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RESEARCH PAPER

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Development of Agrobacterium-mediated transformation technology for mature seed-derived callus tissues of indica rice cultivar IR64

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Keywords: Agrobacterium, indica rice, IR46, mature seed-derived calli, regeneration, rice transformation, transgenic rice

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; MS, Murashige and Skoog; MCS, multiple cloning site; NAA, 1-naphthaleneacetic acid; OD, optical density; PCR, polymerase chain reaction; YEM, yeast extract mannitol

Indica rice cultivar IR64 is most recalcitrant to regenerate, which affects the transformation efficiency especially when mature seed-derived callus tissues are used as explants. Therefore, a simple, rapid and improved genetic transformation protocol has been developed for the indica rice cultivar IR64 using Agrobacterium-mediated genetic transformation. With different hormonal combination tested, the maximum callus induction was observed on MS medium supplemented with 2.5 mg/l 2,4-D and 0.15 mg/l BAP from the scutellum explants. Three weeks old scutellum derived callus explants were immersed in Agrobacterium suspension (strain LBA4404, OD_{600} = 1.0) and co-cultured at 26 ± 2°C in dark for 2 d. The maximum transformation efficiency (12%) was achieved with infection of callus explants for 20 min along with use of 150 μm acetosyringone. The maximum plant regeneration was observed on MS medium supplemented with 3 mg/l BAP, 1 mg/l Kinetin and 0.5 mg/l NAA. The maximum root induction was observed on MS medium along with 10 g/l glucose and 20 g/l sucrose. The integration of the transgene in T1 transgenic plants was confirmed by polymerase chain reaction and Southern blot analyses. The copy number of transgenes has been found to vary from 1 to 2 in transgenic plants. By using this improved method we have successfully raised transgenic rice plants within 3 mo from seed inoculation to plant regeneration.

Introduction

Rice is one of the most important cereal crop in the world and a principal food for the people of tropical region.[1] The increasing demand for rice provide chances to biotechnologists to develop efficient, quick and reproducible transformation protocols for the important rice varieties. Among the rice group, (such as indica, japonica and javanica) the indica rice varieties are in high demand in tropics and subtropics. To fulfill the above demand, more sustained production of indica rice is needed which in turn requires an efficient protocol for its transformation.[2] Various gene transfer protocols were developed for rice, among them the Agrobacterium-mediated transformation is most extensively used method and has been accepted due to its several advantages.[3] Different types of explants are used in this method, such as mature seed derived calli, immature embryo-derived calli, leaf base derived calli and shoot apex.[4-7] The first report on transgenic rice was published in 1988 by three groups, utilizing protoplasts and electroporation.[8,9] Earlier, protocol for Agrobacterium-mediated transformation of mature seed-derived callus tissues of indica rice variety, IR64, was reported with low efficiency.[10-17]

Several approaches have been adopted to improve Agrobacterium-mediated transformation of indica rice.[18,19] It has been suggested that the development of a highly efficient and large-scale transformation system to handle more than 10^3 transformants would be a prerequisite for successful gene transfer.[19] Many authors reported high transformation efficiency in IR64 using immature embryos (explants)[6,20] but the technique has many limitations, such as unavailability of explants throughout the year and their crucial, laborious and time consuming selection. Recently, a simple protocol for transformation of Australian rice varieties using mature embryos has been reported.[21] In rice, many factors are influencing the efficiency of T-DNA delivery to plant cell such as type of explants, cell density of Agrobacterium, infection time, co-cultivation medium and the induction agent of *vir* gene, such as acetosyringone. In this study, we report a simple, rapid, reliable and reproducible protocol for Agrobacterium-mediated transformation from mature seed-derived callus tissues of indica rice cultivar IR64.

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Results

Optimization of conditions for callusing in IR64 rice. We tested different concentration of 2,4-D ranging from 2.0 to 3.0 mg/l in order to determine the optimum concentration on callus induction. The maximum amount of callus induction (65%) was observed in MS medium supplemented with 2.5 mg/l 2,4-D (**Fig. 1A**). Interestingly, the percentage of callus induction was found to be much higher (92%) when 0.15 mg/l BAP was also included along with 2.5 mg/l 2,4-D (**Table 1**) in callus induction medium (**Fig. 1B**). The other concentrations of BAP (0.1 and 0.2 mg/l) along with 2.5 mg/l 2,4-D gave comparatively less percentage of calli (**Fig. 1B**). The different stages of callus induction from mature seed of IR64 indica rice variety are shown in **Figures 2A–C**. Twenty-two days scutellum derived embryogenic calli was found to be better for Agrobacterium transformation (**Fig. 1C**). We selected 15, 22, 25, 30, 40 and 60 d old calli for transformation study; among them 22 d old scutellum derived calli was found to be suitable for Agrobacterium mediated transformation. It was observed that the use of 10 g/l agarose along with 4 g/l phytagel reduces the water potential of the medium, which leads to the formation of hard embryogenic calli that are important for transformation.

Optimization of co-cultivation conditions. Usually the Agrobacterium overgrowth is observed on explants after co-cultivation. In order to prevent Agrobacterium overgrowth on the explants we optimized the conditions. The overgrowth in calli kept in co-cultivation media for 48 h was less with more transformation efficiency as compared with 72 h. We tested different concentration of cefotaxime (200 mg/l, 250 mg/l and 300 mg/l) on selection media to prevent Agrobacterium overgrowth. Cefotaxime at 300 mg/l concentration was found to be efficient concentration for preventing bacterial overgrowth.

Optimization of concentration of acetosyringone. We further tested different concentrations of acetosyringone (100, 150 and 200 μM) on both bacterial suspension and co-cultivation medium with different duration (5, 10, 15 and 20 min). Bacterial infection for 20 min and co-cultivation for 48 h in the presence of 150 μM acetosyringone was found to be suitable for rice transformation (**Table 2**).

Optimization of selection media. The media which were supplemented with 300 mg/l

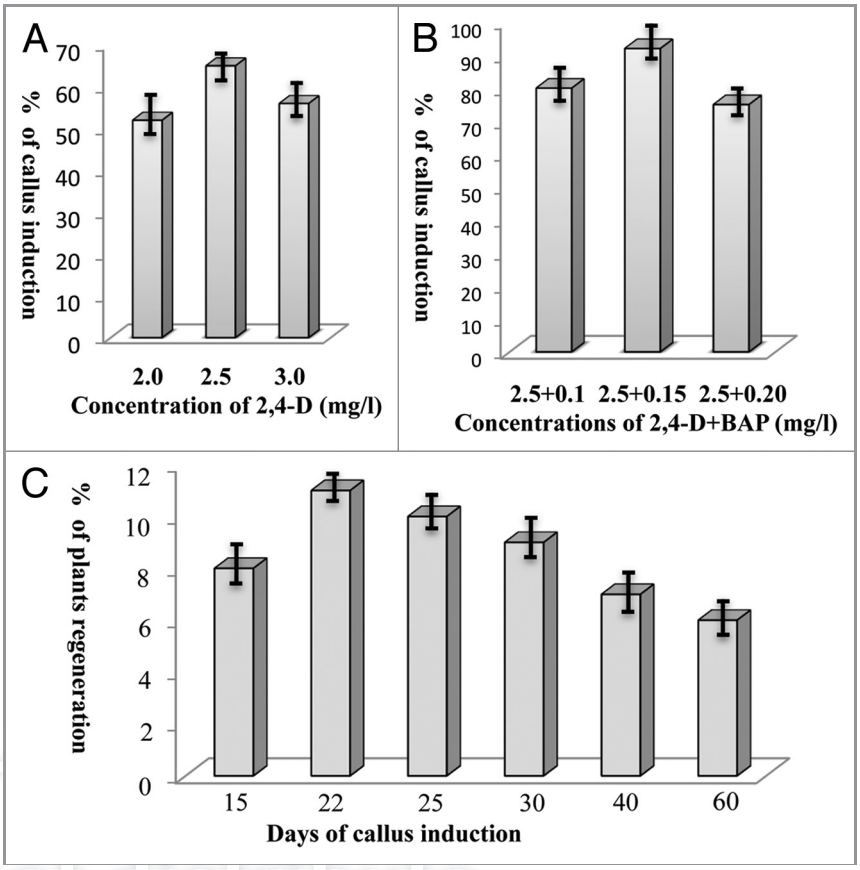

Figure 1. Effect of different concentrations of 2,4-D and BAP on callus induction. (A) Effect of 2,4-D concentrations (2.0, 2.5 and 3.0 mg/l) alone on callus induction. (B) Effect of different concentrations of 2,4-D and BAP together on callus induction medium. (C) Comparison of different days of calli with percentage of plants regenerated. In each case the experiments were replicated five times with 100 mature seeds as explants.

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

Name of the media	Composition
Callus Induction	MS salts and vitamins, Proline 65 mg/l, Casein hydrolysate 30 mg/l, 2,4-D 2.5 mg/l, BAP 0.15 mg/l, Sucrose 30 g/l, Phytagel 4 g/l , Agarose 2 g/l, pH 5.8
Callus sub-culture	MS salts and vitamins, Proline 65 mg/l, Casein hydrolysate 30 mg/l, 2,4-D 2.5 mg /l, BAP 0.15mg /l, Sucrose 30 g/l, Phytagel 4 g/l, Agarose 2 g/l, pH 5.8.
Selection	MS salts and vitamins, Proline 65 mg/l, Casein hydrolysate 30 mg/l, 2,4-D 2.5 mg /l, BAP 0.15 mg/l, Sucrose 30 g/l, Phytagel 4 g/l, Agarose 2 g/l, Hygromycin 50 mg/l, Cefotaxime 300 mg/l, pH 5.8.
Co-cultivation	MS salts and vitamins, Proline 65mg /l, Casein hydrolysate 30 mg/l, 2,4-D 2.5 mg /l, BAP 0.15 mg /l, Sucrose 30 g/l, Phytagel 4 g/l , Agarose 2 g/l, Acetosyringone 150 μM/l, pH 5.8.
Plant regeneration	MS salts and vitamins, BAP 3 mg/l, NAA 0.5 mg/l, Kinetin 1 mg/l, Sucrose 30 g/l, Agarose 10 g/l, Hygromycin 40 mg/l, pH 5.8.
Root induction	MS salts and vitamins, Sucrose 20 g/l , Phytagel 4g/l, Glucose 10 g/l, pH 5.8.
MS liquid	MS salts with vitamins, Sucrose 15 g/l, pH 5.4.

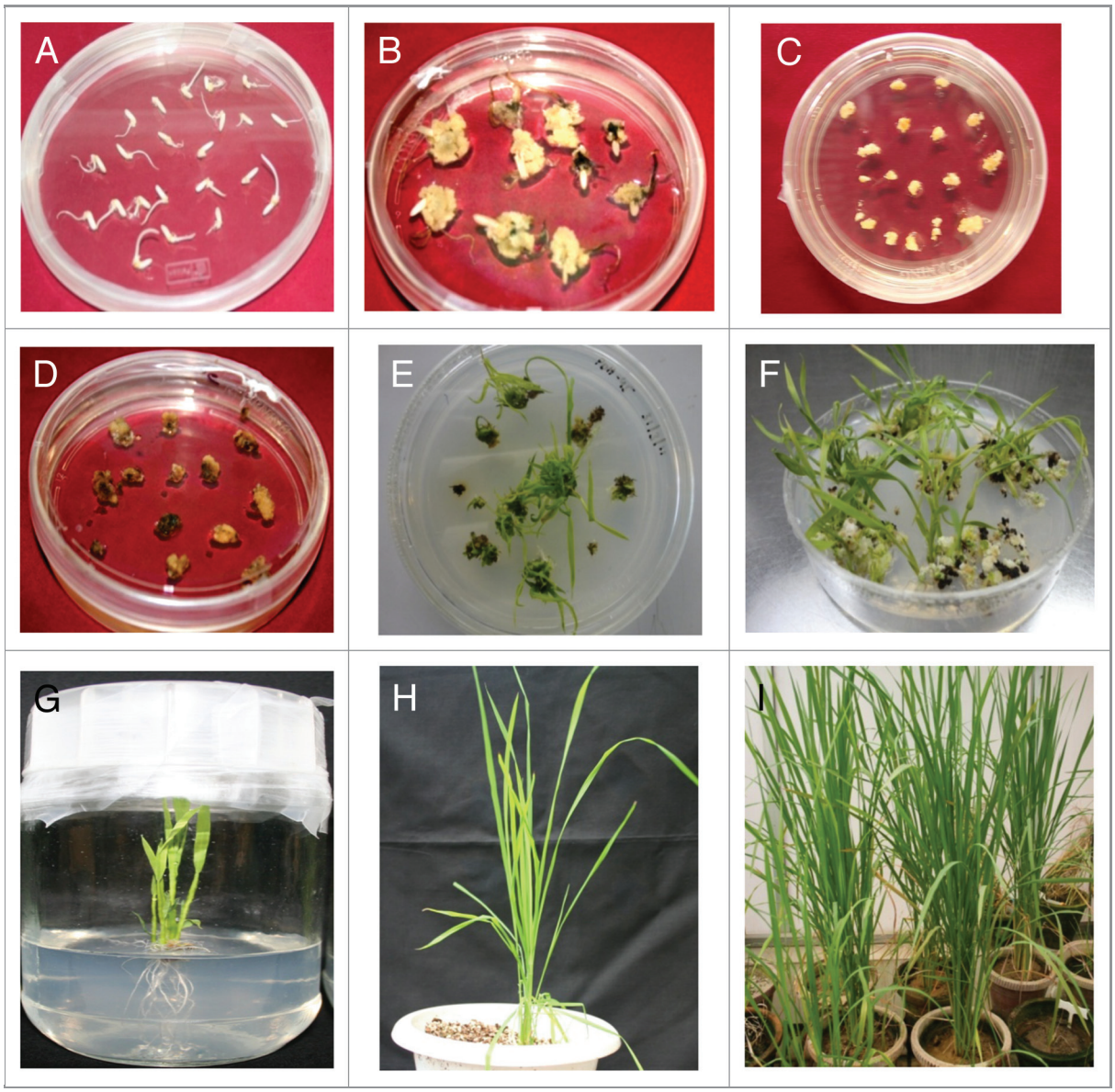

Figure 2. Different stages of callus induction from mature seed and plant regeneration in indica rice variety, IR64. (A) Mature seeds of IR64 in callus induction medium for 20 d. (B) After 20 d of calli initiation. (C) Calli sub-cultured on fresh callus induction medium for 2 d. (D) Selection of transformed calli on selection medium having hygromycin 50 mg/l. (E) The resistant calli in MS regeneration medium. (F) Emerging shoot buds on regeneration medium. (G) Shoots in rooting medium. (H) Hardening of plant in vermiculite. (I) Plants in soil.

cefotaxime along with 50 mg/l of hygromycin showed total killing of the untransformed explants after two round of selection. In the first round of selection the non-transformed parts of the calli died and became black (**Fig. 2D**). In the second round of selection, the transformed calli became more white and proliferated well. The well-proliferated calli were transferred to regeneration medium (**Fig. 2E**).

Standardization of regeneration media and measurement of transformation efficiency. To get better plant regeneration from mature seed-derived calli, various combinations of BAP, kinetin and NAA were used in the regeneration media (**Table 1**). The maximum plant regeneration was observed on MS medium supplemented with 3 mg/l BAP, 0.5 mg/l NAA and 1 mg/l kinetin along with 30 g/l sucrose (**Table 1**). The use of (10 g/l) of agarose instead of phytagel was reported to increase the plant regeneration.[10] We observed that use of (10 g/l) agarose reduces the water content of the media and increases the plant regeneration (**Fig. 2E and F**). In case of rooting media, MS medium along with (20 g/l) sucrose and (10 g/l) glucose produced maximum number of roots. We observed that with the above conditions the root formation started within 4 d and maximum roots were obtained within 7 d (**Fig. 2G–I**). The transformation efficiency of indica rice cultivar IR64 by using this method was observed up to 12% (**Table 3**).

Molecular analysis of T_0 and T_1 transgenic plants. Initially the T_0 putative transgenic plants were confirmed by PCR analysis

Table 2. The effect of concentration of acetosyringone and co-cultivation period on transformation efficiency.

Time(min)	Concentration of acetosyringone(μm)	% of plants regenerated mean ± SE*
5	100	3.36 ± 0.17
	150	4.12 ± 0.11
	200	4.37 ± 0.65
10	100	5.06 ± 0.21
	150	5.37 ± 0.40
	200	6.21 ± 0.32
15	100	6.33 ± 0.51
	150	7.20 ± 0.65
	200	7.06 ± 0.60
20	100	8.74 ± 0.72
	150	11.43 ± 0.31
	200	9.44 ± 0.43

*Data represent Mean ± SE of five replicates.

(data not shown). Further the analysis for the presence of genomic integration of transgene was performed on T1 plants. Plant genomic DNA was isolated and used for PCR analysis by using gene specific primers. The 1.7 kb *SUV3* gene integration was confirmed in PCR analysis as the amplification of expected size of ~1.7 kb was observed in selected lines (**Fig. 3A**). The amplification of transgene was further confirmed by using promoter (CaMV 35S) specific forward and gene specific reverse primers and the expected size (~2.2 kb) fragment was obtained (**Fig. 3B**). The copy number of the integrated transgene in the transgenic lines (T1) was analyzed by Southern hybridization analysis. The Southern analysis results confirmed the integration of transgene (**Fig. 3C**) in T1 plants. We observed that single copy integration was in lines 1 and 2 (**Fig. 3C**, lanes 1 and 2) and two copy number integration was in line 3 (**Fig. 3C**, lane 3). The undigested genomic DNA from line 1 shows the hybridized band of higher size (**Fig. 3C**, lane 4), while no band was observed in case of wild-type (WT) genomic DNA digested with Xba1 (**Fig. 3C**, lane 5). In T1 progeny analysis we observed 3:1 ratio.

Table 3. Transformation efficiency of indica rice cultivar IR 64

Rice cultivar	Experiment Number	Cocultivated (A)	Produced HyR calli	Produced HyR plants (B)	Transformation efficiency (%) (B/A)
IR64	1	200	34	22	11.0
	2	140	19	16	11.4
	3	100	16	11	11.3
	4	100	23	12	12.0
	5	100	26	11	11.0

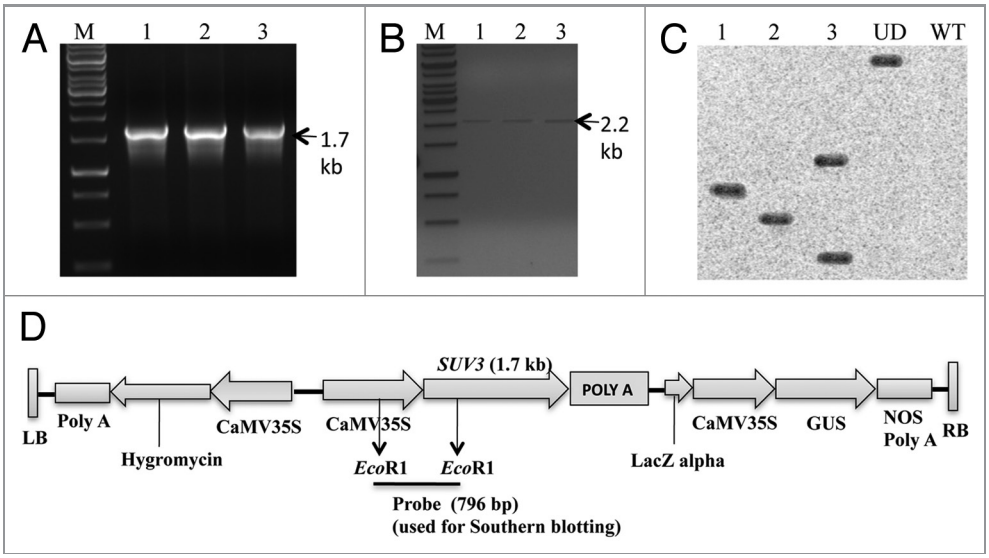

Figure 3. Molecular analysis of transgenic plants (T1). (A) Integration of the rice *SUV3* gene (1.7 kb) in the genome of randomly selected putatively transformed plants. Lane M is 1 kb ladder. Lanes 1–3 are, rice transgenic lines (L1, L2, L3, respectively) showing the gene amplification by PCR with use of gene specific forward and reverse primers. (B) PCR analysis of the transgenic (T1) lines by the use of promoter specific forward and gene specific reverse primers. Lane M is 1 kb ladder. Lanes 1–3 are, rice transgenic lines (L1, L2, L3, respectively) showing the required amplification (2.2 kb). (C) Southern blot analysis of T1 plants obtained from Agrobacterium-mediated transformation of rice variety IR64. Lanes 1–3 are the three transgenic lines (L1, L2, L3). Lane UD is the undigested DNA of transgenic line (L1). Lane WT is the genomic DNA from wild type rice plant. (D) Structure of T-DNA region of pCAMBIA1301 containing *SUV3* gene (1.7 kb) in MCS region with CaMV35S promoter and poly A terminator (pCAMBIA1301-*SUV3*).

Discussion

The starting material such as explants for Agrobacterium-mediated transformation is the most crucial factor for successful rice transformation. The explants should be easy to handle and easily available throughout the year. Here, as starting material for transformation, we have used mature seeds of the indica rice cultivar IR64, which is easily available throughout the year. In the earlier reports the transformation efficiency of indica rice cultivar IR64 from mature seeds was relatively low.[10-17] Though the transformation efficiency by use of immature embryo was reported to be relatively higher,[20] but this technique has many limitations. The starting material i.e., the immature embryos are not available throughout the year, whereas mature seeds are available all the time. The selection of time of immature embryo is very crucial factor in this method. The improper selection of immature embryos may lead to the failure of the experiment. Therefore we put our efforts to improve transformation protocol for IR46 rice variety from mature seed. In an earlier report, 2 mo old calli from mature seeds were used as explants.[10] Here we have improved the method by using 22 d old calli as explants for quick and efficient transformation.

In this study we have improved the method by incubating the calli for 48 h in co-cultivation media, which reduces the Agrobacterium overgrowth and also improved the transformation efficiency. In the earlier reports, cefotaxime (250 mg/l) and carbenicillin (250 mg/l) were used for preventing Agrobacterium overgrowth.[10] In the present study, we have used only cefotaxime (300 mg/l) for preventing Agrobacterium overgrowth. In the earlier reports 100 μM acetosyringone was used in the infection media for 1 to 10 min.[7,10] Here we have used 150 μM acetosyringone in MS liquid as well as in co-cultivation medium, which produced more transformants as compared with the earlier reports. Infection of callus explants for 20 min and co-cultivation of infected explants for 48 h were found to be optimum conditions for Agrobacterium-mediated transformation in rice. In case of plant regeneration, in the present study we have used (10 g/l) agarose as gelling agent, instead of phytagel or agar, which gave better results by reducing the water content of the regeneration medium.[10] High concentration of agarose (10 g/l) imposes the desiccation stress to the calli which triggers more number of shoots.[22] During plant regeneration, we found that higher concentration of BAP (3 mg/l) increases the number of shoot formation as compared with lower concentration used in earlier study.[7] In case of root induction, many researchers used half strength MS salts, but here we have used full strength MS salts with vitamins along with sucrose and glucose as carbon source, the root formation was observed within 4 d as compared with 15 d in earlier reports.[10] This improved protocol is very simple, comparatively rapid and reproducible for Agrobacterium-mediated transformation of mature seed-derived callus tissues of indica rice variety, IR64. This protocol could be a promising transformation method for other rice cultivars but still it needs to be tested in order to determine its feasibility for other cultivars.

Materials and Methods

Establishment of tissue culture. Indica rice cultivars: IR64 was used in the present study. The dehusked, mature, healthy seeds were disinfected with 70% (v/v) ethanol for 1 min, followed by a wash with freshly prepared aqueous solution of 50% bleach (6% sodium hypochlorite commercial grade) for 20 min, and finally rinsed five times with sterile distilled water. The seeds were placed on callus induction media (**Table 1**) at 26 ± 2°C in the dark for 20 d to induce callus development from the scutellum tissue. Then the proliferated embryogenic calli were sub-cultured on the above medium and incubated for 2 d in dark at 26 ± 2°C. The regeneration of calli was performed on MS regeneration medium (**Table 1**). The regenerated plantlets were grown and rooted in MS solid medium and subsequently transferred to the soil.

***Agrobacteruim tumefaciens* strains, plasmid and culture.** The complete 1.7 kb coding region of putative rice RNA helicase (*SUV3*) gene was PCR amplified by using forward primers (5'-GGATCCATGGCGTGGCTGCG-3') and reverse primer (5'-GGATCCTTTTGATCTCACATCAATTTCTTG-3') from rice cDNA library and the sequence was confirmed by sequencing. The amplified fragment was cloned into pGEMTeasy vector. The plasmid was digested with Bam H1 and the fragment was eluted and cloned in pRT100 vector. The entire cassette with CaMV35S promoter-*SUV3*-poly A terminator fragment was digested using Hind III, eluted and cloned into the MCS of pCAMBIA1301 plant expression vector containing the *hpt* gene (hygromycin phosphotransferase) as selectable marker (**Fig. 3D**). The construct was named as pCAMBIA1301-*SUV3.* The plasmid pCAMBIA1301-*SUV3* was introduced into the disarmed Agrobacterium strain LBA4404 by freeze thaw method[22] and confirmed by plasmid rescue and restriction analysis. A single colony of Agrobacterium was taken in 3 ml of liquid culture (primary culture) of YEM medium (yeast extract mannitol) and incubated at 28°C over-night in a rotary shaker. One ml of primary culture was inoculated in 100 ml of YEM liquid medium with Streptomycin 25 mg/l, Rifampicin 10 mg/l and Kanamycin 50 mg/l. The above culture was incubated at 28°C over-night in a rotary shaker. The next day optical density (OD) of Agrobacterium culture was checked at A600 and the culture showing OD in the range of 0.6–1.0 was selected. The culture was centrifuged for 20 min at 4,000 rpm at 20°C to pellet the cells. The resulting pellet was dissolved in 10–15 ml (depending on the pellet size) of liquid MS media containing 150 μM acetosyringone (AS).

Cocultivation, selection and plant regeneration. The scutellum derived calli were immersed and swirled by hand for 20 min in the suspension, then blotted dry on a filter paper and transferred to co-cultivation media (**Table 1**) and incubated in dark at 26° ± 2°C for 48 h. After 48 h, the infected calli were washed with sterile distilled water containing 300 mg/l cefotaxime and blotted dry on sterile Whatman paper, then transferred to selection medium (**Table 1**) containing 50 mg/l hygromycin for 15 d. After 15 d of selection, white portions of calli were isolated and transferred to fresh selection medium and incubated in dark at 26° ± 2°C. After two rounds of selection, the resistant proliferated calli were isolated and transferred to MS regeneration

medium containing 40 mg/l hygromycin (**Table 1**) and kept in dark for 5 d and then transferred to light (16 h photo period) for 5 d. The regenerated shoots were transferred to rooting media (**Table 1**) and kept in light for 5 d. Then the rooted plants were transferred to vermiculite pots for hardening. The hardened plants were transferred to soil pots and kept in green house.

PCR analysis of putative T_0 transgenic plants. PCR analysis was performed using the gene specific primers. These primers amplified a 1.7 kb fragment from the *SUV3* gene. PCR analysis was performed in a reaction volume of 25 µl containing the template genomic DNA (100 ng), 2.5 µl 10X PCR amplification buffer, 0.5 µl 10 mM $dNTP_S$, 1.2 µl 50 mM $MgCl_2$, 3 µM (2.5 µl) of each primer, 13.6 µl sterile distilled water, 1 unit (0.20 µl of Taq DNA polymerase (Genei). The samples were heated to 94°C for 5 min and then subjected to 30 cycles of 30 sec melting at 94°C, 30 sec annealing at 52°C and 1 min synthesis at 72°C and followed by another 10 min final extension at 72°C. The amplified products were assayed by electrophoresis on 0.8% agarose gels, stained with ethidium bromide (EtBr; 0.5 µg/ml), visualized and photographed under UV light.

PCR and Southern blot analysis of T1 plants. T1 transgenic lines were raised in the greenhouse and genomic DNA was isolated from the T1 rice leaves according to procedures described by Murray and Thompson (1980).[23] The PCR analysis was performed to confirm the presence of transgene in the T1 transgenic plants using the gene specific forward and reverse primers as well as promoter (CaMV35S promoter) specific forward and gene (*SUV3*) specific reverse primers. For Southern analysis, 20 µg of DNA was digested with *Xba I* and the digested samples were resolved on 0.8% agarose gels. DNA was transferred to negatively charged nylon membrane (Hybond-N+, Amersham, Inc.) for Southern hybridization analysis as described by Sambrook et al., (1989).[24] The coding sequence of 796 bp was (plasmid digested with EcoRI) probed with [α^{32}P] dCTP-labled probe.

Segregation analysis of the transgene. The inheritance of the *SUV3* gene in selfed T1 generation was analyzed. Here the selfed progenies were evaluated for resistance to hygromycin. T1 seeds of 3 independent transformants of IR64 cultivar were germinated on hygromycin (50 mg/l) containing medium. Seeds showing hygromycin resistance clearly displayed segregation ratio of 3:1 in inoculation analysis (data not shown). The sensitive plants died within two weeks after the treatment, while the resistant plants were healthy as control plants. In Southern hybridization, one independent transformants clearly showed two-copy number of transgene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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