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Agrobacterium Mediated Genetic Transformation of Rice for Salinity Tolerance for Combating Climate Change

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Abstract: *Rice based coastal livelihoods of Bangladesh are now vulnerable for sea-level rising and salinity stress. Transgenic salt tolerant rice were developed by inoculating mature embryos with Agrobacterium tumefaciens strain LBA4404 carrying a binary vector p^{CIPK} with GUS and nptll gene. Transformation was performed with different infection times and co-cultivation periods. Infection was most effective when explants were inoculated for 25 minutes (69.444 % GUS positive) and co-cultivated for 4 days (72.22% GUS positive). Among the varieties, Binadhan-6 showed highest response to GUS assay (65.185% GUS positive). Putative transformed plantlets were also the highest in Binadhan-6 (7.222%). Infection time 25 minutes and 4 days co-cultivation period were effective for regeneration of shoots (9.444 % and 12.222 %, respectively). Binadhan-6 produced the greatest number of rooted shoots (60%). This protocol should help to develop salinity tolerance rice line(s) for combat climate change.*

Keywords: *Rice, Agrobacterium tumefaciens, Genetic transformation and Salinity*

I. Introduction

Rice (Oryza sativa L.) is the staple food in Bangladesh, occupying 11.0 million ha, with a total production of 338 million tons [1]. More than one million hectare of rice land including 53% of the coastal areas is salt affected due to global warming and recent adverse climate change [2]. Soil salinity (4-15 dSm^{-1}) is one of the most brutal environmental factors from seedling to harvesting stage [3]. Though some salinity tolerant varieties are already developed but these varieties are not suitable to overcome higher stresses of salinity in rice production to combat climate change. Beside conventional breeding, genetic transformation plays a vital role to introduce novel genes into crop, will be essential in complementing existing climatic stress [4]. New breeding lines should develop for better adapted varieties for salinity prone area. There is a tremendous variation for salt tolerance within species in rice providing opportunities to improve crop salt-stress tolerance through genetic transformation [5].

Agrobacterium-mediated transformation usually results in the insertion of unrearranged segments of DNA into recipient plant genomes, often at low copy number [6]. Additionally, relatively large DNA fragments with defined ends (i.e. left and right T-DNA borders) can be integrated at high frequency into recipient plant genomes [7]. Agrobacterium-mediated transformation is now the preferred method of gene delivery into rice, as this procedure has several advantages, compared to direct DNA uptake procedures [8] and [9]. It is a powerful and important tool. So an efficient and reproducible transformation protocol is required for successful genetic transformation.

The objectives of this experiment were optimizing efficient, reproducible and powerful genetic transformation protocol of rice using Agrobacterium tumefaciens and transferring salinity tolerance gene into modern rice cultivars.

II. Materials and methods:

The experiments were carried out at the Tissue Culture Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh in 2010 by using Binadhan-5 and Binadhan-6. Agrobacterium tumefaciens strain LBA4404 was used for infecting the embryogenic calli in the transformation process. This strain contains plasmid p^{BI121}, udiA gene [10] encoding GUS gene GUS (β-glucuronidase), driven by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation. The nptll gene [11] encoding neomycin phosphotransferase Il (nptll) conferring Kanamycin resistance. The hpt gene conferring hygromycin resistence driven by NOS promoter and NOS terminator and T-DNA border sequences (RB, LB).

2.1 Media used:

For callus induction, callus proliferation, shoot regeneration and rooting MS medium [12] was used. MS media was used as a basal medium for both callus induction and regeneration of explant.

2.2 Sterilization:

To ensure aseptic condition in in vitro, all instruments, glasswares and culture media were sterilized properly by autoclaving at 121^{o}C for 30 minutes at 1.16 kg cm^{-2} pressure.

2.3 Agrobacterium culture and inoculation:

Two different types of culture media, namely, YMB (Yeast extract Mannitol Broth) medium and LB (Luria Broth) medium were used with kanamycin as antibiotic to grow the strain of genetically engineered Agrobacteriurm tumefaciens. Here two sorts of media were used, such as, Agrobacterium maintenance medium and Agrobacterium working culture medium for transformation.

2.3.1 Agrobacterium Co-cultivation:

T_1: MS medium+1.00 mg L^{-1} 2,4-D + 100 µm acetosyringone
T_2: MS medium + 1.50 mg L^{-1} 2,4-D + 100 µm acetosyringone

2.3.2 Post-cultivation and shoot differentiation:

T_1: MS medium + 6 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA + 200 mg L^{-1} Cefotaxime
T_2: MS medium + 8 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA + 200 mg L^{-1} Cefotaxime

2.3.3 Low selection:

T_1: MS medium + 6 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA + 100 mg L^{-1} Cefotaxime + 10 mg L^{-1} Kanamycin
T_2: MS medium + 8 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA + 100 mg L^{-1} Cefotaxime + 10 mg L^{-1} Kanamycin

2.3.4 Selection and regeneration:

T_1: MS medium + 6 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA + 75 mg L^{-1} Cefotaxime + 15 mg L^{-1} Kanamycin
T_2: MS medium + 8 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA + 75 mg L^{-1} Cefotaxime + 15mg L^{-1} Kanamycin

2.4 Transplantation of plantlets from growth chamber's to earthen pot:

Soil containing 25% garden soil + 50% sand + 25% cowdung in pots placed in growth chamber having 4500 Lux light at 25±1°C for seven days.

2.5 Culture techniques: The following culture techniques were employed in the experiment-

2.5.1 Agrobacterium culture:

Two kinds of culture media were needed for the Agrobacterium strain as stated earlier. One is for maintaining Agrobacterium stock and the other for the infection of explants. One single colony from previously maintained Agrobacterium stocks was streaked into freshly prepared petridish containing YMB medium having kanamycin. The petridish was sealed with parafilm and kept in room temperature for at least 48 hours at 4°C. For infection, single streak from Agrobacterium stock was taken in an inoculation loop and was inoculated in a conical flask containing liquid LB medium with 50 mg L^{-1} kanamycin. The culture was allowed to grow at 28°C to get optimum population of Agrobacterium for infection and co-cultivation of explants.

2.5.2 Infection and incubation:

Following the determination of "Optical Density" by spectrophotometer at 600 nm, to get suitable and sufficient infection of the explants, freshly excised explants were wounded and immersed in a culture of the preinduced bacteria for 20-30 minutes with gentle shaking and then transferred them to co-cultivation medium.

2.5.3 Co-cultivation:

The explants were co-cultured on co-cultivation medium. All the explants were maintained in co-cultivation media for 3-5 days. Co-cultured petridishes containing explants were placed under fluorescent illumination with 16/8 hours light (1500 lux) /dark cycle at 25±2°C.

2.5.4 GUS (β-glucuronidase) histochemical assay:

Co-cultured explants tissues were immersed in X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution and were incubated at 37°C overnight. A characteristics blue color would be the expression of GUS (β-glucuronidase) gene in the plant tissue. Proper control for GUS histochemical assay was done with the explants having no Agrobacterium infection. After X-gluc treatment explants were transferred to 70% alcohol for degreening. The degreening explants were observed under stereomicroscope.

2.5.5 Washing and post-cultivation:

The infected explants were washed twice with distilled water and once with liquid MS media supplemented with 200 mg L^{-1} cefotaxime. Then the explants were transferred onto post-cultivation medium.

2.5.6 Transfer to the selection medium for shoot regeneration:

Following 8-10 days of post-cultivation, the explants were transferred onto low selective medium (LSM). After culture for ten days, the hypocotyls with calli were transferred onto selection and regeneration medium (SRM) for further selection and shoot regeneration. After 10-12 days, the calli were subcultured once on selection and regeneration media.

2.5.7 Transfer of the transgenic shoots for root initiations:

The green shoots (2-3 cm) were separated from each other and again were cultured on petridishes with freshly prepared rooting medium (RTM) to induce root. The vials containing plantlets were incubated at 22±2°C with 16 hrs photoperiod.

2.5.8 Transplantation:

The plantlets were transplanted to pots. Pots were kept in growth chamber for 7 to 15 days under controlled environment, also nourished with Hoagland's solution. After 15 to 20 days they were transferred to the field condition.

2.5.9 Data recording and analysis:

The effect of different treatments data were collected on following parameters: Number of explants positive for GUS, transformed plantlet regeneration, days to shoot initiation, no. transgenic plantlets and average number of shoots with root. The Completely Randomized Design (CRD) was used for this experiment. The analyses of variances for different parameters were performed and means were compared by the Duncan's Multiple Range Test (DMRT) in MSTATC program.

III. Results and discussion:

Agrobacterium- mediated genetic transformation is a powerful and important tool. An efficient and reproducible transformation protocol is required for successful genetic transformation.

3.1 Histochemical GUS (β-glucuroronidase) assay on transformation efficiency:

Histochemical GUS assay was carried out to assess the expression of GUS gene in the calli as described by [13]. Blue patches indicated GUS activity, confirming GUS expression in co-cultivated callus tissues. Transient GUS assay was done at the end of co-cultivation with randomly selected 20% inoculated explants tissue. In the GUS assay, conspicuous GUS positive (blue color) regions were detected in the explants surface (Plate 3.1, 3.2; Table-3.1).

Table 3.1: Effects of infection time and co-cultivation periods on GUS histochemical assay Agrobacterium tumefaciens containing binary plasmid P^{CIPK} of two indica rice varieties

Variety	Co-cultivation period (Day)	Infection Time (minute)	No. of explants incubated	Explants No. assayed for GUS	Explants No. +ve for GUS	% GUS +ve explants
Binadhan-5	3	20	30	10	5	50
		25	30	10	6	60
		30	30	10	4	40
	4	20	30	10	5	50
		25	30	10	7	70
		30	30	10	5	50
	5	20	30	10	3	30
		25	30	10	5	50
		30	30	10	3	30
Binadhan-6	3	20	30	10	5	50
		25	30	10	7	70
		30	30	10	4	40
	4	20	30	10	8	80
		25	30	10	9	90
		30	30	10	8	80
	5	20	30	10	5	50
		25	30	10	7	70
		30	30	10	4	40

3.2 Effects of infection time on transformation efficiency:

The calli were infected for 20, 25 and 30 minutes and was found a wide range of variation of explants for GUS assay. It was observed that GUS response increased with the time of infection. Calli showed the highest (69.444%) response to the GUS assay in case of 25 minutes infection time followed by 25 minutes (90%) of calli and the lowest (50.556%) response to the GUS assay in 30 minutes (Fig.3.1).

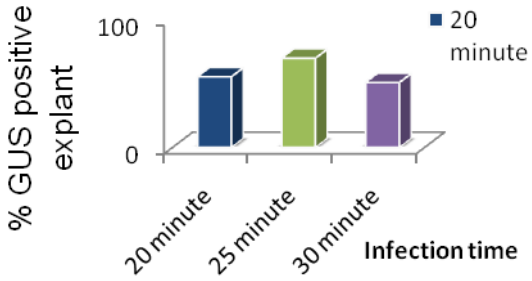


Figure 3. 1: Effects of infection time on transformation efficiency

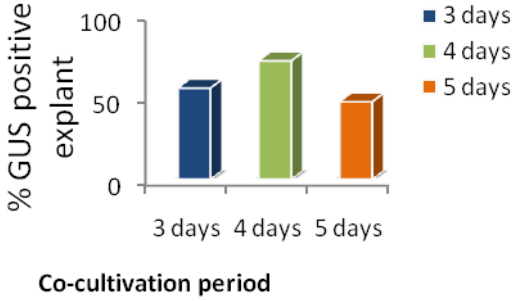


Fig. 3.2: Effects of co-cultivation period on percentage of explants positive (+ve) for GUS

3.3 Effects of co-cultivation periods on transformation efficiency:

Different co-cultivation periods viz. 2, 3, and 4 days were followed using bacterial suspension having constant optical density (OD_{600}=0.6). The highest percentage of GUS positive calli (72.22 %) were found in co-cultivation period of 4 days followed by 3 days (55.556 %) and it was least (47.22 %) in co-cultivation period of 5 days (Fig. 3.2). Also the highest percentage (80%) of calli was stained after 4 days by [14].

3.4 Effects of Variety X co-cultivation period interactions on percentage of explants positive (+ve) for GUS:

Variety x co-cultivation period interactions on GUS histochemical assay showed statistically significant differences for percentage of explants positive (+ve) for GUS. Binadhan-6 showed the highest (83.333 %) percentage of explants positive (+ve) for GUS on 4 days and lowest (38.889%) by Binadhan-5 on 5 days (Table 3.2). Also the level of transient GUS expression after co-cultivation with Agrobacterium in the two indica rice cultivars varied with genotype showed by [15]. . Percent transient GUS expression observed in this study were comparable to earlier reports in indica rice cultivars.

Table 3.2: Effects of variety x co-cultivation period interactions on percentage of GUS (+ve)

Variety	Co-cultivation period (Day)	%GUS (+ve) explants
	3	54.444 b
Binadhan-5	4	61.111 b
	5	38.889 c
	3	56.667 b
Binadhan-6	4	83.333 a
	5	55.556 b
CV (%)		11.66
$LSD_{(0.05)}$		6.505

Table 3.3: Effects of variety x infection time interactions on percentage of GUS (+ve)

Variety	Infection time (min.)	% GUS (+ve) explants
Binadhan-5	20	48.889 c
	25	63.333 b
	30	42.222 d
Binadhan-6	20	61.111 b
	25	75.556 a
	30	58.889 b
CV (%)		11.66
$LSD_{(0.05)}$		6.505

In the column figures followed by same letter(s) do not statistically significant

3.5 Effects of variety x infection time interactions on percentage of explants positive (+ve) for GUS:

Variety x infection time interactions on GUS histochemical assay were found statistically significant for percentage of explants positive (+ve) for GUS. Between the varieties Binadhan-6 showed the highest percentage (75.556%) of GUS positive explants followed by Binadhan-5 (63.333 %), on 25 minutes of infection time and the lowest (42.222) by Binadhan-5 on 30 minutes of infection time (Table 3.3).

3.6 Effects of Variety x co-cultivation period x Infection time interactions on percentage of explants positive (+ve) for GUS:

Variety x co-cultivation period x Infection time interactions on GUS histochemical assay showed statistically significant differences for percentage of explants positive (+ve) for GUS. Binadhan-6 showed the

highest (96.667%) percentage of explants positive (+ve) for GUS on 25 minutes of infection time and 4 days of co-cultivation period and the lowest percentage(33.333%) of explants showed positive (+ve) for GUS was on 30 minutes infection time and 5 days co-cultivation period (Table 3. 4).

Table 3.4: Effects of Variety x infection time x co-cultivation period interactions on percentage of explants positive (+ve) for GUS

Variety × Co-cultivation period × Infection time			
Variety	**Co-cultivation period (Days)**	**Infection Time (min.)**	**Percent of GUS (+ve) explants**
Binadhan-5	3	20	53.333 def
		25	66.667 bc
		30	43.333 fgh
	4	20	56.667 cde
		25	76.667 b
		30	50.000 ef
	5	20	36.667 gh
		25	46.667 efg
		30	33.333 h
Binadhan-6	3	20	56.667 cde
		25	66.667 bc
		30	46.667 efg
	4	20	76.667 b
		25	96.667 a
		30	76.667 b
	5	20	50.000 ef
		25	63.333 cd
		30	53.333 def
CV (%)			11.66
$LSD_{(0.05)}$			11.270

In the column figures followed by same letter(s) do not differ significantly

Binadhan-6

Binadhan-5

Plate 3.1 : Calli infected with Agrobaterium tumefaciens contain p^{CIPK}

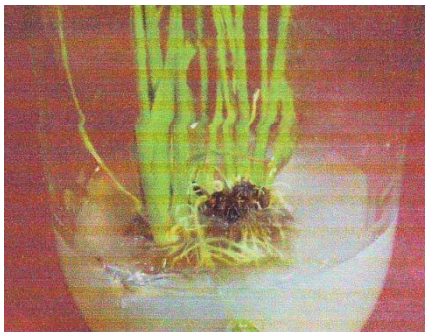
3.7 Shoot initiation:

After culturing on low selection media, calli were transferred to selection and regeneration media for shoot initiation. Calli started to initiate shoot buds after 23-45 days of incubation. The percentage of plant regeneration showed variation depending on different varieties, infection time and co-cultivation periods (Table 3.5). A few of the calli showed green spots and continued to differentiation into shoots (Plate 3.3). Among the two varieties Binadhan-6 (7.22 %) showed higher shoot initiation followed by Binadhan-5 (5.926 %) (Fig.3.3). Regeneration of putative transformed shoot was the highest (12.22 %) when calli were co-cultivated for 4 days. Calli showed higher (9.444 %) shoots regeneration when infected for 25 minutes (Fig. 3.4). The two genotypic mean values were found statistically significant for number of callus producing shoot but of two varieties tested Binadhan-6 showed higher percentage (7.222%) of transgenic shoots followed by Binadhan-5 (5.926%) presented in Table 3.5. and Fig. 3.3. Transformation efficiency greatly influenced by variety also reported by [16].

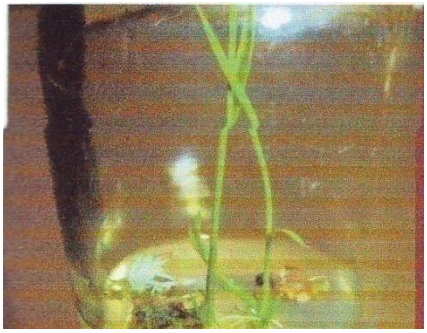
Binadhan-6

Binadhan-5

.2: Histochemical localization of GUS activity (blue zone) at the entire surface of infected calli (upper two calli of each petridish)



Binadhan-6



Binadhan-5

Plate 3.3: Transgenic plantlets of Binadhan-6 and Binadhan-5

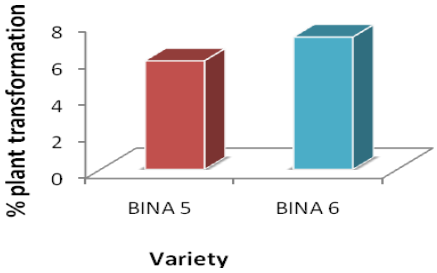


Fig. 3.3: Response of varieties towards plant transformation

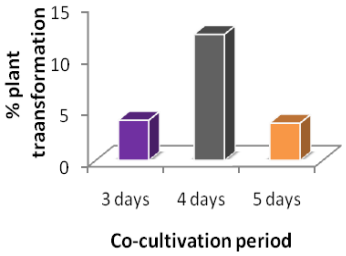


Fig. 3.4: Effects of co-cultivation periods on transformation efficiency

3.8 Effects of co-cultivation periods on transformation efficiency for shoot regeneration:

Duration of co-cultivation (mean square) showed significant variation for percentage of callus showing shoot. It was observed that among the various co-cultivation periods tested the highest percentage (12.222%) of transgenic shoots showed on 4 days followed by (3.889%) on 3 days and the lowest (3.611%) was found on 5 days which was not statistically significant (Fig.3.4). These finding are similar to [17], [18] and [19].

Table 3.5: Response of two rice varieties towards regeneration of transgenic shoots i.e., plant transformation

Variety	Co-cultivation period (Days)	Infection Time (minutes)	Number of explants incubated	Number of shoot produced	%plant transformation	Days required for shoot initiation
Binadhan-5	3	20	20	0	0	0
		25	20	2	10	22-18
		30	20	1	5	30-35
	4	20	20	3	15	26-30
		25	20	3	15	21-27
		30	20	2	10	29-36
	5	20	20	0	0	0
		25	20	2	10	30-40
		30	20	1	5	235-45
Binadhan-6	3	20	20	1	5	24-29
		25	20	2	10	25-30
		30	20	0	0	0
	4	20	20	3	15	22-25
		25	20	4	20	21-24
		30	20	3	15	25-30
	5	20	20	1	5	31-38
		25	20	1	1	30-35
		30	20	0	0	0

3.9 Effects of infection time on transformation efficiency for shoot regeneration

The various levels (mean square) of infection time showed significant variation for number of callus showing shoot. It was observed that among the various infection times tested the highest percentage (9.44%) of transgenic shoots showed on 25 minutes followed by (5.0%) number of shoots per callus on 30 minutes and the lowest (5.278%) was found on 20 minutes which was not statistically significant (Fig. 3.5). These results are concordance with [20] who stated that prolonged infection time adversely affects the callus growth and subsequent regeneration.

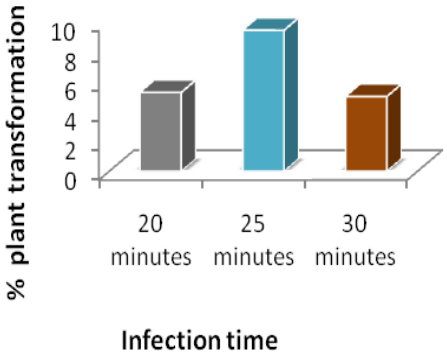


Figure 3.5: Effects of infection time on transformation efficiency for shoot regeneration

3.6: Effects of Co-cultivation period × Infection time interaction on transformation efficiency of two indica rice varieties Same letter(s) do not statistically significant

Co-cultivation period (Days)	Infection time (minutes)	% putative transgenic shoot regenerated
3	20	2.500 d
	25	5.833 bcd
	30	3.333 d
4	20	9.167 bc
	25	17.500 a
	30	10.000 b
5	20	4.167 cd
	25	5.000 bcd
	30	1.667 d
CV (%)		60.35
$LSD_{(0.05)}$		4.646

3.10 Effects of Co-cultivation period × Infection time interaction on transformation efficiency

Co-cultivation period × Infection time interactions on shoot regeneration were found statistically significant for percentage of transgenic shoots regeneration. Out of three (3,4,5 days) Co-cultivation period 4 days was shown the highest (17.500 %) percentage of transgenic shoots on 25 minutes of infection time and the lowest (1.667 %) in the variety 5 days of Co-cultivation period on 30 minutes of infection time (Table 3.6).

3.11 Root induction:

Putative transgenic shoot regenerated from Agrobacterium infected calli were transferred to root induction medium (Table-3.7). Binadhan-6 produced the highest percentage (60%) of rooted shoots. The lowest (50%) transformed rooted shoots found in Binadhan-5.

Table 3.7: Number of transgenic shoots produced roots

Variety	Number of shoots incubated	Number of shoots showing roots	Percent root initiation
Binadhan-6	15	9	60
Binadhan-5	12	6	50

IV. Summary and Conclusion:

Agrobacterium mediated genetic transformation of two indica rice varieties was carried out in this experiment. Agrobacterium tumefaciens strain LBA4404 contains the GUS (fl-glucuronidase) gene produces conspicuous blue color with X-glue through GUS histochemical assay. This blue color successfully confirmed the integration of GUS gene from bacterial plasmid into the plant cell. Among the two varieties, Binadhan-6 showed the highest percent 65.185% GUS (+ve) response to GUS assay. The 25 minutes infection time and 4 days of co-cultivation period were most effective for producing best response (69.444 and 72.222%, respectively) in GUS assay. After inoculation, calli were cultured on low selection medium and finally on selection and regeneration medium containing Kanamycin, supplemented with Cefotaxime and different phytohormones. Agrobacterium tumefaciens strain LBA4404 has p^{CIPK} gene within its T-DNA, which confirms Kanamycin resistance of transformed cells.

Regeneration of Kanamycin resistant putative transgenic shoot from induced calli greatly varied depending on infection time, co-cultivation periods and variety. The highest rate of putative transgenic shoots was found in Binadhan-6 (7.222%), in case of 25 minutes infection time (9.444 %) and four days of co-cultivation (12.222 %). After the shoot initiation, the transformed plants were established in MS medium supplemented with 0.5 mg L^{-1} IAA to induce root of shoots. For further selection against Kanamycin and to check bacterial overgrowth, 75 mgL^{-1} Cefotaxime and 20 mgL^{-1} Kanamycin were used in the media. Among two varieties, Binadhan-6 produced the highest percentage (60%) of rooted shoots. The lowest (50%) transformed rooted shoots were found in Binadhan-5. This protocol can be followed for genetic transformation of indica rice. An efficient protocol for the transformation has been developed by this procedure, which can help to develop salinity tolerance rice variety(s) to combat climate change.

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