

# Regeneration and *Agrobacterium*-mediated transformation of a popular *indica* rice variety, ADT39

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**A simple and efficient protocol for the regeneration and *Agrobacterium*-mediated transformation of an agronomically useful *indica* rice variety, ADT39 has been standardized. Initiation of callusing and its subculture were best achieved in MS medium supplemented with 300 mg/l casein enzymatic hydrolysate. The best callusing and regeneration responses were observed at concentrations of 2 mg/l 2,4-D and 1.5 mg/l BAP. Optimal transformation efficiency of 22.2% was obtained using high concentrations of augmentin as a bacteriostatic agent for a short period to inhibit the growth/persistence of *Agrobacterium*, without compromising the regeneration potential of the tissue. Using a viral promoter-driven GUS reporter gene, morphologically normal fertile plants were obtained. Molecular analysis of the above transgenic plants indicated several independent, single-copy transgene insertion events. Expression of the reporter gene was detected in the above plants. Mendelian inheritance of the transgene in the progeny was also observed.**

**Keywords:** *Agrobacterium*, GUS activity, rice transformation, segregation.

RICE is the world's single most important food crop and a primary food for more than a third of the world's population, mainly in the tropics<sup>1</sup>. To meet the growing demand for rice, biotechnological intervention for its improvement using genetic engineering is becoming increasingly important. Such interventions hinge on the development of efficient and reproducible transformation protocols for agronomically superior and popular rice varieties grown in rice-consuming countries like India. Of the two major types of cultivated rice *indica* and *japonica*, wide difference exists between their tissue culturability; the former being less responsive than the latter. Thus, it is important to establish parameters for the transformation of popular *indica* rice varieties<sup>2</sup>. Two transformation protocols are generally available for rice, namely *Agrobacterium*-mediated and biotics. *Agrobacterium*-mediated transformation has several advantages, such as higher transformation efficiency, the ability to transfer large pieces of

DNA, minimal re-arrangement of transferred DNA, integration in low copy numbers and low cost, etc. Amongst *indica* rice, reproducible transformation protocols have been reported only in a few varieties<sup>3–11</sup>, including the recently reported<sup>11</sup> *Agrobacterium*-mediated transformation protocol for *indica*-type rice varieties, BR29 and IR64.

Most of the high-yielding and agronomically superior varieties of *indica* rice grown in India do not have reproducible transformation protocols available for them. ADT39 is a popular, semi-dwarf *indica* rice variety, of IR8 × IR20 parentage, yielding 5000 kg/ha of medium, slender grains in 120–125 days; it is widely cultivated in the South Indian states, especially Tamil Nadu<sup>12</sup>. To develop transformation protocols for one such variety (ADT39), an *Agrobacterium*-mediated co-cultivation has been described in this article. Molecular evidences of transgene integration, its expression and Mendelian inheritance are also presented.

## Materials and methods

### Plant material

Seeds of rice variety ADT39 were obtained from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

### Bacterial strain and plasmid

The binary plasmid<sup>13</sup> pCAMBIA RTBVDD7 (Figure 1) was constructed by inserting the 289 bp *Rice tungro bacilliform virus* (RTBV) promoter fragment upstream of a GUS reporter gene carried in plasmid<sup>14</sup> pCAMBIA 1381Z. pCAMBIA RTBVDD7 was then introduced into *Agrobacterium tumefaciens* strain EHA 105 by the freeze-thaw method<sup>15</sup>.

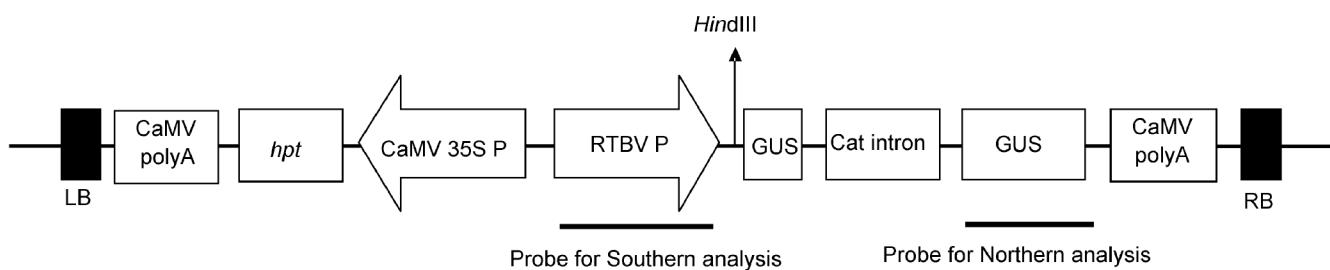
### Callus induction

Mature seeds of var. ADT39 were dehusked manually and rinsed with water containing 3–4 drops of Teepol. Seeds

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**Table 1.** Composition of different media used in the study

Medium	Composition
MS basal	MS salts and vitamins, 30 g/l sucrose, 8 g/l agar, pH 5.8
2MSCEH	MS basal, 2 mg/l 2,4-D, 300 mg/l CEH, 30 g/l sucrose, 8 g/l agar, pH 5.8
2MSCEH-AS	2MS-CEH, 100 $\mu$ M acetosyringone
2MSCEH-Sel I	2MS-CEH, 150 mg/l augmentin, 30 mg/l hygromycin
2MSCEH-Sel II	2MS-CEH, 30 mg/l hygromycin
MSCEH-Reg	MS basal, 300 mg/l CEH, 1.5 mg/l BAP, 30 mg/l hygromycin, 30 g/l sucrose, 8 g/l agar, pH 5.8
MS-Root I	MS basal (1/2 strength), 30 g/l sucrose, pH 5.8
MS-Root II	MS basal (1/2 strength), pH 5.8

**Figure 1.** Structure of T-DNA region of plasmid pCAMBIA RTBVDD7 containing GUS reporter gene driven by RTBVDD7 promoter.

were soaked in sterile water for 16 h and subsequently blotted dry on a sterile filter paper and inoculated on 2MS-CEH and incubated at 28°C in dark for 21 days. The compositions of various media are given in Table 1. The proliferating calluses were dissected out and subcultured on fresh medium for another 21 days, followed by another 2 days on fresh medium. Nodular, creamish-white embryogenic calluses were used for transformation.

#### Co-cultivation and regeneration of transgenic plants

The co-cultivation of calluses with *Agrobacterium* and regeneration to mature plants was done essentially according to the procedure described earlier<sup>16</sup>, with modifications as given below. About 100 ml culture of *A. tumefaciens* strain EHA105 containing the binary plasmid pCAMBIA RTBVDD7 was grown in liquid LB medium containing rifampicin and kanamycin (50 mg/l) each till the optical density of the culture reached 0.3 at 600 nm. Following centrifugation, the pellet was resuspended in 11 ml 2MSCEH-AS. Calluses were swirled for 10–20 min in this suspension, blotted dry on a filter paper and transferred to co-cultivation medium 2MSCEH-AS for two and half days at 28°C in dark. This was followed by thorough washing in sterile water and then with sterile water containing 300 mg/l augmentin. The washed calluses were blotted on a sterile filter paper to remove excess moisture and transferred to selection medium 2MSCEH-Sel I at 28°C in dark, for 14–15 days. Hygromycin-resistant calluses were transferred to fresh selection medium 2MSCEH-Sel II every 15 days, 2–3 times. White

proliferating calluses were transferred to regeneration medium 2MSCEH-Reg at 28°C under a 16 h photoperiod. Following regeneration, approximately 3–4 cm plantlets were transferred from tubes containing regeneration medium for rooting to MS-Root I, followed by transfer to MS-Root II. Subsequently, plants were transferred to pots containing a mixture of soil and SOILRITE™ and grown to maturity in a greenhouse at 32°C and 70–80% humidity. The plants were supplied with MS basal<sup>17</sup> without sucrose, whenever required.

#### Molecular analyses for presence, activity and inheritance pattern of the transgene

Total genomic DNA was isolated from the transgenic plants following the protocol described earlier<sup>18</sup>. PCR, restriction enzyme digestion, electrophoresis, Southern hybridization and Northern hybridization were performed according to standard protocols<sup>19</sup>. For PCR analysis, oligonucleotide primers specific for RTBV promoter were designed from the clone pRTBV203 (EMBL accession no. AJ314596). The primers used were the following: FP1 (28 mer) 5' CGG AAT TCT GTC CTG CAC CAC CTC AAT G 3' between positions 7165 and 7182, and RP8 (28 mer) 5' AAC TGC AGC TCT CTT GCT CTT GTG GAA G 3' between positions 7424 and 7449 of the RTBV genome capable of amplifying a fragment of 289 bp specifying the RTBV promoter. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and synthesis at 72°C for 45 s,

with a final extension step of 72°C for 7 min. For Southern analysis, 5–10 µg each of genomic DNA extracted from the leaves of mature GUS-positive, regenerated plants and control, untransformed plants was completely digested by *Hind*III and electrophoresed in a 0.8% agarose gel. To visualize the copy number of the transgene, the blotted gel was probed using a radioactively labelled 0.8 kb RTBV promoter fragment. For Northern blotting, total RNA was isolated from the above plants using guanidine thiocyanate<sup>20</sup>, and 12–15 µg of it was electrophoresed and blotted according to standard methods<sup>19</sup>. The probe used was 1 kb fragment encoding the GUS gene. Probes were labelled using Megaprime Labeling kit (Amersham International) according to the manufacturer's instructions, using  $\alpha^{32}\text{P}$ -dATP from BRIT, Mumbai. To test for GUS activity, appropriately cut sections of the tissue were stained using methyl umbelliferon<sup>21</sup>. Photographs of the above stained sections were obtained using Nikon Optiphot-2 microscope (Nikon Corporation, Tokyo, Japan).

The inheritance pattern of GUS transgene was studied by germinating seeds obtained following selfing of three transgenic lines and checking for GUS activity as described earlier<sup>21</sup>.

## Results

### Optimization of conditions for callusing and regeneration in ADT39

To determine the optimum conditions for callusing and regeneration, both MS as well as NB medium with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 1.0, 1.5, 2.0 and 2.5 mg/l) were tried. Results presented in Table 2 indicate that MS was a better medium than NB and the best calluses were obtained in the presence of 2.0 mg/l 2,4-D. In order to study the time requirement for obtaining embryogenic calluses, seeds were incubated in the medium for 21 days and the calluses thus obtained were then subcultured for four more days on the same medium before testing for their regeneration potential. In another set of experiments, seeds were incubated in the medium for 21 days, followed by subculturing for another 21 days and finally for two more days before putting them on regeneration medium. As shown in Table 2, the second treatment (45 days) was found to be better than the first. Frequency of callusing was found to be higher (50%) in case of 2MS compared to 2NB (30%; data not shown). The addition of supplements like casein enzymatic hydrolysate (CEH) significantly improved the quality of the calluses, but did not improve the callusing frequency of the seeds, which remained approximately 50%. Calluses obtained on 2MS + CEH were nodular and creamy-white as against the pale yellow obtained on 2MS without CEH.

On testing regeneration with increasing concentrations of the cytokinin 6-benzylaminopurine (BAP) ranging from 0.5 to 1.5 mg/l, it was observed that 45-day-old calluses uniformly showed higher regeneration as against the 25-day-old calluses. The best regeneration frequency was at 1.5 mg/l BAP, which for the 45-day-old calluses was close to 67% (Figure 2). Greening was observed within one week following subculture in MS medium supplemented with 1.5 mg/l BAP, when the 45-day-old callus initiated on MS supplemented with 2.0 mg/l 2,4-D was used. Such calluses were used for genetic transformation in subsequent experiments.

### Optimization of co-cultivation conditions

To find out the optimum conditions for co-cultivation, different concentrations of acetosyringone and the duration of co-cultivation were tested. For optimizing the concentration of acetosyringone, the frequency of hygromycin-resistant calluses obtained following each variation in co-cultivation condition was taken as the transformation frequency. It was found that 100 µM acetosyringone

**Table 2.** Optimization of medium, 2,4-D concentration and time for callus formation in the rice variety ADT39

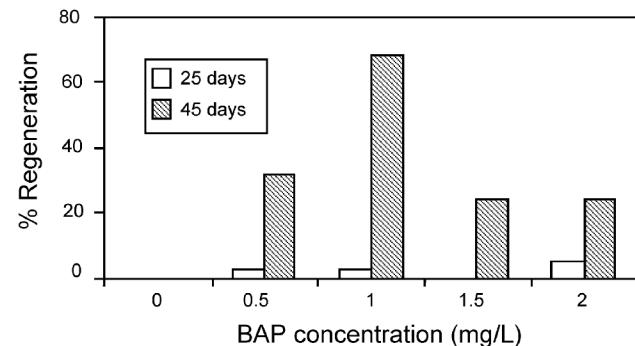
Medium	2,4-D (mg/l)	Response*	
		25 days <sup>a</sup>	45 days <sup>b</sup>
MS	1	+	+
MS	1.5	++	+
MS	2	++	+++
MS	2.5	++	++
NB	1	++	++
NB	1.5	++	++
NB	2	++	+
NB	2.5	++	++

\*Characteristics of calluses.

<sup>a</sup>Slimy, light brown, non proliferating. <sup>++</sup>Slimy, yellow, poor proliferation. <sup>+++</sup>Pale yellow, nodular hard callus, proliferating.

<sup>a</sup>Culture for 21 days, followed by transfer to fresh medium for 4 days.

<sup>b</sup>Culture for 21 days, followed by transfer to a fresh medium for another 21 days and then to a fresh medium for 4 days.



**Figure 2.** Relation of regeneration frequency with BAP concentration and duration of subculturing.

was ideal for transformation. Approximately 60% of the co-cultivated calluses proliferated following incubation in 2MSCEH-Sel I. Co-cultivation for more than two and half days resulted in the calluses becoming prone to repeated *Agrobacterium* infection, which ultimately resulted in the loss of regeneration potential. Thus, co-cultivation for two and half days in the presence of 100 µM acetosy-ringone was found to be the most suitable for optimum transformation. It was observed that by increasing the concentration of augmentin during washing, *Agrobacterium* contamination in the calluses was reduced by substantial proportions.

#### Regeneration and characterization of transgenic plants

Out of a total of 90 co-cultivated calluses transferred to the selection, 20 survived the selection (22.2%). Following transfer to regeneration medium, 18 shoots were observed within 10 days and the plantlets were transferred to the rooting medium and, thereafter, to the pots.

Under glasshouse conditions, the putative transgenic plants flowered in approximately seven months following transfer to pots. The average yield of seeds from each plant was about 75. Analysis for the presence and genomic integration of the *trans*-genes was carried out on T0 plants. As an initial test, the plants were analysed by PCR for the presence of 289 bp transgene. All the 18 hygromycin-resistant regenerants tested amplified the expected 289 bp product (Figure 3 a). Subsequently, the above PCR-positive plants were analysed by Southern hybridization for the integration of the transgene (Figure 3 b). Using a RTBV promoter-specific probe, *Hind*III digestion of genomic DNA extracted from eight PCR-positive lines showed a single hybridizing band in four lines (Figure 3 b, lanes 1–4) and two bands in the remaining four lines (Figure 3 b, lanes 5–8). Since *Hind*III cleaves the T-DNA region once (Figure 1), the number of hybridizing fragments obtained indicates the same number of transgene copies. The patterns indicated several independent integration events of the transgene in the rice genome. Northern analysis of nine randomly selected PCR-positive plants showed that all of them tested were actively transcribing GUS transgene, reflected by the accumulation of transcripts hybridizing with the probe (Figure 3 c). Lack of signal in the control untransformed plant indicated the specificity of the signal for the *trans*-gene. To reveal the presence of the marker enzyme, bacterial  $\beta$ -glucuronidase, five tissues, representing independent transformation events were stained and observed under light microscope. Development of deep blue colour in three tissues, following histochemical staining for GUS activity further confirmed the transgenic status of the plants tested. One such representative stained tissue section showing blue staining is shown in Figure 3 d.

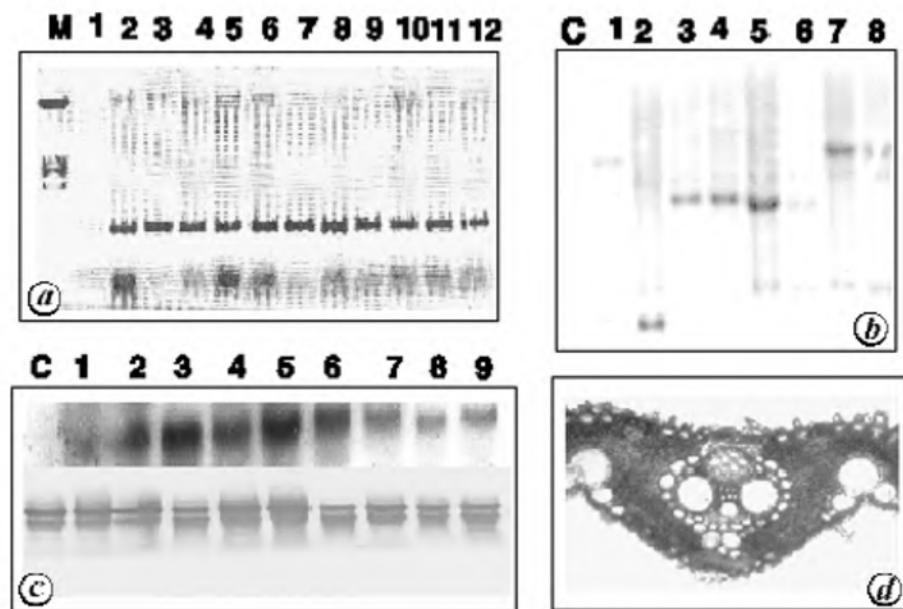
#### Analysis of T1 progeny for inheritance of the transgene

Approximately 31–40 T1 progeny plants, each of three T0 single-copy transgenic lines were checked for GUS activity to determine the inheritance pattern of the transgene. In two of the lines (lines 1 and 3), the transgene expressed in three-fourths of the progeny plants, whereas in the third line (line 2), it was expressed by almost half of the progeny plants (Table 3). Thus, the first two lines showed Mendelian segregation, whereas the third line demonstrated non-Mendelian ratios.

#### Discussion

In this article, we describe a method for *Agrobacterium*-mediated genetic transformation of a popular *indica* rice variety ADT39. Studies have indicated that in rice, efficient transformation and subsequent regeneration using *Agrobacterium*-mediated methods are dependent on several factors. These include, among others, choice of explant, hormonal composition of the medium used, nutritional supplements, temperature and duration of co-cultivation with *Agrobacterium*, virulence of the *Agrobacterium* strain, concentration and composition of the bacteriostatic agent used, duration of selection and concentration of antibiotic selection marker<sup>22–27</sup>. Among the several types of explants<sup>28–30</sup>, scutellum-derived callus has been found to be the most amenable to transformation<sup>16</sup>. It was recently reported that the successful regeneration of transgenic plants within a month of the start of the aseptic culture of mature seeds using scutellum could be obtained from 1-day precultured seeds as explants, thus avoiding the risk of somaclonal variations<sup>31</sup>. The potential for callus formation, transformation and regeneration in rice is a varietal characteristic<sup>32,33</sup> and *indica* rice is reported to be inferior to *japonica* in this respect<sup>34</sup>. For ADT39, we tested two media, MS and NB, each with four different concentrations of BAP, for callusing response, involving two different incubation periods. Our results indicating 2MS containing 2.0 mg/l 2,4-D with added CEH, was in agreement with earlier reports for most of the *indica* varieties<sup>2–10</sup>.

On analysing parameters like callusing, co-cultivation and regeneration, it was seen that best callusing response was observed on MS medium having 2 mg/l 2,4-D and 300 mg/l CEH, following sub-culturing for a total of 45 days. Our results support the recent report that *indica* varieties respond better to MS compared to NB<sup>3</sup>. Although some reports recommend increased 2,4-D concentration<sup>3,9</sup>, up to 6 mg/l, most reports recommend 1.5–2.0 mg/l for callus induction, and our results also confirm the same. Nutritional supplements like CEH, proline and glutamine have earlier been reported to enhance the callusing response<sup>3,8</sup>. However, in our case, although CEH led to significant improvement in the quality of the calluses, there



**Figure 3.** Molecular analysis of transgenic ADT39 plants. *a*, PCR analysis of transgenic plants. Lane 1, Untransformed control; lanes 2–11, Putative transgenic plants, and lane 12, Positive control (plasmid containing RTBV promoter). M, Marker  $\lambda$  DNA digested with *Hind*III. *b*, DNA gel blot analysis of transgenic plants. Lane C, Untransformed control; lanes 1–8, Transgenic lines; probe used was 0.8 kb RTBVDD7 promoter fragment. *c*, RNA gel blot analysis of the transgenic plants (upper panel). Lane C, Untransformed control; lanes 1–9, Putative transgenic lines, probe used was 1 kb GUS gene (lower panel). Loading control showing methylene blue-stained ribosomal RNA bands. *d*, Transverse section of a leaf from a transgenic plant stained for GUS activity.

**Table 3.** Segregation pattern of GUS gene as assayed by  $\beta$ -glucuronidase (GUS) activity in the T1 progeny of single copy transgenic rice plants

Transgenic line no.	No. of plants tested	No. showing GUS activity	No. not showing GUS activity	Ratio GUS <sup>+</sup> ve/GUS <sup>-</sup> ve	$\chi^2$ value
1	40	30	10	3:1	0.00
2	32	18	14	1:1	6.00
3	31	25	6	4:1	0.54

was no improvement in the callusing frequency which remained approximately 50%. Optimization of the duration of co-cultivation showed that two and half days of co-cultivation was the best and resulted in optimal transfer of DNA without resulting in superfluous *Agrobacterium* infection. It has been reported previously that the use of high concentration of bacteriostatic agents like cefotaxime, carbenicillin, augmentin, etc. may reduce the regenerability of the calluses as they resemble auxins in their structure and in combination with other callus-inducing hormones like 2,4-D, may cause loss in regeneration potential<sup>22–24</sup>. In this study it was found that reduction of the time period for which the calluses were subjected to the bacteriostatic agent led to a substantial increase in the regeneration potential of the transformed calluses. Regeneration was best achieved in the presence of 1.5 mg/l BAP, which is consistent with earlier reports that describe the use of 0.5–2.0 mg/l of cytokinin for different varieties of rice<sup>3–10</sup>.

Southern analysis indicated that the *trans*-gene was stable and was integrated independently at low copy numbers in the genome. Northern analysis showed that the reporter gene transcribed at different levels in different transgenic lines (Figure 3 *b*). The promoter used to drive the reporter gene has been demonstrated to be constitutively expressed in rice<sup>13</sup>. In this context, the differential expression of the reporter gene might be attributed to position effects<sup>35</sup>. Histochemical staining, coupled with Mendelian segregation of the heterologous gene in two out of the three lines tested confirmed the stable transgenic nature of these plants. One of the transgenic lines showed a segregation ratio of 1:1, which is a deviation from expected 3:1 ratio for a single-copy integration event. This type of anomalous behaviour has also been reported earlier<sup>8</sup>, and is considered to be normal in transgenic plants.

Thus, we demonstrate stable genetic transformation by *Agrobacterium*-mediated transformation protocol of the popular *indica* rice variety, ADT39. Availability of this

transformation protocol for ADT39, which is already resistant to devastating diseases like bacterial blast and sheath blight<sup>36,37</sup>, will allow the introduction of several agronomically important genes, leading to its further genetic enhancement.

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