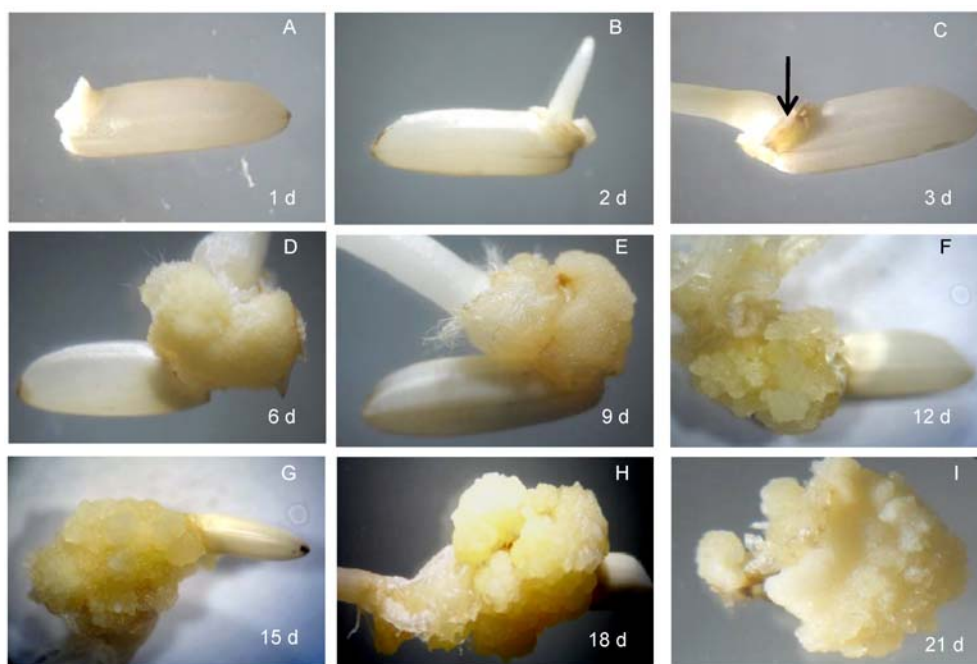
Fig. 3. Callus development in indica rice BPT 5204.
      A and B, No callus formation; C, Callus initiated (black arrow); D, Compact primary callus on the 6th day after inoculation; E to I, Secondary callus; I, Callus removed from seeds. Leica EZ4d stereo microscope, 10 × 1.6 magnification.

embryogenic calli by altering phytohormone, combinations of kinetin, 6-Benzylaminopurine (BAP) and NAA in three different RM was used to ensure any changes in regeneration potential. The concentration of growth regulator NAA (0.3 mg/L) was constant in all the three media tested where RM I was supplemented with kinetin (2 mg/L), RM II with BAP (2 mg/L) and RM III with kinetin (2 mg/L) and BAP (1 mg/L) respectively. Among the two different aged calli, 6-day-old calli showed the highest regeneration frequency of 60.0% compared with 21-day-old ones (48.0%) (Fig. 4). Of the three different RM tested, low regeneration frequency was observed in RM III (1–2 plants/callus), 45.0% in 6-day-old and 39.0% in 21-day-old calli. Since RM II produced multiple shoots (4–6 plants/per callus) BUT root initiation was very slow, RM I was

selected for regeneration of transgenic plants from transformed calli. A maximum of 2–3 fully grown plantlets per callus was regenerated in RM I.

High transformation frequency in young callus of indica rice BPT 5204

To study the competency of different aged calli (3- to 30-day-old) from BPT 5204, transformation was performed with *Agrobacterium* strain LBA4404 (pSB1) harboring binary plasmid pCAMBIA1301 with *gus* and *hpt* as reporter and plant selection marker genes (Fig. 1). For each experiment, 200 calli were infected

Table 1. Frequency of callusing and regeneration of different aged non-transformed calli of indica rice BPT 5204.

Callus age (d)	No. of seed inoculated	Callusing freq (%) (mean ± *SE*)	No. of calli in reg	Reg freq (%) (mean ± *SE*)
3	200	16.7 ± 3.0	25	22.3 ± 1.9
6	200	43.0 ± 1.8	50	61.3 ± 1.8
10	200	57.8 ± 1.2	50	55.3 ± 2.4
15	200	64.5 ± 2.3	50	52.0 ± 1.2
21	200	85.8 ± 2.2	50	48.7 ± 0.7
30	200	94.5 ± 0.3	50	42.0 ± 1.2

   Means were calculated from three independent replications.
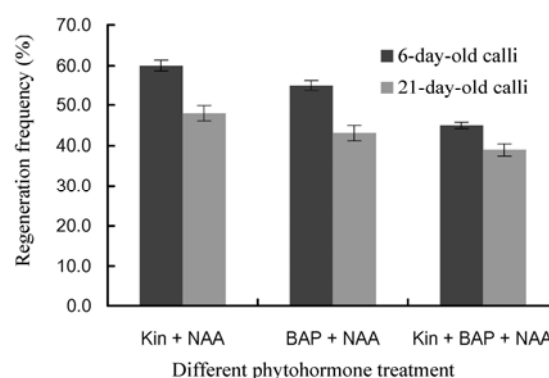   Freq, Frequency; Reg, Regeneration.

Fig. 4. Regeneration frequencies of 6- and 21-day-old non-transformed calli in different regeneration media.
      Kin, Kinetin; NAA, Naphthyl acetic acid; BAP, 6-Benzylaminopurine.

with *Agrobacterium* suspended with MS-AS solution for 15 min followed by co-cultivation in dark for 3 d. After co-cultivation, all the transformed calli were then washed in sterile water followed by MS callus induction liquid medium supplemented with 250 mg/L of cefatoxime and checked for transient *GUS* expression. A few calli showed GUS expression in 3, 10-, 15-, 21- and 30-day old calli (Table 2), while considerable number of blue spots was observed in most of the 6-day-old calli. The experiment was repeated three times and checked number of *GUS* positive calli for assessing the transformation frequency. There were no changes in transformation frequency (i.e., transient transformation) when 6-day-old calli were used for transformation without supplement of *hygromycin*.

The mean transformation frequency based on transient *GUS* expression was observed as (14.3 ± 1.2) % in 6-day-old calli while it was lesser in other aged calli used for transformation (Table 2). Using *t*-test, the mean transformation frequency of different aged calli was compared and found to be highly significant at 5% level. For stable transformation, we studied 6- and 21-day-old calli by *Agrobacterium* transformation (Fig. 5). The transformed calli were selected on the medium containing 50 mg/L *hygromycin* (Fig. 5-C). The mean transformation frequency after the 3rd selection on *hygromycin* was observed as 16.9% in 6-day-old calli while it was 12.7% in 21-day-old calli (Table 3). The *hygromycin* resistant calli were transferred to regeneration medium supplemented with 25 mg/L hygromycin (Fig. 5-D). The mean regeneration frequency after transformation was 11.7% in 6-day-old calli, which was about two-fold higher than 21-day-old calli (Table 3). The *hygromycin* resistant putative transgenic plants were tested for *GUS* expression analysis (Fig. 5-E, I). Out of 72 plants regenerated in 6-day-old calli,

Table 2. Comparison of transformation of BPT 5204 calli of different ages with LBA4404 (pSB1, pCAMBIA1301).

Days after inoculation (d)	No. of calli infected	Transformation frequency (%) (Mean ± SE)
3	200	1.2 ± 0.2
6	200	14.3 ± 1.2
10	200	4.3 ± 0.6
15	200	5.3 ± 0.4
21	200	7.2 ± 0.7
30	200	3.8 ± 0.4

Means were calculated from three independent replications.
After co-cultivation for 3 d, all the calli were checked for *GUS* expression analysis.

39.0% of the plants were *GUS* positive where number of *GUS* positive plants was 14.6% in 21-day-old calli. The *GUS* positive plants were screened by PCR using *GUS* gene primers which showed an amplicon of 606 bp (Fig. 6-A). DNAs from selected PCR positive plants were digested with *Xho* I enzyme to confirm for its selection marker gene (*hpt*) of fragment length 1.1 kb by Southern hybridization (Fig. 6-B). DNAs of selective Southern positive plants were subsequently digested by single cutter enzyme (*Hind* III) used for identifying the independent transformation events (Fig. 6-C). Most of the plants had single copy T-DNA insertion and a few had two copy T-DNA insertions. All the confirmed transgenics were fertile and set seed normally. The overall transformation efficiency of BPT 5204 was improved from 0.8% to 5.9% by using 6-day-old calli (Table 3). Overall, the development of transgenic plants required 109 d for 6-day-old calli and 132 d for 21-day-old calli (Fig. 2).

Segregation analysis

All the transgenic T_0 plants obtained from early calli were fertile and selfed T_1 seeds were harvested. From the T_0 plants, six BPT 5204 (#1 to #6) independent transgenic lines were selected for segregation analysis

Table 3. Transformation of 6- and 21-day-old calli and its transformation efficiency.

Callus age	Expt	No of calli infected (A)	HygR calli after III sel (B)	Mean TF (%) (B/A×100)	No. of calli reg (C)	No. of plant obtained	Mean RF after trans (%) (C/A×100)	No. of *GUS*+ plants	No. of PCR+	No. of S+ (D)	Mean TE (%) (D/A×100)
6	1	125	23		16	15		8	8	8	
	2	119	19		13	19		6	6	6	
	3	115	18		13	22		9	9	9	
	4	120	21		14	16		5	5	5	
Total		479	81	16.9	56	72	11.7	28	28	28	5.9 ± 0.8
21	1	121	11		7	10		1	1	1	
	2	117	15		8	5		1	1	1	
	3	123	20		10	7		2	2	2	
	4	104	13		4	5		0	0	0	
Total		465	59	12.7	29	27	6.2	4	4	4	0.8 ± 0.3

R, Resistant; sel, Selection; reg, Regeneration; RF, Regeneration frequency; trans, Transformation; *GUS*+, GUS positive; PCR+, PCR positive; S+, Southern positive; TE, Transformation efficiency; TF, Transformation frequency.
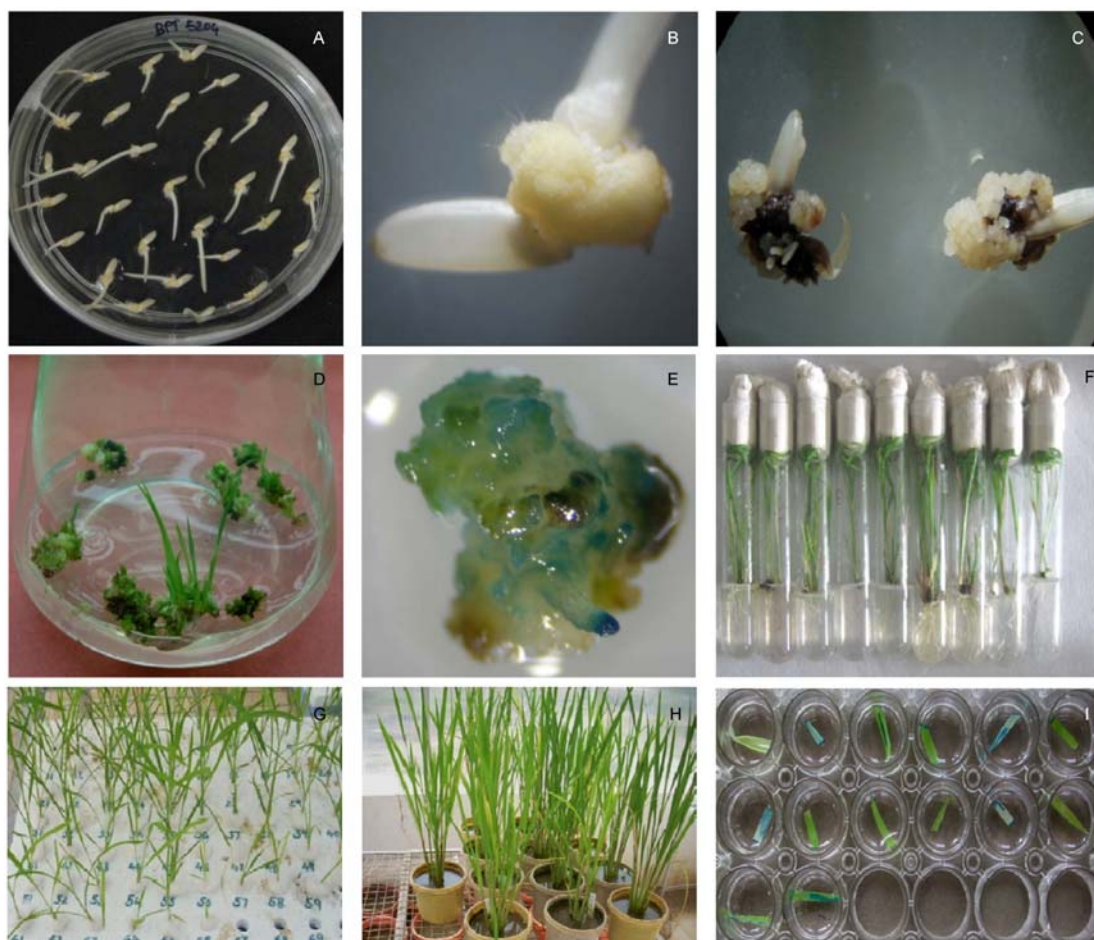
Fig. 5. *Agrobacterium*-mediated transformation of seed-derived 6-day-old calli from BPT 5204.

A, Seeds after 6 d on MS callus induction medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin; B, Embryogenic callus; C, Proliferation of resistant calli after onset on selection medium containing 50 mg/L hygromycin; D, Regeneration of putative transgenic plants; E, *GUS* expression in regenerating putative transformed callus; F, Putative transgenic plants in rooting medium; G, Plants hardening; H, Plants transferred to earthen pots at transgenic biosafety glass house; I, *GUS* expression in selected transgenic leaf tissues.

of T_1 progenies. Thirty seeds (T_1) from each selected Southern positive T_0 plants were inoculated in ½ MS basal medium with 50 mg/L *hygromycin* for two weeks under light conditions. Non-transformed seeds were not germinated in the medium containing *hygromycin*. Based on the number of transformed seedlings germinated on the medium containing *hygromycin*, we confirmed that the transgene inherited as mendelian ratio 3:1 for single copy insertion (#2, #3 and #4) and 15:1 for two copy insertions (#1, #5 and #6) (Table 4). All the *hygromycin*-resistant plants were *GUS* positive. Further, *GUS* positive progenies were confirmed by PCR and Southern analysis.

DISCUSSION

The aim of the present investigation was to establish the efficient transformation with improved transformation efficiency in indica rice BPT 5204. The rice variety BPT 5204 was selected for its unique qualities like high yielding, cooking quality, but was highly susceptible to biotic and abiotic stress (Kumari et al, 2007; Sundaram et al, 2008). In order to increase the transformation frequency of this popular indica rice variety for crop improvement, we attempted transformation with two different aged calli, i.e., 6- and 21-day-old calli.

For successful genetic transformation, a reliable and repeatable plant tissue culture is essential. Among the three subspecies of rice, japonica genotype was more amenable to transformation than indica genotype (Hiei et al, 1997). So many factors were reported to influence callus induction and regeneration frequency in rice (Aldemita and Hodges, 1996; Mohanty et al, 1999; Shrawat and Lorz, 2006; Ratnayake and Hettiarachchi,
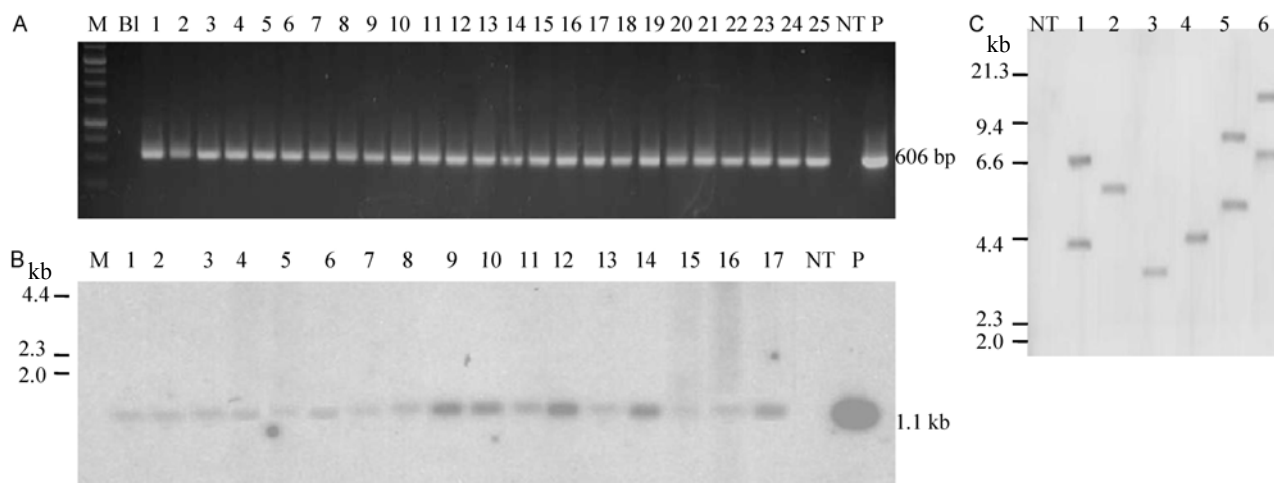
Fig. 6. Molecular analysis of T_0 transgenic plants obtained from 6- and 21-day-old calli.

A, PCR analysis of selected *GUS* positive plants using *GUS* gene primers. M, 1 kb ladder DNA marker; Lanes 1 to 21, 6-day-old callus; Lanes 22 to 25, 21-day-old callus; Bl, Water as template; NT, Non-transformed control DNA; P, Plasmid pCAMBIA1301. B and C, Southern hybridization of selective PCR positive transgenic BPT 5204 (T_0) plants. About 5 µg of genomic DNA from transformed and non-transformed plants was digested with *Xho* I (B) and *Hind* III (C) probed with 1.1 kb of *hpt* gene fragment as shown in Fig. 1.

2010). A highly efficient transformation protocol in japonica rice cultivars using scutellum-derived calli was developed by Hiei et al (1994). Subsequently, efforts were made to develop transgenic indica rice (Aldemita and Hodges, 1996; Rashid et al, 1996; Khanna and Raina, 1999; Datta et al, 2000; Sridevi et al, 2005). These reports suggested that optimization of callus induction and regeneration was a prerequisite for actual process of transgenic plant development.

In the present study, the callus induction frequency of different aged calli (i.e., 3 to 30 days) was assessed. There was no change in callus induction medium composition. Among the six different aged calli, the highest callus induction frequency was obtained on 30 days (94.5%) whereas less than 50.0% of the first one week old calli were induced (Table 1). The frequency was gradually increased as the age of the callus increased. The morphology of 6-day-old calli was compact, globular, yellow and embryogenic in appearance. However, as the age of calli increased, the embryogenic portion got reduced leading to appearance

of watery callus characterized by non-embryogenic tissues. Mostly indica rice was considered as recalcitrant to tissue culture and poor responsive to transformation (Pandey et al, 1994; Lin and Zhang, 2005; Nishimura et al, 2005). Ge et al (2006) reported that the optimization of phytohormones, organic and salt concentrations in the tissue culture media on different indica rice germplasms would enhance the production of high quality embryogenic calli which could be useful for the *Agrobacterium* mediated transformation. It was clearly indicated that the tissue culture response of indica rice was genotype-dependent, other than a single medium composition for all indica rice genotypes.

In the present study, the calli obtained on different days were checked for regeneration capacity, and the highest regeneration frequency was obtained in 6-day-old calli (61.3%), whereas 48.7% was observed in routine 21-day-old calli (Table 1). Based on the high regeneration potential, both 6- and 21-day-old calli which obtained from common callus induction medium were further assessed for its regeneration

Table 4. Segregation analysis of transgenic plants obtained from transformation of young calli of BPT 5204.

Plant #	No. of T-DNA copy	Seeds inoculated	Hyg[R]	Hyg[S]	*GUS*[+]	Ratio	χ^2 value	*P*-value
1	2	30	27	3	27	15:1	0.720	0.396
2	1	30	22	8	22	3:1	0.186	0.666
3	1	30	23	7	23	3:1	0.000	1.000
4	1	30	21	9	21	3:1	0.745	0.388
5	2	30	27	3	27	15:1	0.720	0.396
6	2	30	28	2	28	15:1	0.009	0.924

Hyg, *Hygromycin*; R, Resistant; S, Sensitive. [+], Positive.

enhancement by altering different phytohormones concentration in three different RM. Previously, regeneration medium (i.e., RM I) with kinetin (2 mg/L) and NAA (0.3 mg/L) for 21-day-old calli was optimized and used for transformation (Balachandran et al, 2009). Embryogenic calli were transferred to RM I which showed higher regeneration frequency in 6-day-old callus than 21-day-old callus. Similarly, calli in RM II regenerated with multiple plantlets from single callus hence the regeneration frequency was observed as 55.0% in 6-day-old calli while 43.0% in 21-day-old calli (Fig. 4). Moreover, low regeneration frequency was observed in calli cultured on RM III with a frequency of 39.0% in 21-day-old calli and 45.0% in 6-day-old calli. Though there were reports of BAP and NAA for regenerating rice plants in different indica rice varieties (Mohanty et al, 1999; Lin and Zhang, 2005; Sridevi et al, 2005; Tyagi et al, 2007; Tariq et al, 2008; Karthikeyan et al, 2011b), but combination of kinetin and NAA regenerated more plants with good number of roots which is in agreement with a few reports of transformation and regeneration of indica varieties (Saika and Toki, 2010; Sahoo et al, 2011). Our study clearly showed that 6-day-old young calli regenerated significantly higher number of plants than 21-day-old calli.

There were different approaches for indica rice transformation and a few varieties were successfully demonstrated with high rate of transformation efficiency, but *Agrobacterium*-mediated transformation in indica rice variety is still an obstacle due to genotype-dependent and poor tissue culture response especially in high-yielding rice varieties (Nishimura et al, 2005). Other improved method for *Agrobacterium*-mediated transformation was reported by using mature indica seeds (Kumar et al, 2005). However, the tissue culture procedures of this method take a long time (about six months) to obtain transgenic plants due to prolonged culture selections. To shorten the tissue culture duration, Toki et al (2006) used 5-day precultured seeds on callus induction medium and successfully developed transgenic rice plants in Nipponbare. Later, Saika and Toki (2010) reported that selection of matured seed-derived primary callus (i.e < 10 d) was more competent than the secondary callus or three-week-old callus in indica cv. Kasalath. Still three-week-old calli or subculturing of calli up to 45–66 days were used as explants for indica rice transformation (Pipatpanukul et al, 2004; Saharan et al, 2004; Ramesh and Gupta, 2005; Wanichananan et al, 2010). Thus, age of the

callus is an important factor which influences the tissue culture response in indica cultivars and also transformation frequency.

In present study, we selected 6-day-old young callus derived from scutellum with the help of stereo microscope and performed transformation. During incubation, there was no callus formation in 1- or 2-day-old pre-cultured seeds. The callus initiation started from 3 day onwards. For *Agrobacterium* competency, 3- to 30-day-old calli were transformed with LBA 4404 (pSB1, pCAMBIA1301) with OD_{600} value of 0.5. During transient *GUS* expression analysis, 6-day-old callus showed higher transformation frequency (14.3%) than the other aged calli (Table 2). Lower transformation frequency rate observed in other aged calli of BPT 5204 may have less embryogenic portion which could not be competent for *Agrobacterium* infection. Moreover, randomly selected 6-day-old calli used for transformation resulted in very low transformation frequency (data not shown) compared to selected callus. Though 6-day-old calli were compact, not all the calli were embryogenic. However, 60% of the calli were embryogenic in nature than 21-day-old calli which were used for transformation. In Nipponbare, infection of 1-day pre-cultured seeds resulted in successful regeneration of transgenic plants. Still, more transgenic events were achieved in 5-day-old pre-cultured seeds (Toki et al, 2006). We observed that prolonged tissue culture condition of 3–4 weeks old calli showed less embroygenic portion and mostly the calli appeared translucent and watery. Our aim was to shorten the tissue culture duration with high quality embryogenic calli and high regeneration which would improve the transformation frequency than routine 21-day-old or regular subcultured calli used for transformation. During co-cultivation period, most of the 21-day-old calli turned brown after infection with *Agrobacterium*. At the same time, transformation frequency was 14.3% (without *hygromycin*) in 6-day-old calli. Browning of calli after infection was less in 6-day-old callus than in 21-day-old callus. There are reports that suggested tissue browning after infection was influenced by factors like genotypes susceptibility to *Agrobacterium* (Khanna et al, 2007; Dan, 2008), inhibitory metabolites to *Agrobacterium* sensory machinery (Zhang et al, 2000; Liu and Nester, 2006; Maresh et al, 2006) or susceptibility of plant cells to *Agrobacterium* infection might have influenced the stimulation of plant host defense mechanism (Veena et al, 2003; Ditt et al, 2005; Zipfel et al, 2006; Anand

et al, 2008). In our study, the 6-day-old calli possessed more embryogenic, dry, compact, globular tissues in appreance with more actively dividing cells that might be highly competent and easy for *Agrobacterium* infection than other aged calli which were soft, translucent and unorganised in apperance. Hence the transformation frequency was higher in 6-day-old than 21-day-old calli. The final stable transformation efficiency after selection on *hygromycin* was 5.9% in 6-day-old callus, whereas it was 0.8% in 21-day-old callus (Table 3). Transformation efficiency of BPT 5204 was 1.7% when one-month-old scutellum derived callus was used as explants (Kumari et al, 2007). However, in the present study, we observed that transformation frequency was higher in 6-day-old calli, while it was reduced in stable transformation. The difference in transient vs stable transformation frequecy of present method was similar with Tie et al (2012). They reported that the transient transformation frequency was 41.2% while stable transformation was 2.2% in indica rice Zhenshan 97. Overall, the transformation efficiency of indica rice BPT 5204 was improved by using young callus though it was low compared to other reported indica rice varieties (Karthikeyan et al, 2011b), which could be due to genotype specificity. Thus the demonstrated protocol using young calli of BPT 5204 has advantages like shortened callus induction period, no subculture of callus, high regeneration frequency, high transformation frequency and reduced period for developement of transgenic plants than 21-day-old calli.

In summary, this study demonstrated the efficient *Agrobacterium*-mediated transformation in BPT 5204 young calli and improved transformation efficiency when compared to three weeks old calli. Further, this protocol also minimizes time to get transgenic plants. This work has paved the way further to transform other trait improvement/enhancement genes or functional genomics studies in rice variety BPT 5204.

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