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Optimization of *Agrobacterium tumefaciens* mediated genetic transformation protocol for aromatic rice

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Abstract

A rapid, efficient and reproducible genetic transformation protocol was optimized for four aromatic rice varieties by using the established plant regeneration protocol. Mature embryos were inoculated with *Agrobacterium tumefaciens* strain EHA105 carrying a binary vector pIG121-Hm with *GUS* (reporter gene) and *hpt* (hygromycin resistance) gene and the transformation experiment was performed by optimizing two important parameters *viz*. infection times and co-cultivation periods. The highest response to *GUS* assay was showed by Kalizira (70% *GUS* positive) followed by Pusa Basmati 1 (66.67%) when the explants were inoculated for 25 minutes and co-cultivated for three days. Twenty five minutes infection time (9.44%) and three day co- cultivation period (8.06%) were found effective for percentage of transgenic shoot regeneration. The highest percentage of putative transgenic shoots was regenerated by variety Kalizira (33.33%) followed by Pusa Basmati 1 (20.0%) and Radhunipagol (13.33%). Among the varieties, Kalizira produced the highest percentage (60%) of rooted shoots. Kalizira also showed the highest survival rate in growth chamber (75%) and in field condition (60%). The performance of Tulsimala was poor for almost all the cases.

Keywords: *Agrobacterium*, *GUS*, *hpt* gene, Transformation and Aromatic rice

Introduction

In Bangladesh, rice has been the traditional source of carbohydrates and proteins since the prehistoric days. Among the 150 different crops grown in Bangladesh, rice alone occupies about 77% of the total cultivated area, of which aromatic rice is cultivated only on the 10% of the rice growing area (BBS 2006). Aromatic rice is closely related to the social and cultural heritage of Bangladesh and is consumed during different social and religious festivals by all class of people including high demand to classed hotels. Aromatic rice is a profitable farming venture and a good source of livelihood since it does not normally require additional expenditure for fertilizer, pesticides and irrigation. Moreover, due to high consumer's demand reportedly, Bangladesh imports around 50 thousand tons of fine aromatic rice each year from neighboring countries. But due to several problems such as lack of high yielding variety, biotic stress (yellow stem borer, stripped stem borer, leaf folders, blast and blight) resistant and abiotic stress (salinity and drought) tolerant variety, aromatic rice cultivation is becoming limited gradually. Hence the improvement of the existing genotypes in respect of yield and aroma is of paramount importance. To achieve this goal, conventional breeding method merely is not sufficient and therefore, new strategies must be devised to elevate aromatic rice productivity to accommodate the growing demand. Incorporation of gene(s) of interest *viz.* salinity tolerant, drought and disease pest resistant gene(s) through modern biotechnological approach may combat the situation better. The genetic transformation approach is thus, now-a-days, considered as a powerful technique in plant molecular genetics. Efficient induction of *Agrobacterium vir* genes holds the key for successful plant transformation. Aromatic rice has got a little practice, but more effort should be given to develop an efficient, stable and reproducible DNA delivery protocol. Realizing the importance of such imperative tools and giving priority to the above facts, the present piece of research work was framed and materialized to standardize an efficient, high frequency and reproducible genetic transformation protocol for aromatic rice using *Agrobacterium tumefaciens* as a vector.

Materials and Methods

Plant materials and explants: Sterilized mature embryos along with endosperm (dehusked grain) of four *aromatic* rice varieties *viz.* Kalizira, Radhunipagol, Tulsimala and Pusa Basmati-1 were placed horizontally with gentle press onto the surface of the sterilized callus induction medium.

Agrobacterium strain and plasmid: Genetically engineered *Agrobacterium tumefaciens* strain EHA105 (Fig. 1) consists of plasmid pIG121-Hm, a binary vector (Ohta *et al*., 1994).

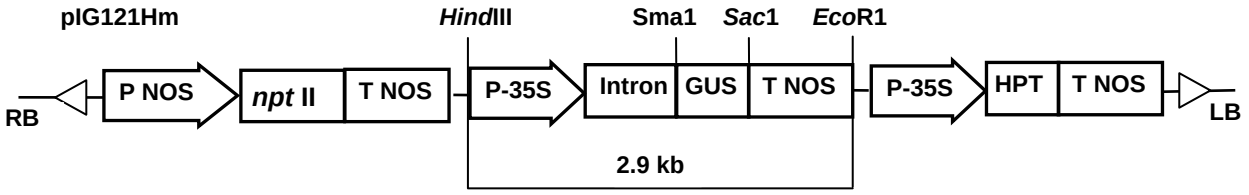

Fig 1. Schematic representation of the plant expression vector pIG121Hm

This binary vector contains the *udiA* gene (Jefferson *et al*., 1987) encoding *GUS* (*β-glucuronidase*) reporter gene, driven by CaMV 35S promoter, the *(nptII) gene* (Herrera-Estrella *et al*., 1983) encoding *neomycin phosphotransferase* II conferring kanamycin resistance driven by NOS promoter and NOS terminator in its right and left border region of the construct. The vector also contains the *hpt* gene. In this study, one way selection was done using kanamycin.

Table1. Composition of different media used in the study

Media	Composition
Callus induction	MS basal +1.5 mgL^{-1} 2, 4-D, 0.5 mgL^{-1} BAP and 50 mgL^{-1} L-tryptophan, pH 5.8
Agrobacterium stock	YMB medium
Infection medium	LB medium + 50 mgL^{-1} Kanamycin
Co-cultivation	MS, 1.5 mgL^{-1} 2,4-D, 0.5 mgL^{-1} BAP, 50 mgL^{-1} L-tryptophan, 100 µm cetosyringone
Washing	MS basal + 200 mg L^{-1} Cefotaxime, pH 5.8
Post-cultivation	MS, 6 mgL^{-1} kinetin, 0.5 mgL^{-1} NAA, 100 mgL^{-1} Cefotaxime, 10 mgL^{-1} Kanamycin
Selection and regeneration	MS, 6 mgL^{-1} kinetin, 0.5 mgL^{-1} NAA, 75 mgL^{-1} cefotaxime, 15 mgL^{-1} kanamycin, pH 5.8
Root initiation	MS, 6 mgL^{-1} kinetin, 0.5 mgL^{-1} NAA, 75 mgL^{-1} cefotaxime, 15 mgL^{-1} kanamycin, pH 5.8
Transplanting of plantlets	Soil containing 25% garden soil + 50% sand + 25% cow dung
watering the plantlets	Hoagland's solution

Culture technique: A single streak from previously maintained *Agrobacterium* stock was inoculated in liquid LB medium + 50 mgL^{-1} kanamycin. The culture was allowed to grow at 28^0C to get optimum population of *Agrobacterium* for infection. Freshly excised calli were immersed into bacterial suspension (OD_{600}=0.6) for 20, 25 and 30 minutes with gentle shaking to get suitable and sufficient infection. The infected calli were blotted dry to remove excess bacterial suspension and were co-cultivated for 2, 3 and 4 days on co-cultivation media and incubated under fluorescent illumination with 16/8 hours light/dark cycle at 25±2^0C for 2-4 days. The intensity of light was maintained at 1500 lux. Transient *GUS* assay was done at the end of co-cultivation with randomly selected fifteen inoculated calli according to the method described by Jefferson (1987). The infected explants were washed three times with sterile distilled water and once with liquid MS media supplemented with 200 mg l^{-1} of Cefotaxime and then were transferred onto post-cultivation medium for 15 days. For shoot regeneration, 10 calli were transferred to selection and regeneration medium. The aseptically separated shoots were again cultured on conical vials for rooting on rooting medium. When the plantlets become 5-8 cm in length with sufficient root system, they were taken out from the vials and placed in hardening chamber and the survived plants were transferred to pot to acclimatize in field condition. Daily observation was carried out at every step to note the responses and the contaminated vials were discarded immediately to avoid contamination.

Statistical analysis: The analyses of variances were done and means were compared by the Duncan's Multiple Range Test (DMRT) using MSTATC program.

Results and Discussion

Histochemical *GUS* (*ß*-glucuroronidase) assay

Varietal response: The highest percentage of *GUS* positive explants (Plate 1.c was recorded in variety Kalizira followed by Pusa Basmati 1 and Radhunipagol and the lowest response was found in Tulsimala (Table 2).

Effects of infection times: Calli showed the highest response to the percent *GUS* positive explants in case of 25 minutes infection time followed by 20 minutes and 30 minutes infection time (Table 2).

Effects of co-cultivation periods: The highest percentage of *GUS* positive calli was found for the co-cultivation period of 3 days followed by that of 2 days and it was least for the co-cultivation period of 4 days (Table 2).

Table 2. Varietal responses and effects of infection times and co-cultivation periods to *GUS* assay and shoot regeneration

	Percent *GUS* positive (+ve) explants	Percent transgenic shoot regeneration
Varietal response		
Kalizira	47.22 a	8.52 a
Pusa Basmati 1	37.78 b	4.44 ab
Radhunipagol	37.78 b	3.33 a
Tulsimala	0.3065 c	0.37 b
Effects of infection time (min)		
20	37.78 b	2.78 b
25	47.22 a	9.44 a
30	37.78 b	0.28 b
Effects of co-cultivation period (day)		
2	47.22 b	3.89 ab
3	53.30 a	8.06 a
4	37.78 c	0.56 b

Figures followed by different letter(s) in a column are not statistically similar

Effects of variety × infection time interactions: Among the four varieties, Kalizira showed the highest percentage of *GUS* positive explants followed by Pusa Basmati 1, Radhunipagol and Tulsimala all on 25 minutes of infection time (Table 3).

Effects of infection time × co-cultivation period: Maximum *GUS* positive explants was found for 3 days co-cultivation period and 25 minutes infection time followed by 2 days co-cultivation period and 20 minutes infection time (Table 4).

Effects of Variety × co-cultivation period interactions: Kalizira showed the highest response to *GUS* assay for 3 days co-cultivation period followed by Pusa Basmati 1, Radhunipagol for 3 days (Table 5).

Effects of Variety × infection time × co-cultivation period interactions: Kalizira showed the highest percentage of *GUS* positive explants followed by Pusa Basmati 1, Radhunipagol for 25 minutes of infection time and 3 days of co-cultivation period and the lowest response was showed by Tulsimala for 30 minutes infection time and 4 days co-cultivation period (data not shown).

Regeneration of transgenic shoot

Varietal response: Kalizira showed the highest percentage of transgenic shoot regeneration followed by Pusa Basmati 1, Radhunipagol and Tulsimala (Table 2). The present result supports the findings of Aldemita and Hodges (1996) who reported influenced of varieties on transformation efficiency to be greatly.

Effects of infection times: The maximum percentage of transgenic shoots (9.44%) was regenerated from 25 minutes of infection time followed by 20 minutes (2.78%) and 30 minutes (0.28%). Kumaria *et al.* (2001) also reported that prolonged infection time adversely affects the callus growth and subsequent regeneration.

Effects of co-cultivation periods: Maximum percentage of putative transgenic shoots was found when calli were co-cultivated for 4 days followed by 2 days and 3 days (Table 2). Das (2004), Bhalla and Smith (1998), Hiei *et al.,* (1997) also reported similar trend of result in rice regeneration.

Effects of variety × infection time interactions: Kalizira showed the highest percentage of shoots followed by Pusa Basmati 1, Radhunipagol and Tulsimala for 25 minutes infection time (Table 3).

Table 3. Effects of variety × infection time interactions on percentage of explants positive (+ve) for *GUS*

Variety × Infection time		Percent *GUS* positive (+ve) explants	Percent transgenic shoot regeneration
Variety	Infection time (min)		
Kalizira	20	66.67 b	5.56 b-d
	25	70.00 a	18.8 a
	30	37.78 e	1.11 d
Pusa Basmati 1	20	37.78 e	3.33 cd
	25	55.42 c	10.0 b
	30	53.30 c	0.0 d
Radhunipagol	20	36.67 e	2.22 cd
	25	0.3065 f	7.78 bc
	30	47.22 d	0.0 d
Tulsimala	20	38.33 e	0.0 d
	25	36.67 e	1.11 d
	30	0.2655 f	0.0 d

Figures followed by different letter(s) in a column are not statistically similar

Effects of infection time × co-cultivation period interactions: The highest percentage of regenerated shoots was found for 3 days co-cultivation period and 25 minutes infection time and the least was found for 4 days in both 20 and 30 minutes infection time (Table 4).

Table 4. Effects of infection time × co-cultivation interactions on percentage of explants positive (+ve) for *GUS*

Infection time × co-cultivation period		Percent *GUS* positive (+ve) explants	Percent transgenic shoot regeneration
Infection time (min)	co-cultivation period (days)		
20	2	55.42 b	2.5 c
	3	37.78 d	5.83 bc
	4	37.78 d	0.0 c
25	2	0.3065 e	9.16 b
	3	70.00 a	17.5 a
	4	36.67 d	1.67 c
30	2	0.2655 e	0.0 c
	3	47.22 c	1.66 c
	4	38.33 d	0.0 c

Figures followed by different letter(s) in a column are not statistically similar

Effects of Variety × co-cultivation period interactions: Kalizira regenerated the highest percentage of transgenic shoots followed by Pusa Basmati 1 and Radhunipagol for 3 days of co-cultivation period (Table 5).

Effects of Variety × infection time × co-cultivation period interactions: The variety Kalizira showed the highest percentage of regenerated shoots followed by Pusa Basmati 1, Radhunipagol and Tulsimala in case of 25 minutes infection time and 3 days co-cultivation period (data not shown).

Root induction

Among the four varieties examined, Kalizira produced the highest percentage of rooted shoots followed by Pusa Basmati 1. Radhunipagol and Tulsimala both produced the lower percentage of rooted shoots (data not shown).

Table 5. Effects of variety × co-cultivation period interactions on percentage of explants positive (+ve) for *GUS*

Variety × Co-cultivation		Percent *GUS* positive (+ve) explants	Percent transgenic shoot regeneration
Variety	Co-cultivation period (days)		
Kalizira	2	53.30 c	7.78 b
	3	70.00 a	16.67 a
	4	37.78 e	1.11 c
Pusa Basmati 1	2	37.78 e	4.44 bc
	3	66.67 b	8.89 b
	4	47.22 d	0.0 c
Radhunipagol	2	36.67 e	3.33 bc
	3	55.42 c	5.56 c
	4	0.2655 f	1.11 c
Tulsimala	2	38.33 e	0.0 c
	3	36.67 e	1.11c
	4	0.3065 f	0.0 c

Figures followed by different letter(s) in a column are not statistically similar

Establishment of plantlets

In growth chamber's pot, Kalizira showed the maximum survival rate (Table 6). In field condition (earthen pot), Kalizira showed the highest survival rate followed by Radhunipagol and Tulsimala and Pusa Basmati 1.

Table 6. Survival rate of transgenic rice plants after transfer to growth chamber and field condition (earthen pot)

Genotype	No. of plantlets transplanted			No. of plants survived			Survival rate (%)	
	In growth chamber	In earthen pot		In growth chamber	In earthen pot		In growth chamber	In earthen pot
Kalizira	20	15		15	9		75	60
Radhunipagol	15	12		10	7		66.67	58.33
Tulsimala	15	10		9	5		60	50
Pusa Basmati 1	12	10		7	4		58.33	40

The present study thus found Kalizira to be best variety in terms of *Agrobacterium* mediated genetic transformation. It can be recommended that Kalizira will be a potential variety if any gene(s) of interest *viz*. salinity tolerant &/or disease resistant is aimed to transfer to aromatic rice variety.

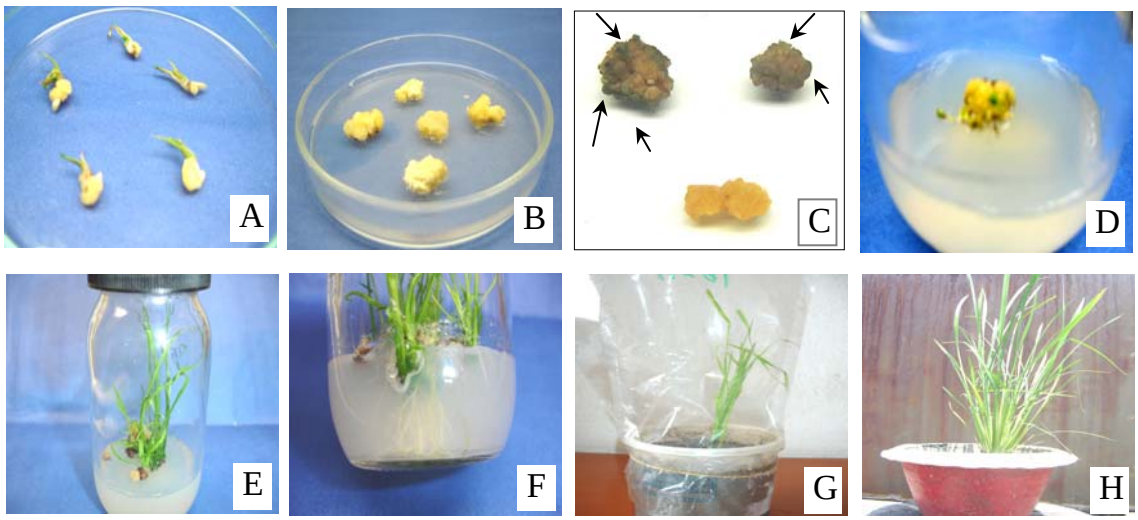

Plate 1. Callus initiation (A), maintenance of callus through subculture (B), histochemical *GUS* activity (blue zone indicated by arrow) at the infected callus with control (C), shoot initiation from *agrobacterium* infected callus on selection medium (D), putative transgenic shoot (E), root initiation on rooting medum (F), plant in growth chamber for hardening (G) and transgenic rice plant on earthen pot (H).

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