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Agrobacterium mediated genetic transformation of popular Indica rice Ratna (IET 1411)

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This is the first report of genetic transformation of indica rice cultivar Ratna (IET 1411). *Agrobacterium tumefaciens* strain LBA 4404 carrying a binary vector pCAMBIA1301 harboring the CaMV35S promoter driven modified GUS gene was used. Various parameters critical to rice transformation were optimized including callus induction medium, bacterial concentration, co-cultivation conditions, concentration of the plant growth regulator 2,4-D and the concentration of acetosyringone. The transformed lines were analyzed using PCR for marker sequence and gusA gene expression by GUS activity assays. The regeneration and transformation frequency were calculated to be 70 and 47% respectively. This is a reproducible high efficiency transformation technique for the Indica rice cultivar Ratna (IET 1411).

Key words: Indica rice, *Agrobacterium tumefaciens,* callus induction, transformation, regeneration, GUS.

INTRODUCTION

Rice (*Oryza sativa* L.), the staple primary food crop for more than one third of the world's population, has emerged as a model crop for genome and proteome analysis (Goff, 2002). It serves more than two third of the world population and more than 90% of the Asian population (Khush and Brar, 2001), particularly in tropical and sub-tropical regions. The population of the world is increasing at the rate of 1.17% per year (World Bank, World Development Indicators). The unceasingly expanding population and decrease in arable land area have caused difficulty in meeting people's need. In the same light, the consumption of rice is increasing every year at the rate of 1.8%. But the production of rice has slowed. It is estimated that rice production has to increase by 50% by 2025 (Khush and Virk, 2000) to be enough to feed the people. In order to solve this problem,

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Abbreviations: MS, Murashige and Skoog, 2,4-D, 2,4-dichlorophenoxyacetic acid, **CH,** casein hydrolysate.

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development of rice varieties with higher yields, excellent grain quality and resistance to biotic/abiotic stresses is the only option. The production of high yielding disease resistant varieties through breeding has reached a plateau and the only viable option left is through transgenic approach (Duan et al., 2012).

The first genetic transformation of rice was carried out by using poly ethylene glycol (PEG) (Uchimiya et al., 1986; Zhang and Wu, 1988; Datta et al., 1990). Subsequently, gene transfer through electroporation and micro projectile bombardment became popular (Christou and Ford, 1995). Various genetic systems have been employed for transformation of rice *via* particle gun delivery (Ghosh et al., 2013). However, *Agrobacterium* mediated transformation of rice is still the method of choice because of its higher transformation efficiency, ability to transfer large fragments of DNA, minimal re-arrangement of the host DNA, and low cost. *Agrobacterium* mediated transformation is a superior method also because it results in stable and low copy number integration of foreign genes into the plant chromosomes (Sahoo et al., 2011).

Rice cultivated in Asia has been classified into two groups: Indica *(Oryza sativa* subsp *indica)* and *japonica (Oryza sativa* subsp *japonica),* of which *indica* rice is more popular (Agrama et al., 2010). But compared to Japonica most of the Indica rice cultivars are recalcitrant and less responsive in tissue culture as well as to *Agrobacterium* mediated transformation. Till date *Agrobacterium* mediated transformation protocols are available for only a few *indica* rice cultivars. Previous reports of Indica rice transformation include the cultivars IR64, IR72 (Kumar et al., 2005), Pusa Basmati1, Swarna (Ramesh et al., 2004) Chaitanya, Phalguna (Ramesh et al., 2004), DS20, OMCS97, ADT 39 (Tyagi et al., 2007). Saika and Toki (2010) reported a new protocol for transformation of *indica* rice cultivar Kasalath while Sahoo et al. (2011) standardized *Agrobacterium* mediated transformation of IR64. In spite of all these efforts, rice being recalcitrant and non-uniform in response to *Agrobacterium* mediated gene transfer, high efficiency protocols for *Agrobacterium*-mediated transformation is still not available for most of the Indica rice cultivars including cultivar Ratna (IET 1411).

Ratna (IET 1411) is a very popular elite Indica rice cultivar of TKM-6 × IR-8 parentage with dwarf size, long slender grains, grain yield of 45 to 50 ton ha^{-1} with a cultivation time of 130 to 135 days, grown vastly throughout India and Southeast Asia. The major problem for its cultivation is that it is susceptible to most of the rice insects, bacterial and fungal diseases, resulting in the high yield loss every year. To overcome this problem, transgenesis is the most obvious solution by which multiple traits like insect /fungal /bacterial resistance genes can be incorporated. But at present there is no available protocol for *Agrobacterium* mediated transformation for Ratna.

Here we present the first transformation protocol for Ratna. We have standardized the critical parameters of transformation, like callus induction prior to infection by *Agrobacterium*, co-cultivation period with *Agrobacterium*, concentrations of Acetosyringone in co-cultivation medium, callus induction medium, Hygromycin concentration and concentration of plant growth regulator 2,4-D. This protocol gave us high regeneration and transformation efficiencies. The transformed Ratna rice lines have been tested by PCR for hygromycin phosphotransferase gene sequence and GUS assay. The regeneration and transformation frequency are 70 and 47% respectively.

MATERIALS AND METHODS

Plant material

Seeds of *indica* rice cultivar Ratna (IET1411) were obtained from Rice Research station, Chinsurah, West Bengal, India.

Surface sterilization of rice seeds and callus induction

Dehusked rice seeds are surface sterilized with 30% (v/v) sodium hypochlorite with 0.01% (v/v) of Tween 20 (Merck, Germany) for 30 min. Seeds were rinsed thoroughly with sterilized distilled water five times. Surface sterilized seeds are transferred onto callus induction medium (CIM) that is MS medium (Murashige and Skoog, 1962) supplemented with 2,4-Dichlorophenoxy acetic acid concentrations of 2 mg L^{-1}, 2.5 mg L^{-1} or 3 mg L^{-1} . Different concentrations of casein hydrolysates were used, pH was adjusted to 5.2-5.6, then finally incubated at 28°C in the dark. Within 7 days, callus development was observed from the scutellum tissue. After calli grew to a size 4 to 5 mm in diameter, they were separated from the seeds and transferred to the fresh media for further proliferation. The calli were sub cultured every 10 days interval up to 2 months before infection with *Agrobacterium*.

Agrobacterium strain and binary vector

A. tumefaciens strain LBA4404 harboring pCAMBIA 1301 binary vector was used for rice transformation. The T-DNA construct of the vector contains hygromycin phospho transferase gene (*hpt)* as the plant selection marker. It also contains *GUS* as a reporter gene under CaMV35S promoter and *nos* terminater. Figure 1 shows the T-DNA region of the vector. The vector confers kanamycin resistance as bacterial selectable marker under the control of CaMV35S promoter.

Agrobacterium culture, infection, co-cultivation and callus selection

A. tumefaciens strain LBA4404 harboring pCAMBIA 1301 was maintained on Luria Bertani (LB) agar (1%) medium. A single colony of bacteria was inoculated in 50 ml of liquid AB medium (Hiei et al., 1994) containing 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} rifampicin and allowed to grow for two days at 28°C on an orbital shaker at 160 rpm. The culture was centrifuged at 4 000 rpm for 10 min and resuspended in AAM media (Hiei et al., 1994). OD_{600} was adjusted to 0.9 to 1.0. The proliferating rice calli were transferred to fresh media and cultured for 3 to 5 days before the *Agrobacterium*

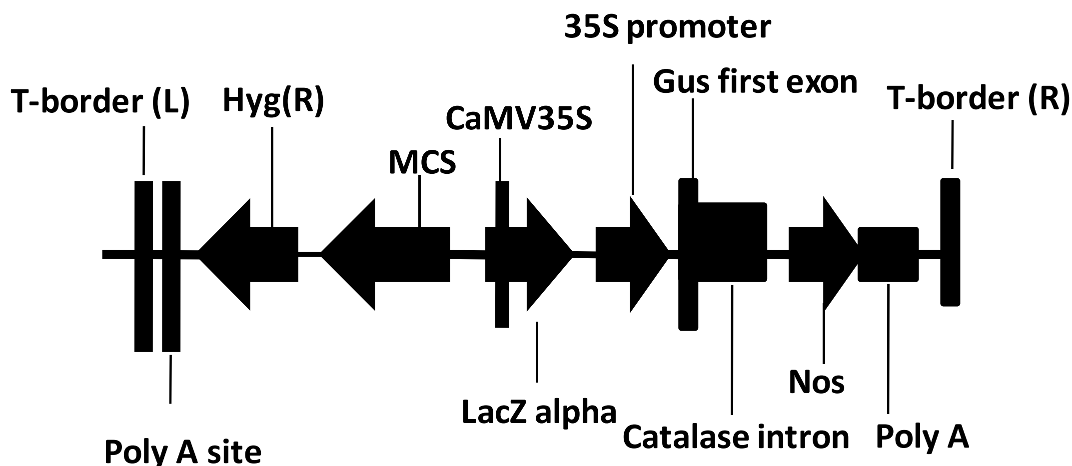

Figure 1. Partial map of binary vector pCAMBIA 1301. T-DNA region containing intron-*gus* and *hpt* plant expression cassettes.

infection. The calli were immersed in the bacterial suspension for 10 min. Excess bacteria were soaked on sterilized filter paper (Whatman no.1).

For standardization of co-cultivation, the inoculated calli were transferred either directly onto the co-cultivation media (CCM) or placed on a sterilized filter paper soaked in 1 ml of AAM media, the paper being placed on co-cultivation media (CCM) in 9 cm diameter Petri plates (Rashid et al., 1996). Co-cultivation was carried out in the dark at 25°C for 3 days. Two different concentrations (100 and 200 µg ml^{-1}) of acetosyringone (Sigma-Aldrich) were used in this experiment in AAM as well as in the CCM.

After 3 days of co-cultivation under 25°C, infected calli were washed three times in sterilized distilled water followed by a final rinse in 250 µg ml^{-1} cefotaxime solution. Calli were dried on sterilized filter paper and transferred onto selection medium 1 (SM-1). After 15 to 20 days on the selection medium, proliferating portions of the calli were excised with sterile scalpel and transferred onto SM-2 and incubated for 15 days before these were transferred onto SM-3 media.

Plantlet regeneration

The proliferating nodular cream-white calli from SM-3 are transferred onto the regeneration medium MMS with or without 20 µg/ml Hygromycin and 250 µg ml^{-1} cefotaxime and incubated at 28±1°C under 16/8 h light/dark photoperiod. After 4 to 5 weeks of incubation, shoot development started from putative transformed calli.

Genomic DNA isolation

Genomic DNA was isolated from the callus and leaf tissue using CTAB method with slight modifications (Saghai et al., 1984). 200 mg of leaf tissue was crushed with 1ml CTAB buffer in a sterile mortar. 1 ml of this slurry was taken in a microfuge tube and kept in a water bath set at 65°C for 30 min with occasional mixing. The slurry was centrifuged at 12 000 rpm for 10 min and the supernatant was collected. Equal volume of chloroform: isoamyl alcohol (24:1) was mixed thoroughly with the supernatant by vigorous shaking and centrifuged at 12 000 rpm for 10 min. The upper aqueous layer was collected and 0.8 volume of isopropyl alcohol was mixed with it. Following 10 min of incubation in ice, the mixture was centrifuged at 12 000 rpm for 10 min. The pellet of genomic DNA thus obtained was washed with nuclease free 70% ethanol, dried and dissolved in 50 µL of nuclease free deionised water.

PCR analysis for presence of hygromycin phosphotransferase gene

PCR was done to confirm the integration of the T-DNA into the genomic DNA of rice. The forward primers for hpt sequence were 5'GCTCCTACAAATGCCATCA3' and the reverse primer used was 5'GATAGTGGGATTGTGCGTCA3'. PCR reaction cycles was carried out as follows- initial denaturation for 5 min at 94°C, 35 cycles of 30s at 94°C, 45s at 55°C and 1min at 72°C; final extension at 72°C for 10 min. PCR products were visualized in 1% agarose gel stained with 0.5 µg ml^{-1} ethidium bromide.

Gus activity assay

The expression of GUS was assayed in rice calli essentially as described by Rueb et al. (1994) with 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) as substrate. Segments of rice calli were incubated in phosphate buffer (50 mM $NaPO_4$, pH 6.8) at 37°C for 1h. The buffer was decanted and fresh phosphate buffer containing 1.0 mM X-Gluc and 20% methanol was added to the calli. The mixture was vacuum infiltrated into the tissues for 5 min and then the tissues were incubated in that mixture at 37°C overnight. Finally, the tissues were visually examined under stereo microscope (Radical, India). The GUS positive calli showed dark blue patches.

RESULTS

Overview of *Agrobacterium* mediated transformation of Ratna (IET 1411) rice

Healthy mature dehusked IET 1411 rice seeds were surface sterilized with 30% aqueous solution of sodium hypochlorite for 30 min, washed with sterile distilled water and placed on MS medium supplemented with 0.5 gL^{-1} CH and 4 mgL^{-1} of 2, 4-D for callus induction. In this method, we have used a simple callus induction medium

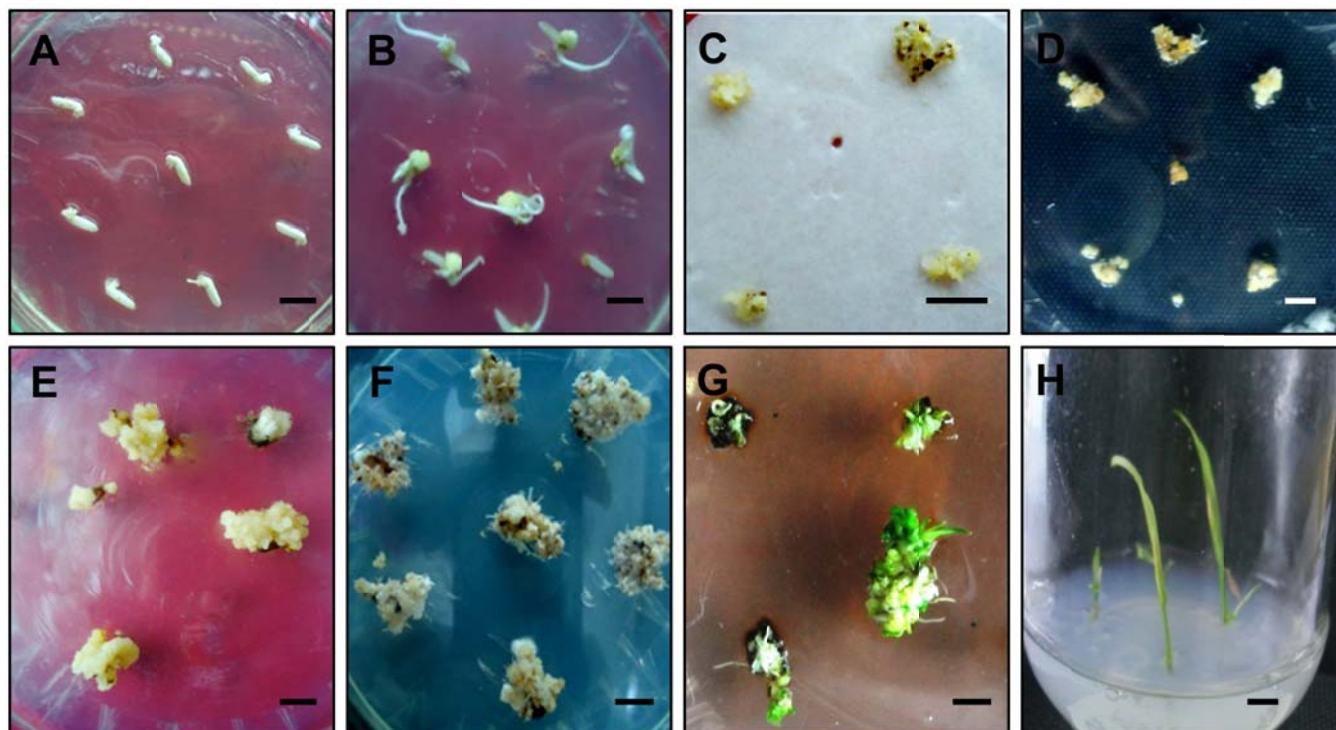

Figure 2. Steps for *Agrobacterium* mediated transformation and regeneration from rice calli. (A) Sterilized seed explants; **(B)** callus initiation on callus induction medium (CIM). **(C)** co-cultivation of calli with *Agrobacterium tumefaciens* (LBA4404). **(D, E, F)** Putatively transformed calli on first, second and third selection media that is SIM1, SIM2, SIM3 respectively. (G) Shoots regenerated from transformed calli on regeneration media. (H) Transformed shoots of rice on ½ strength MS agar medium for initiation of roots. Steps A-C was followed in the dark. Bars represents 3 mm.

with only 2, 4-D and without the use of any other hormones or plant growth regulators. These seed explants were sub-cultured onto fresh callus induction media (CIM) with the same composition every 10 days for 2 months. Non-viable brown parts of the calli were removed with scalpel during each transfer. Figure 2A shows the initial stage of callus induction from rice seed explants 2 days after placement on media and Figure 2B shows 10 day old calli. Actively proliferating parts of the calli were used subsequently for infection with *Agrobacterium*.

For preparation of bacterial culture, *A. tumefaciens* strain LBA4404 harboring pCAMBIA 1301 binary vector was cultured in liquid AB media supplemented with 50 µg ml^{-1} Kanamycin. Rice calli were infected using *Agrobacterium* culture with OD_{600} value of 0.35-0.66 resuspended in AAM media. This optimized concentration of *Agrobacterium* is much lower than previous protocols for other idica rice varieties. The inoculated calli were transferred on a sterile filter paper soaked in AAM media which was placed on co-cultivation media (CCM) containing 100 µg mL^{-1} acetosyringone (Figure 2C). Co-cultivation was carried out in the dark at 25°C for 3 days. Infected calli were washed with cefotaxime and transferred to the selection medium SM-1 (Figure 2D). After 15 to 20 day of incubation the proliferating calli were transferred onto SM-2 (Figure 2E) and subsequently onto SM-3 (Figure 2F). The viable calli were transferred onto the regeneration medium MMS with 20 µg ml^{-1} hygromycin and 250 µg ml^{-1} cefotaxime and incubated at 28±1°C under 16/8h light/dark photoperiod (Figure 2G). To induce root initiation, putatively transformed regenerated shoots were separated from the callus tissues, placed in MS basal media of half the strength with 1% agar, pH adjusted to 5.8 for rooting (Figure 2H). The whole work flow for the *Agrobacterium* mediated transformation of IET 1411 rice is summarized in Figure 3.

Standardization of callus inducing medium

Callus induction as well as regeneration potential of tissue is affected not only by type of explants but also the by the culture conditions and composition of the culture medium including concentration of plant growth regulators (Revathi and Pillai, 2011). It has been reported that the particular genotype of explants used was an important factor for successful embryogenic callus induction and regeneration (Rueb et al., 1994).

MS-CH media was used as callus induction medium along with the best suited hormone supplement that is, 4 mgL^{-1} 2-4D (Figure 4A to L). For standardization of callus induction, four different media compositions were used as

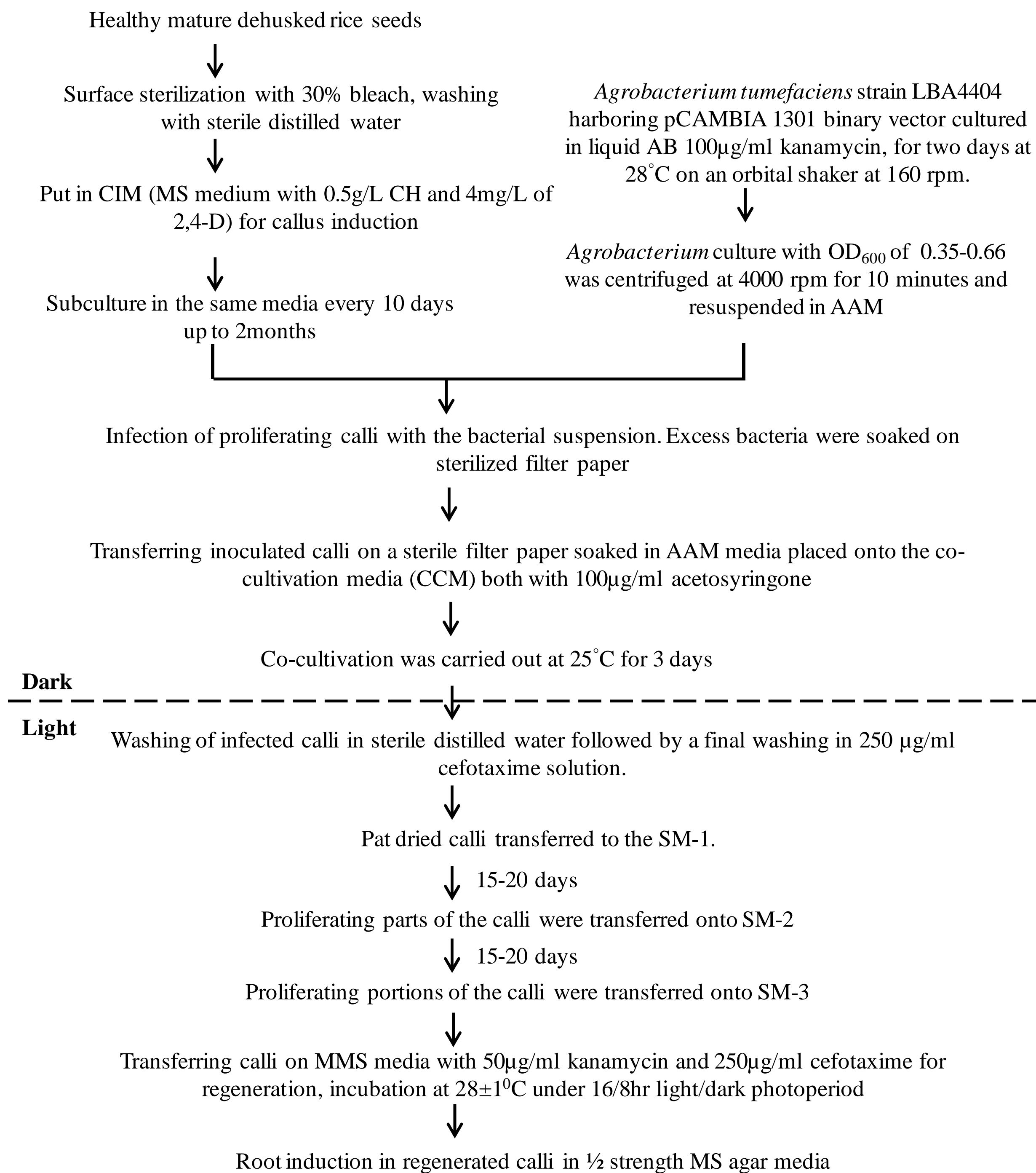

Figure 3. Schematic workflow of the protocol**.** The flow chart briefly describes the steps of *Agrobacterium* mediated transformation of IET 1411 rice Ratna.

shown in Table 2. In the case of CIM2 where proline was added, lower percentage of callusing of about 72% was obtained with respect to CIM1. This is in accordance with earlier reports (Rahman et al., 2011). Here the best callus induction frequency was about 85% with CIM1 (Figure 5C). The average callus size was larger of about 6 mm in diameter.

The presence of proline or maltose or the absence of CH reduced the frequency of callusing. After placement of the seeds on this media, callus development started within 3 to 4 days from the scutellum tissue. This primary callus developed for 5 to 10 days growing to 2 to 6 mm in diameter. Approximately, 85% seeds developed mature embryogenic callus from primary callus. This mature

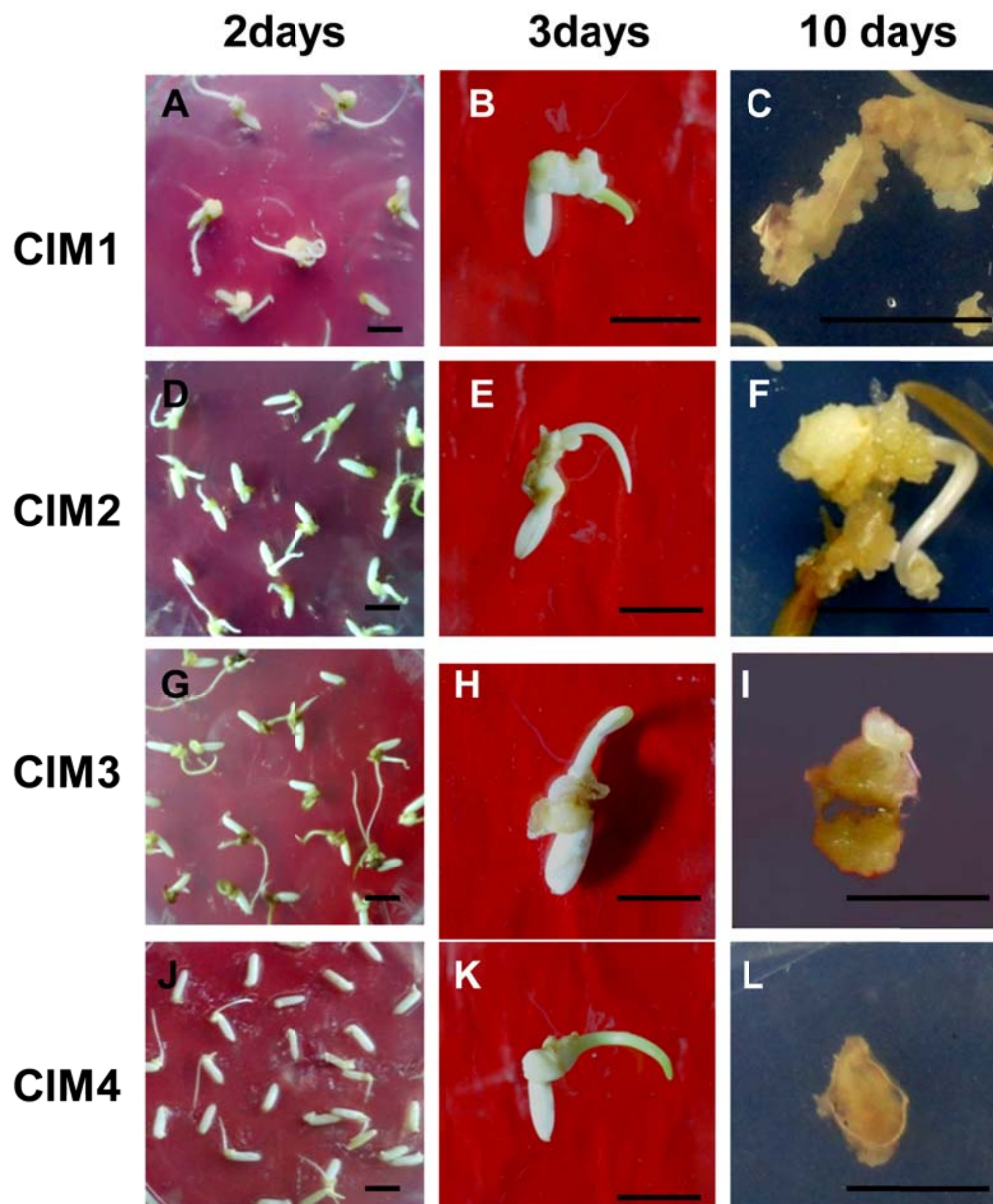

Figure 4. Morphological differences between rice calli in different callus inducing media. Left column shows multiple explants at 2 days after inoculation. Middle and right panel shows morphology of an individual callus at 3 days and 10 days after inoculation in callus inducing media; A, B, C, CIM1; D, E, F, CIM2; G, H, I, CIM3; J, K, L, CIM4. Bars represent 3 mm.

Table 2. List of different callus inducing media (CIM) used.

Media	Composition
CIM 1	MS-CH, 2-4D 2.5 mg L^{-1}, pH~5.8.
CIM 2	MS-CH, Proline (2.5 g L^{-1}), pH~5.8.
CIM 3	MS, Maltose (30 g L^{-1}), 2-4D 2.5 mg L^{-1}, pH~5.8.
CIM 4	MS, 2-4 D 2.5 mg L^{-1}.

embryogenic, nodular, creamish white, friable calli were maintained for 5 days before infection with *Agrobacterium.*

2, 4-D is known to facilitate callusing from rice seed explants. Different concentrations of 2,4-D were used for optimization. Development of calli from seeds were more frequent on the callus inducing media containing 4 mgL^{-1} 2,4-D in comparison to those containing 2.5 and 3 mgL^{-1} 2,4-D (Figure 5C). Moreover these calli were proliferative and bigger in size. The colour of these compact calli was creamish white. But further increase in the concentrations of 2,4-D influenced negatively on the compactness and colour of calli. Therefore 2, 4-D at 4 mgL^{-1} concentration was found to be most suitable and induced the highest frequency (87.7±4.6%) of callusing from the explants

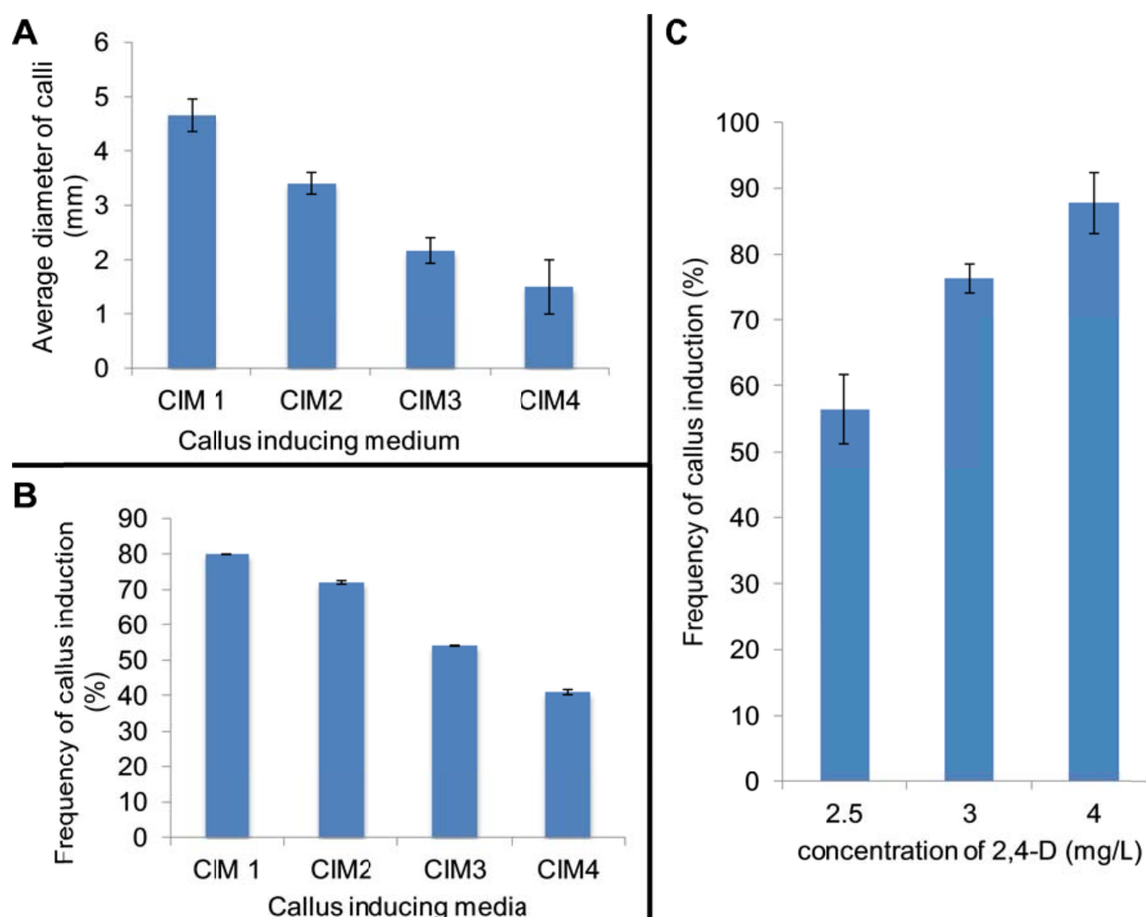

Figure 5. Optimization of media components for callus induction. Effect of different media compositions (CIM1, CIM2 CIM3 and CIM4) on (A) size of calli and (B) frequency of callus induction. (C) Effect of concentration of 2,4-D on frequency of callus induction.

(Figure 5C). Further increase in the concentration of 2, 4-D resulted in decrease and non-uniformity in the frequency of callusing (data not shown). The intermediate concentrations of 2.5 and 3 mgL^{-1} induced 56.47±5.2 and 76.3±2.2% callusing (Figure 5C). Hence, subsequent experiments were done with 4 mgL^{-1} 2, 4-D.

Regeneration of transformed rice plantlets

Mature embryogenic calli were used to standardize the regeneration medium for this variety of rice. Older calli more than 7 days became non-embryogenic. Such calli went on proliferating, when subcultured on regeneration medium but failed to form plantlets. In our study 5 days old calli were most suitable for regeneration.

Regeneration was successful in the modified MS media (MMS) containing MS salts, B_5 vitamins, CH 2 gL^{-1}, maltose 30 gL^{-1}, sorbitol 30 gL^{-1}, BAP 4 mgL^{-1}, NAA 0.2 mgL^{-1}. After placement of the calli on regeneration media and incubated at 28±1°C, 70% of the calli regenerated small shoots within 2 to 3 weeks (Figure 2G).

Effect of concentration of *Agrobacterium* for inoculation of calli, co-cultivation condition and co-cultivation period on transformation efficiency

Prior to infection, the *Agrobacterium* was cultured in liquid AB medium containing inorganic salts and glucose as carbon source to increase its virulence (Hiei et al., 1994). An experiment was designed using seven different densities of *Agrobacterium* cells, OD_{600} being 0.1, 0.13, 0.15, 0.20, 0.38, 0.66 and 0.69 (Rahman et al., 2011). Different durations of co-cultivation periods (2, 3 and 5 days) were also used to optimize the conditions. Calli cocultivated for 2 days showed negligible GUS expression (0.6 to 1% of calli) and excess period that is, 5 days of cocultivation resulted into damage and browning of calli. Hence, 3 days of cocultivation period was optimum for regeneration from transformed calli. GUS expression was observed and its percentage was quantified as a measure of transformation frequency. The result shows that with 0.66 optical density of *Agrobacterium* culture, the transformation efficiency was maximized (Figure 6A to H). During *Agrobacterium* infection, OD600 of the bacterial

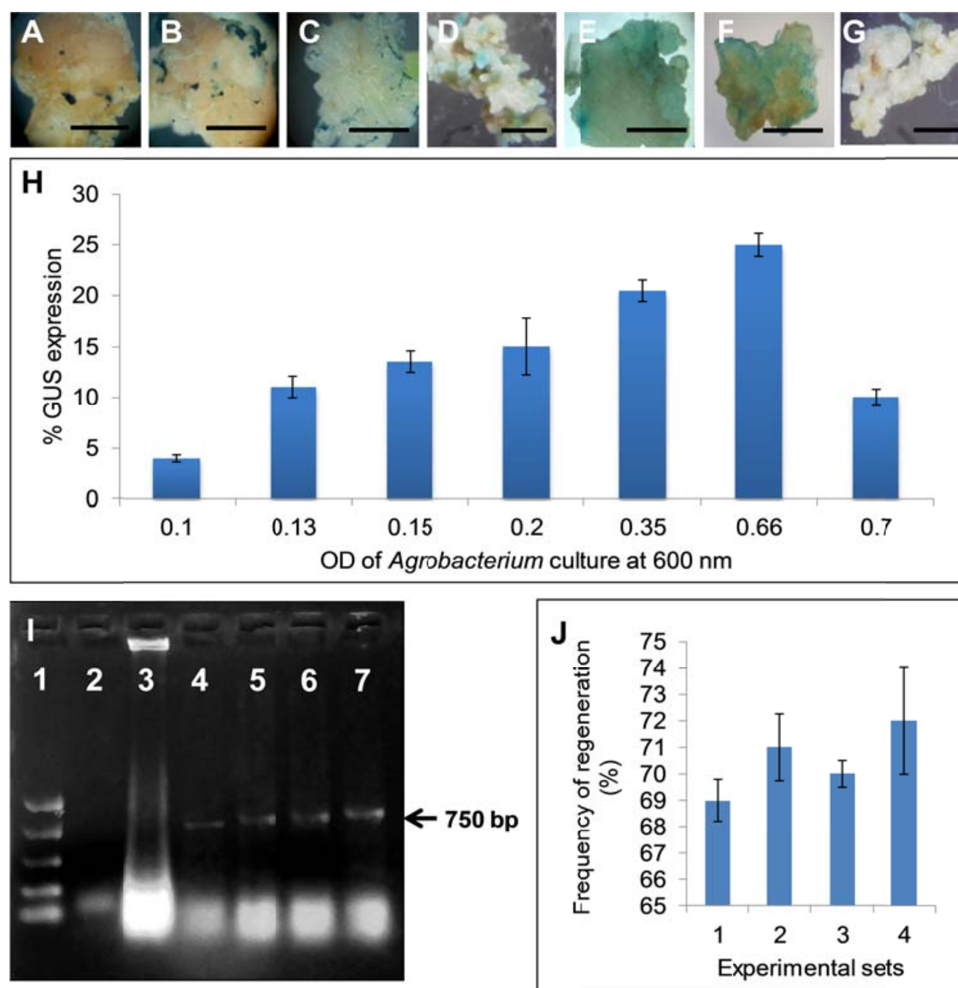

Figure 6. Effect of *Agrobacterium* concentrations on transformation efficiency of rice calli and analysis of transformed rice plants. GUS expression in individual calli transformed with *Agrobacterium* cultures having OD_{600} = 0.1 (A), 0.13 (B), 0.15 (C), 0.2 (D), 0.38 (E), 0.66 (F), 0.69 (G). Bars =2mm (A-G). (H) Percentage of GUS expression in calli transformed using different concentration of *Agrobacterium*. Each bar represents mean from three experiments ± standard deviation. (I) PCR analysis of DNA isolated from transformed rice plantlets. Lane 1, DNA marker; lane 2, PCR of genomic DNA from untransformed rice plantlet as negative control; lane 3, positive control; genomic DNA of transformed rice tissue; lanes 4 to 7; PCR products from genomic DNA of transgenic rice calli carrying hpt gene (750 bp) (Line 1-4). (J) Regeneration frequencies in transformed rice of different experimental sets.

suspension must be adjusted within 1.0 because high concentrations of bacteria caused serious injury of the callus thus lowering the transforming efficiency. Co-cultivation with filter paper (Ozawa, 2009) increased transformation frequency because the filter paper prevented overgrowth of bacteria. In the co-cultivation media we used cysteine which reduces browning of the calli (Ozawa, 2009), aceto-syringone and glucose to induce the *vir* gene activity.

Selections of hygromycin resistant calli

After *Agrobacterium* infection, infected calli were selected on the callus induction media with hygromycin as selection agent. Hygromycin clearly demarcated the transformed calli from non-transformed tissues which failed to regenerate. After first round of selection, only proliferating portions of the calli were transferred onto the fresh selection medium for second round of selection and so on (Figure 2E to G).

GUS positive shoots indicated correct integration and expression of the T-DNA

Competence was assured by having an established totipotency while expression of gusA gene immediately confirmed that these calli were also competent for transformation. High frequency of GUS expression was

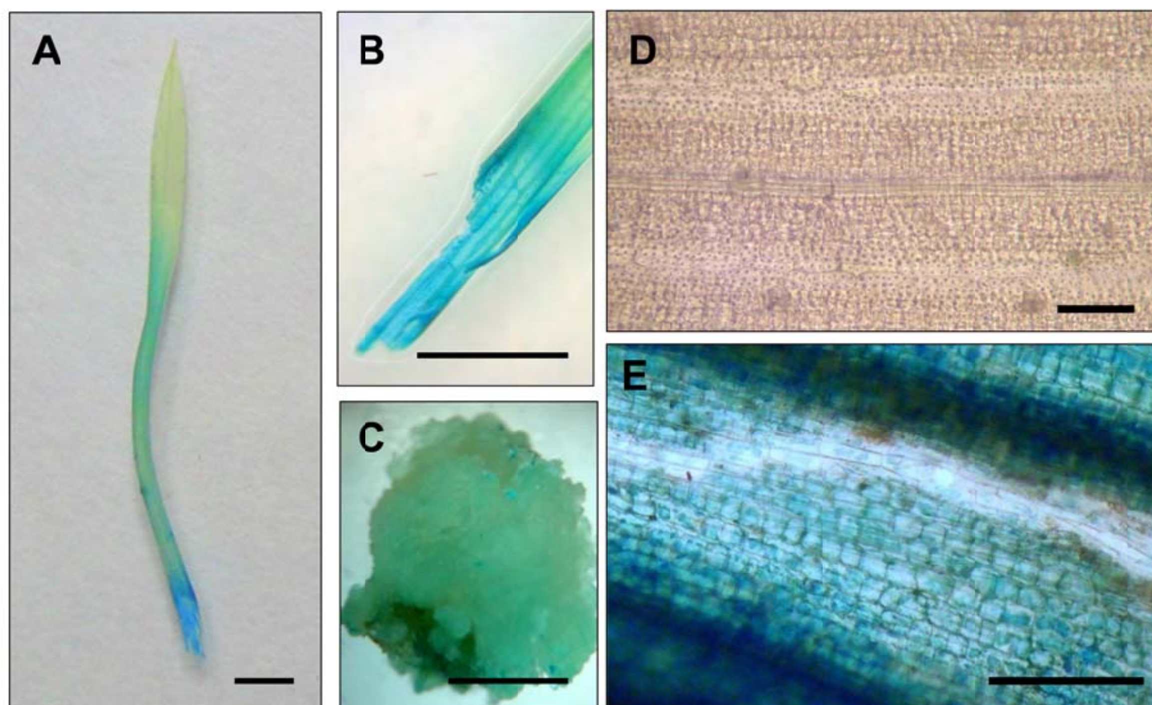

Figure 7. Histochemical assay for expression of the *gus*A gene in calli and mature leaf tissues of *Agrobacterium*-transformed rice. (A) Gus expression in transformed rice leaf. **(B)** a leaf sheath. (C) transformed callus of rice. **(D)** Foliar histology of untransformed control. **(E)** Transformed rice plant after GUS histochemical assay. Bars in A, B and C represent 3mm and bars in D, E represent 50 µm.

routinely observed between 4 to 25% (Figure 6H). The expression of GUS reporter gene was found to be a reliable indicator for subsequent production of stable transgenic lines. The shoots that developed from transformed GUS positive rice calli were also GUS positive. Uniform blue colouration in the leaf tissues of transformed plantlets signified the stable integration and expression of the T-DNA in the plant genome (Figure 7A, B). Figure 7D, E shows clearly difference between the untransformed control and the GUS positive leaf cells of rice. Figure 7C shows GUS expression in a transformed callus.

Regenerated shoots that developed from the hygromycin resistant calli, were all PCR positive for presence of *hpt* gene sequence. This data indicate that T-DNA was properly integrated into the genomic DNA of these plantlets. GUS assay was performed for all the hygromycin resistant calli as well as the first leaves of regenerated shoots. All the calli and the leaves showed blue coloration when incubated in the X-gluc solution indicated expression of the GUS transgene.

Confirmation of presence of hygromycin phosphotrasferase transgene in rice calli by PCR

In the present study hpt gene was used as the selectable marker and hygromycin B as the selective agent for screening of transformed calli. The transformation efficiency of this experiment was measured by the proportion of *hpt* positive calli to the total number of rice calli produced. Genomic DNA of transformed calli was analysed by PCR using specific primers for hpt gene. PCR analysis confirmed the integration of a fragment of expected size 750 bp in the transformed lines (Figure 6I). Out of 22 calli of rice *hpt* gene was integrated in 11 PCR positive calli. Hence the transformation frequency was 47%.

DISCUSSION

Rice transformation systems have relied on the use of protoplast, microprojectile bombardment and *A. tumefaciens* (Cheng et al., 1998; Repellin et al., 2001; Panahi et al., 2004; Vila et al., 2005; Zaidi et al., 2006; Ramesh et al., 2009). Immature embryos were the first explants to be used for successful transformation of rice back in 1993 which resulted in embryogenic calli (Li et al., 1993). Subsequently many attempts resulted in successful transformation of Japonica rice. On the other hand, transformation protocols for *Agrobacterium* mediated transformation Indica varieties are few. The transformation efficiency of the indica rice variety using *Agrobacterium* is much lower than that of japonica cultivars and leaves room for improvement (Tie et al., 2012). Paucity of reports of successful development of transgenic indica rice indicates the difficulties in the tissue culture and transformation of indica. There are multiple

causes behind the sensitivity of indica rice to tissue culture systems and its poor response in transformation (Ge et al., 2006; Zaidi et al., 2006). The main reason being the resistance of Indica rice to infection by *Agrobacterium* which has been directly linked to defense related gene expression (Tie et al., 2012). Manipulation of plant growth regulators as well as organic and inorganic components and salts within the culture media was done in previous reports for establishment of a media suitable for callus induction of indica rice (Ge et al., 2006).

In this study, a reproducible transformation protocol was standardized using seed explants of indica rice cultivar Ratna (IET 1411). For induction of callus, we have optimized a media with minimum number of components. In this callusing medium, 2,4-D was used as the only growth regulator which induced callusing in 87.7% explants. In previous reports a number of growth regulators including Phenyl acetic acid (NAA, BAP, PAA), kinetin in different combinations with 2,4-D have been used but with less efficiency (Tiwari et al., 2012; Ge et al., 2006). In various systems of rice tissue culture supplements like proline, piclorum, maltose etc. were an inevitable part of the callus inducing media. The present protocol includes a new a callus inducing media (CIM 1) with casein hydrolysate as a supplement and 2,4-D. Though addition of either proline or maltose in the MS basal media showed callus induction as well as proliferation of the calli, the maximum callus induction was observed in CIM 1 without these components.

The modified transient GUS expression assay (Rahman et al., 2011) proved to be a rapid method for standardization of concentration of the *A. tumefaciens* cell suspension and optimization of the transformation efficiency. The optimum OD_{600} of *Agrobacterium* cell suspension deduced with this protocol partially supports the result of other reports of efficient *Agrobacterium* mediated transformation system (Sahoo et al., 2012). Though previous studies (Rahman et al., 2011) considered the *Agrobacterium* cell suspension with OD_{600} value 0.8 to 1 as optimum for rice transformation, here the transformation efficiency obtained during transient GUS expression assay gradually increased with increase in OD_{600} value of bacterial cell suspension up to 0.66 but higher concentration of bacterial probably resulted in damage of explant tissues.

Several factors influence the successful transfer of T-DNA into rice of which Acetosyringone, being the induction agent of *vir* genes, is a crucial one. Earlier research used 150 µM Acetosyringone for transforming indica rice and obtained the maximum transformation frequency of 12% and 200 µM Acetosyringone decreased the percentage of regeneration (Sahoo et al., 2012). The regeneration frequency of Ratna (IET 1411) was 4 times higher when 100 µM acetosyringone was used. Higher concentration of Acetosyringone (200 µM) in cocultivation media resulted in lower frequency of regeneration likely due to necrotic effect of acetosyringone (Sreeramanan et al., 2009).

As the Indica rice Ratna (IET 1411) used in the present report is regularly grown in rice producing fields of India and other parts of south East Asia, this protocol will be useful in transgenesis using superior traits. Moreover it seems likely that the transformation system established here would help in transgenic development of closely related genotypes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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