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Investigations for Improved Genetic Transformation Mediated by *Agrobacterium tumefaciens* in Two Rice Cultivars

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Abstract: In this study, we established an efficient *Agrobacterium*-mediated transgenic system in rice (*Oryza sativa* L.). During the studies in the japonica (Xiushui-11) and indica rice cultivars (XC-95), the role of various factors responsible for virulence of *Agrobacterium* was screened more carefully to establish a better transformation system for the rice cultivars. MS and AB media (as VIM) proved to be efficient for Xiushui-11 and XC95, respectively. 100 μM AS (acetosyringone), pH 6.0 (VIM and COM) and use of either sucrose, glucose (Xiushui-11) or sucrose (XC-95) produced the best results for both of japonica and indica rice. Bacterial cell density A_{600} 0.4-0.6 for 10-15 min was the most favorable optimized condition. Completely dark (Xiuishui-11) and light (XC-95) conditions during co-cultivation period were found to be ideal for transformation process. The significance of achieved results has been discussed properly.

Key words: Factors, gene transformation, *Agrobacterium tumefaciens*, *Oryza sativa* L.

INTRODUCTION

Among the various transformation methods such as direct gene transfer methods, transformation by *Agrobacterium tumefaciens* is popularizing more extensively now a days in crop plants (Wimmer, 2003; Ramesh *et al*., 2004). During the process of transformation mediated by *Agrobacterium tumefaciens*, a specific segment of the vector, T- DNA, which can be engineered to contain a selectable marker, reporter gene and/or genes of interest is transferred from the bacterium to host plant and inserted into the nuclear genome. These functions are mediated by a set of *Vir* (virulence) genes such as *VirA*, *VirG*, *VirE*, *VirD* and a complex of *VirB*, with optimal expression occurring at acidic pH and in the presence of phenolic inducers more, such as acetosyringone, that are released by wounded plant cells (Park *et al*., 2000). Though improvements in the *Agrobacterium*-mediated transformation technology of monocots more especially rice have been remarkable in the past, yet still further scope exists (Kumria *et al*., 2001; Li *et al*., 2002; Akutsu *et al*., 2004). One of the key factors involved in the transformation hindrance of monocots was the lower virulence gene expression of *Agrobacterium* compared to dicots.

Monocotyledons, in particular members of *gramineae* family, appear not to produce these compounds, or if they do, the levels are insufficient to serve as signals (Smith and Hood, 1995). To achieve more competent transgenic lines for a particular trait and overcome the incident of gene silencing which is posing a threat to transgenic research, there should be higher number of transgenic lines achieved which is possible through improved tissue culture and transformation system. The former system has been studied comprehensively by the authors of this study earlier (Ali *et al*., 2004). The lower transformation efficiency mediated by *Agrobacterium*, especially in rice, is a continuous hindrance to obtain higher number of transgenics and further efforts are still needed to improve this drawback. A more refined and improved transformation protocol will aid to attain above mentioned objectives. In such an effort, the authors of this research have investigated various factors in more detail to explore the way by which genetic transformation system mediated by *Agrobacterium* can be improved further in both sub-classes i.e., japonica and indica rice.

MATERIALS AND METHODS

This study was conducted during 2004-05 at Zhejiang University, P.R. China.

Plant materials and tissue culture conditions: Two rice genotypes, Xiushui-11, a leading japonica variety growing in China and an indica breeding line XC-95 were employed in the transformation experiments.

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Table 1a: Proposed tissue culture media identified for the transformation of japonica rice (Xiushui-11)

Medium	Utilization	Components
CIM	Callus induction	Basal N6, 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D, 3 g L^{-1} phyta gel, pH 5.8
PCM	Pre-culture	Basal N6, 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D, 3 g L^{-1} phyta gel, pH 5.8
VIM	Bacterium activation	Basal MS, 30 g L^{-1} sucrose, 20 mM MES, 100 μM Acetosyringone (AS), pH 6.0.
COM	Co-cultivation	Basal N6,30 g L^{-1} sucrose, 2 mg L^{-1} 2, 4-D, 20 mM MES, 100 μM Acetosyringone (AS), 3 g L^{-1} phyta gel, pH 6.0
BWM	Washing	Basal MS, 500 mg L^{-1} carbencillin, pH 5.8
TSM	Selection	Basal N6, 30 g L^{-1} sucrose, 2 mg L^{-1} 2, 4-D, 3 g L^{-1} phyta gel, 50 mg L^{-1} hygromycin, 500 mg L^{-1} carbencillin, pH 5.8.
TRM	Regeneration	Basal MS, 30 g L^{-1} sucrose, 2 mg L^{-1} Kinetin, 0.5 mg L^{-1} NAA, 50 mg L^{-1} hygromycin, 5 g L^{-1} Phyta gel, pH 5.8.
RTM	Rooting and further growth	Basal MS, 30 g L^{-1} sucrose, 0.5 mg L^{-1} NAA, 50 mg L^{-1} hygromycin, 3 g L^{-1} Phyta gel , pH 5.8.

Table 1b: Proposed culture media identified for the transformation of indica rice (XC-95)

Medium	Utilization	Components
CIM	Callus induction	Basal N6, 30 g L^{-1} maltose, 2 mg L^{-1} 2, 4-D, 0.5 mg L^{-1} BAP, 0.5 mg L^{-1} NAA, casein hydrolysate 500 mg L^{-1}, 3 g L^{-1} phyta gel, pH 5.8
PCM	Pre-culture	Basal MS, 30 g L^{-1} maltose, casein hydrolysate 500 mg L^{-1}, 3 g L^{-1} phyta gel, pH 5.8
VIM	Bacterium activation	AB minimal components, 20 mM MES, 100 μM Acetosyringone (AS), pH 6.0.
COM	Co-cultivation	Basal N6, 30 g L^{-1} sucrose, 2 mg L^{-1} 2, 4-D, 0.5 mg L^{-1}LBAP, 0.5 mg L^{-1} NAA, 20 mM MES, 100 μM Acetosyringone (AS), casein hydrolysate 500 mg L^{-1}, 3 g L^{-1} phyta gel, pH 6.0
BWM	Washing	Basal MS, 500 mg L^{-1} carrbencillin, pH 5.8
TSM	Selection	Basal N6, 30 g L^{-1} sucrose, 2 mg L^{-1} 2, 4-D, 0.5 mg L^{-1} BAP, 0.5 mg L^{-1} NAA, casein hydrolysate 500 mg L^{-1}, 3 g L^{-1} phyta gel, 50 mg L^{-1} hygromycin, 500 mg L^{-1} carbencillin, pH 5.8.
TRM	Regeneration	Basal MS, 30 g L^{-1} sucrose, 3 mg L^{-1} Kinetin, 0.5 mg L^{-1} NAA, casein hydrolysate 500 mg L^{-1}, 50 mg L^{-1} hygromycin, 6 g L^{-1} Phyta gel , pH 5.8.
RTM	Rooting and further growth	Basal MS, 30 g L^{-1} sucrose, 0.5 mg L^{-1} NAA, 50 mg L^{-1} hygromycin, 3 g L^{-1} Phyta gel , pH 5.8.

CIM-Callus Induction Medium, PCM- Pre- culture Medium, VIM- Virulence Induction Medium, COM- Co-cultivation Medium, BWM- Bacterium Washing Medium, TSM- Transformant Selection Medium, TRM- Transformant Regeneration Medium, RTM- Rooting Medium

Mature seeds were de-husked, sterilized with 70% ethanol for 2-3 min, followed by immersion in 0.1% $HgCl_2$ for 10-15 min and finally washed 3-4 times consecutively with sterilized water. The seeds were placed on blot paper to blot excess of water and then placed afterwards under light conditions on callus induction medium as shown in Table 1a and 1b for a period of 10 and 14 days for Xiushui-11 and XC-95, respectively. Embryogenic calli induced from the mature seeds were excised. Before co-cultured with *Agrobacterium*, calli were precultured on preinduction medium from 60 to 72 h to exploit the maximum physiological potential (Khanna and Raina, 1999).

Transformation vectors: Vector used to transform was based on a pCAMBIA1301 binary vector. The *A. tumefaciens* strain employed in the experiments was an agropine strain *EHA105* and both of these were kindly donated by Genetic Institute of China. The T-DNA regions of pCAMBIA1301 derivative is shown in Fig. 1, which contains the hygromycin phosphotransferase (hyg)-coding sequence and *uidA* gene under control of the cauliflower mosaic virus (CaMV) 35S promoter

Transformation of plasmid DNA into *Agrobacterium*: Preparation of competent bacterium cells for the transformation of plasmid DNA was performed using $CaCl_2$ method according to Sambrook and Russel (2001). The transformation step of competent cells with plasmid DNA was carried out employing liquid nitrogen method as described by Tzfira *et al*. (1997).

Transformation strategy; co-cultivation, selection and plant regeneration: Various media involved in the transformation process have been shown in Table 1a and b. The *Agrobacterium* strain EHA-105 harboring the vectors based on pCAMBIA1301 was streaked on agar solidified YEP medium supplemented with rifampicin (20 mg L^{-1}), kanamycin (50 mg L^{-1}) and hygromycin (50 mg L^{-1}) and grown at 28°C for colonies to appear. A single clone was sorted out and transferred to 3 mL YEP medium containing the same selective antibiotics and the culture was allowed to shake for 12-16 h at 28°C with 250 rpm to obtain an A_{600} of 1.0-1.5 (A_{600} corresponds to 1×10^8). The bacterial cells were pelleted at 5000 rpm for 5 min at room temperature and then re-suspended in 100 mL flask containing 25-30 mL Virulence Induction Medium (VIM) supplemented with 3% sucrose along with 100 uM acetosyringone (Fluka) to an A600 of 0.3-0.35. The 100 mL flask containing the culture was allowed to shake for a period of 12 h until an A_{600} of 0.4-0.6 was reached. The primary calli subcultured for 3-4 days were immersed in the VIM containing bacterial suspension for 10-15 min and then transferred to sterilized filter paper to remove excess of bacterial liquid. The infected calli were transferred on to co-cultivation medium at 26°C for 60-72 h in dark. After co-cultivation, the calli were first washed 3-4 times with sterilized double distilled water followed by 2 times washing with liquid MS medium containing 500 mg L^{-1} carbencillin to decontaminate the left over bacteria and placed on to filter paper to blot excess of liquid. In the next phase, the calli were

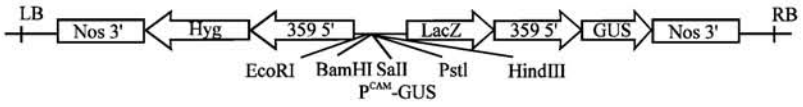

Fig. 1: Diagram of the plasmid construct LB: Left border; RB: Right border

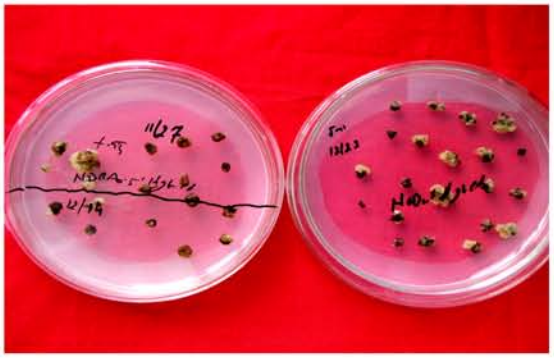

Fig. 2: Development of bright yellow resistant callus from brown, dead calli; left-XC-95; right-Xiushui-11

transferred to selection medium and incubated at 26°C in the dark. Three- five weeks later, bright yellow resistant calli started appearing (Fig. 2). These calli were transferred to regeneration medium. In approximately 3-4 weeks, shoots or plantlets were recovered. When the shoots attained a leaf height of 2-3 cm, these were transferred to rooting medium for a further period of 2-3 weeks. The independent T_0 transgenic plants with a height of 10-15 cm with well-developed, vigorous roots were first temporarily acclimatized in tubes containing tap water for one week, later transferred to potted soil and grown to reproductive stage until the seeds were obtained.

Factor evaluation: To assess the influence of various factors on transformation efficiency, an investigation was designed in the following way;

- Three independent experiments were set to study the effect of virulence induction medium on transformation efficiency using three different media, MS, AB and N6. A minimum of 45 calli was used in each experiment. Data were collected for appearance of resistance calli and calli producing green plantlets.
- Regarding the effect of acetosyringone concentrations, six MS virulence induction and co-cultivation media were set containing different concentrations of Acetosyringone, (1) 0 µM (2) 50 µM (3) 100 µM (4) 150 µM (5) 200 µM and (6) 300 µM. Two experiments were performed employing 25 calli per experiment. Data were collected for appearance of resistance calli.
- For the study of interactive effect between cell density and co-cultivation time on transformation, four inoculum sizes at A600, 0.4, 0.6, 0.8 and 1.0, each one tested for four time periods, 5, 10, 15 and 20 min, were examined. Data were collected for appearance of resistance and contaminated calli. The experiment was repeated four times.
- For the parameters like impact of pH, carbon source, different MS virulence induction and co-cultivation media were set with five pH levels i.e., 5.5.5, 6.0, 6.5 and 7.0 and various sugars (no sugar, glucose, maltose and sucrose). Co-cultivation was also performed under light conditions to examine the effect of light, histochemical GUS assay for transient expression was performed and there were four experiments for each factor using 20 calli per experiment.

Each experiment for all the factors studied was treated as a replicate in the data analysis process.

GUS histochemical assay: The infected calli, leaves, stem and roots of putative transgenic plants were subjected to GUS (X-Gluc staining) expression assay using X-Gluc (5-bromo-4-chloro-3-indoly-glucuronide) as followed by Rueb and Hensgens (1989) with some modifications. The tissues (calli, leaves, stem and roots) were directly incubated in the solution at 37°C for 12 h. In case of leaves and stem, when incubation was completed, they were washed with ddH_2O and incubated in 95% ethanol for 1-2 h for the bleaching of chlorophyll contents. The materials with GUS expression display the blue coloration (Fig. 3).

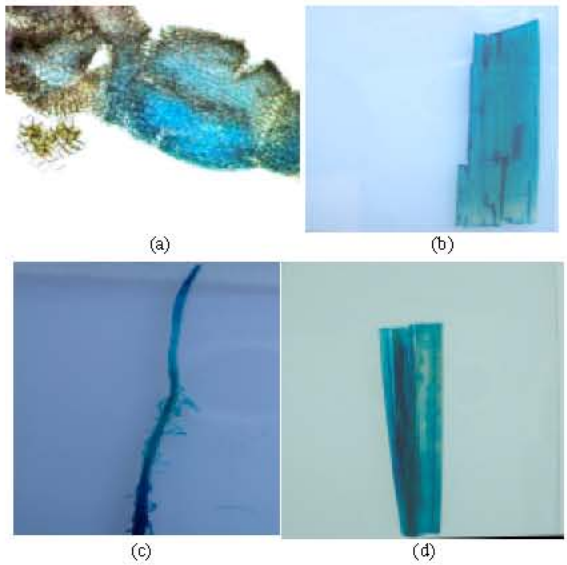

Fig. 3: GUS staining assay with different transgenic tissues. a-) Cell structure of histochemical staining of GUS activity in transformed callus (b) leaf (c) root (d) stem

Data analysis: The mean values of data were tested to evaluate the significance at 5% level using SAS statistical software.

RESULTS

Plasmid DNA transformation frequency studies in *Agrobacterium:* Different protocols for *Agrobacterium* transformation are available including triparental mating, direct elecroprotation etc. Unlike the lengthy tri-parental procedure, which requires complicated media and selection methods and for electroprotation method, a suitable device is required which is not always available, we evaluated an alternative, efficient, direct transformation procedure employing the liquid nitrogen and compared its efficiency with the protocol devoid of liquid nitrogen. The results shown in Table 2 evidenced that the use of liquid nitrogen was quite efficient and boosted up remarkably the transformation efficiency of plasmid DNA into *Agrobacterium* in contrast to the method without the involvement of liquid nitrogen.

Table 2: Effect of liquid nitrogen on plasmid transformation into *Agrobacterium*

	No. clones appeared	
Experiment	M-1	M-2
1	20	240
2	8	123
3	18	>300

M-1 = Without liquid nitrogen, M-2 = With liquid nitrogen

Factors affecting plant tissue transformation efficiency mediated by *Agrobacterium*

Effect of virulence induction medium (VIM): To test whether medium constitution has any significance during the initial virulence activation of bacterium, three different media were tested, namely, AB, MS and N6, during the transformation process. The response of *Agrobacterium* to these media was found to be variable for indica and japonica genotypes in terms of transformation efficiency apart from observed variable growth rates (Table 3a-c). A positive correlation was found between the number of resistant calli appeared and number of calli producing green plantlets in all of the cases. As it is clear from the data in Table 3a and b, MS and AB were not able to induce a pronounced difference in case of indica rice (with average values of 26.0 and 32.6 resistant calli, respectively), yet AB medium seemed to function better still compared to MS. Contrarily, the two media came into action variably while dealing with the transformation of japonica rice (Table 3a and b). AB medium here generated a similar value (32.3 resistant calli) of transformation efficiency to indica rice. MS medium, in this case, pushed up the transformation efficiency (45.1 resistant calli) to a remarkable level. Regarding the effect of N6, a considerable poor transformation level was detected irrespective of genotype while comparing with the influence of MS and AB (Table 3c).

Significance of acetosyringone, a phenolic inducer compound: In the virulence system of *Agrobacterium*,

Table 3a: Effect of MS virulence induction medium on the transformation efficiency

Experiment		Calli No. (a)	No. of Hyg$^+$ calli (b)	No. of calli producing plants (c)	No. of Resistant plants	Transformation efficiency (%) b/a	Transformation efficiency (%) c/a
Xiushui-11	1	64	31	22	34	48.4	34.3
	2	80	35	26	29	43.75	32.5
	3	175	76	52	41	43.4	29.7
Total		319	142	100	104	45.1[a]	32.1[b]
XC-95	1	45	10	6	0	22.2	13.3
	2	45	16	5	10	32.0	10.0
	3	50	12	8	6	24.0	16.0
Total		145	38	19	16	26.0[a]	13.1[b]

Table 3b: Effect of AB virulence induction medium on the transformation efficiency

Experiment		No. of calli (a)	No. of Hyg$^+$ calli (b)	No. of calli producing plants (c)	No. of Resistant plants	Transformation efficiency (%) b/a	Transformation efficiency (%) c/a
Xiushui-11	1	50	17	11	9	34.0	22.0
	2	90	29	16	21	32.2	17.7
	3	71	22	9	14	30.9	12.6
Total		211	68	36	44	32.3[a]	17.4[b]
XC-95	1	30	8	3	0	26.6	10.0
	2	60	17	9	13	28.3	15.0
	3	72	31	16	10	43.05	22.2
Total		162	56	28	23	32.65[a]	15.7[b]

Table 3c: Effect of N6 virulence induction medium on the transformation efficiency

Experiment		No. of calli (a)	No. of Hyg$^+$ calli (b)	No. of calli producing plants (c)	No. of resistant plants	Transformation Efficiency (%) b/a	Transformation Efficiency (%) c/a
Xiushui-11	1	45	10	4	5	22.2	8.8
	2	50	12	7	11	24.0	14.0
	3	50	8	0	0	16.0	0.0
Total		145	30	11	16	20.7[a]	7.6[b]
XC-95	1	30	5	0	0	16.6	0.0
	2	55	4	3	1	7.2	5.4
	3	48	6	0	0	12.5	0.0
Total		133	15	3	1	12.1[a]	1.8[b]

[a,b]Average transformation efficiencies

unit *VirA* acts as sensor of phenolic compounds like acetosyringone. To have an insight whether the *Agropine* strain EHA-105 exhibited differential virulence sensitivity towards different levels of phenolic compounds in terms of plant transformation efficiency, we set five different concentrations of acetosyringone i.e., 0, 50, 100, 150, 200 and 300 uM. The results in Table 4 demonstrated clearly the differential behavior of bacterium regarding the genesis of resistant calli in the two classes of rice material used. The concentration of 100 uM proved to be more efficient for stimulating the virulence system of bacterium generating a maximum value of resistant calli i.e., 12.5, being significantly higher and with an efficiency of 50% as compared to 12, 18, 38, 24 and 22% efficiencies for 0, 50, 150, 200 and 300 uM concentrations, respectively, in japonica rice. Although the transformation values were lower, yet the same trend was noticed in case of XC-95. No and almost negligible transformation level was observed at 0 and 50 uM (1.5 resistant calli). 100 uM again did well (7.5 resistant calli) with an efficiency of 30%, but the later concentrations of 150, 200 and 300 uM could not be able to maintain/ enhance this transformation efficiency.

Impact of relationship between bacterial density and co-cultivation time: To assess the impact of inoculum's density and time for suspension of explant in bacterial culture, a bi-factorial experiment was designed using four levels of cell densities i.e., A_{600} = 0.4, 0.6, 0.8 and 1.0 and time periods i.e., 5, 10, 15 and 20 min. The results have been summarized in Table 5. The minimum and maximum time limits (5 and 20 min, respectively) combined with all levels of densities proved to be not more effective with an efficiency ranging from 1-9.5 resistant calli per 30 calli compared to 10 and 15 min. The later mentioned timings together with inoculum size of 0.4 and 0.6 produced the significantly highest efficiencies, 12, 14.5 and 16, 14.0, for A0.4 and A0.6, respectively. The overall lowest values of

Table 4: Effect of Acetosyringone concentrations on the transformation efficiency of *Agrobacterium*

	Xiushui-11		XC-95	
Concentration (μM)	Resistant calli[a]	Efficiency (%)	Resistant calli[a]	Efficiency (%)
0	2.5±0.5a	10	0	0
50	4.5±1.5b	18	1.5±1.0a	6
100	12.5±1.5c	50	7.5±0.5b	30
150	9.5±1.5c	38	6.5±1.3b	26
200	6.0±1.0d	24	3.5±2.0c	14
300	5.5±0.5d	22	3.5±1.5c	14

Table 5: Effect of interaction between bacterial density and co-cultivation time on the transformation efficiency of *Agrobacterium* in Xiushui-11

Timex density	No. of calli	Resistant calli[a]	No. of contaminated calli
5×0.4	30	9.5±0.5cd	0.0
5×0.6	30	8.0±1.0d	0.0
5×0.8	30	2.5±0.5fg	2.0
5×1.0	30	2.5±0.5fg	4.0
10×0.4	30	12.0±1.0bc	0.0
10×0.6	30	16.0±1.0a	1.0
10×0.8	30	4.5±0.5ef	3.5
10×1.0	30	2.0±1.0fg	4.5
15×0.4	30	14.5±2.5ab	0.0
15×0.6	30	14.0±2.0ab	2.0
15×0.8	30	2.00±1.46fg	5.5
15×1.0	30	1.00±1.0g	5.0
20×0.4	30	6.0±0.5de	1.5
20×0.6	30	4.5±0.5ef	4.5
20×0.8	30	1.00±1.0g	9.0
20×1.0	30	1.00±1.0g	7.5

[a]Data within the column followed by the same letter indicate no significance at 5% level

resistant calli, for all the time periods, were observed for A0.8 and A1.0 cell densities i.e., 2, 4.5, 2.0 ,1.0 and 2.5, 2.0, 1.0, 1.0, for 5, 10, 15 and 20 min, respectively. In addition to that, the contamination of infected calli during the co-cultivation period was more pronounced for inoculum size of 0.8 and 1.0 with values of 2, 3.5, 5.5, 9.0 and 4.0, 6.5, 5, 7.5, for 5, 10, 15 and 20 min, respectively.

Effect of pH: The necessity of an acidic pH during co-cultivation was also tested for its influence on transformation efficiency. Five different levels of pH i.e., 5.0, 5.5, 6.0, 6.5 and 7.0 were maintained in the virulence induction and co-cultivation media in order to determine the effect of these levels on the transformation efficiency of *Agrobacterium* strain EHA-105. It is important to note that the pH of all media in this experiment was maintained by addition of 20 mM MES to ensure a stable pH, if it m ay change during the co-cultivation period. Transient GUS expression of infected calli was analyzed to have an idea about the sensitivity of different pH levels in system. Table 6 shows that in case of Xiushui-11, pH levels of 5.0, 6.5 and 7.0 seemed to prevent gene transfer in most of the calli (mean the values being 9.0, 11 and 7.0 GUS^+ calli, respectively). On the other hand, pH levels of 5.5 and 6.0 appeared to facilitate the transformation with values of 14.0 and 15.0 GUS positive calli, the values being non significant, but remarkably differ from the outcome values of pH 5.0, 6.5 and 7.0. It was visually observed that the GUS expressing zones of calli were larger in size at pH 6.0 in comparison to other levels. Lower time for GUS staining appearance in case of pH 6.0 also gives an indication of higher gene transfer. The same result was almost reproducible in case of XC-95 except for the difference for GUS^+ calli was non significant for levels 6.0 and 6.5. However, still, relatively, lower time (10 h) was required for the development of calli at level 6.0.

In order to ensure whether MES (Methoxysulfonylethane) exerts its role in maintaining a stable pH of media, that ultimately has an important impact on transformation based upon the above experiment, the calli were inoculated on two media, with and without the inclusion of 20 mM MES, while initially maintaining the pH 6.0 of media. A significant difference was observed with respect to GUS activity between the tested media (Table 7). The medium with MES was more efficient (22 GUS^+ calli) than without MES.

Effect of carbon source type: Carbon source also acts as supplementary factor for the maximum levels of *vir* gene induction. The *Agrobacterium* strains may exhibit differential sensitivity towards various sugary sources. To test these hypotheses, four experiments were set. Each experiment was supplemented with a different carbon source i.e., without sugar, glucose, maltose and sucrose with 3% concentration for each one in the medium. Table 8 proves that sugars definitely have some certain role in the transformation system as all generated significantly higher values from the medium with no sugar in the transformation system. For Xiushui-11, glucose and sucrose were found to be equally competitive with non-significant values of 14.0 and 15.5 GUS positive calli. Maltose displayed a significantly lower value of 10.5 GUS^+ calli compared to glucose and sucrose, but still produced a much higher value than the medium without carbon source (5.5 GUS^+calli). Sucrose still worked well in case of XC-95 producing the highest efficiency (40%).

Effect of photoperiod conditions during co-cultivation: To test the effect of light conditions during co-cultivation on the gene transfer, we co-cultivated calli either in

Table 6: Effect of various pH levels on the transformation efficiency of *Agrobacterium*

		Xiushui-11			XC-95		
Level	No. of inoculated calli	*GUS+	Efficiency (%)	Time period (h)	*GUS+	Efficiency (%)	Time period (h)
5.0	20	9±4.0b	45.00	25-30	3.5±0.3c	15.00	20-30
5.5	20	14±1.0a	70.00	12-16	7±0.5b	35.00	16-20
6.0	20	15±1.0a	75.00	12	11±1.0a	55.00	10
6.5	20	11±1.0ab	55.00	15-24	11±1.0a	55.00	12-24
7.0	20	7±1.0b	35.00	>35	4.5±0.3c	20.00	>35

[a]Data within the column followed by the same letter indicate no significance at 5% level. *Calli showing any degree of blue coloration were counted as GUS positive

Table 7: Role of MES on transformation efficiency (Xiushui-11)

Medium	No. of Calli	*GUS+ Calli	Efficiency (%)
With MES	30	22±2.0a	73.30
Without MES	30	14.5±2.5b	51.67

*Calli showing any degree of blue coloration were counted as GUS positive

Table 8: Effect of various carbohydrate sources on the transformation efficiency of *Agrobacterium* in Xiushui-11

		Xiushui-11		XC-95	
Sugar (3%)	No. of inoculated Calli	[ab]GUS+ Calli	Efficiency (%)	[ab]GUS+ Calli	Efficiency (%)
No sugar	20	5.5±0.5c	27.5	2.4±0.2b	12.0
Glucose	20	14.0±1.0a	70.0	3.0±0.5b	15.0
Maltose	20	10.5±0.5b	52.5	1.5±0.01c	7.5
Sucrose	20	15.5±0.5a	77.5	8.0±1.0a	40.0

[a]Data within the column followed by the same letter indicate no significance at 5% level. [b]Calli showing any degree of blue coloration were counted as GUS positive

Table 9: Effect of light conditions on the transformation efficiency of *Agrobacrerium* in Xiushui-11 and XC-95

		Xiushui-11			XC-95		
Condition	No. of inoculated Calli	GUS+ Calli	Efficiency (%)	Time period (h)	GUS+ Calli	Efficiency (%)	Time period (h)
L1	20	12.5±0.5ab	62.5	20-24	8.5±0.5b	42.7	12-16
L2	20	10.0±1.0a	50.0	> 30	12.6±1.0a	63.0	10-15
Darkness	20	15.5±0.5a	77.5	12-14	7.5±2.0b	37.5	>20

[a]Data within the column followed by the same letter indicate no significance at 5% level. [b]Calli showing any degree of blue coloration were counted as GUS positive

darkness (24 h) or under a 16/8 h (light/dark) photoperiod and complete light (24 h) designated as L1 and L2, respectively. Darkness seemed to promote the level of gene transfer. The detected GUS activity (15.5 calli) and transformation efficiency (77.5%) under 24 h period of dark was higher than conditions involving photoperiod (Table 9). Aside from it, very few and week spots of staining under light conditions were observed after a much longer time, indicating a lower level of gene transfer. The results showed that light might have some inhibitory effect in the gene transfer mechanism in case of japonica class. However such an effect could not be observed in case of XC-95, where light produced the best effect (Table 9).

DISCUSSION

The advantages of *Agrobacterium*-mediated gene transfer over other methods that can be used for transformation of higher plants include the higher efficiency of transformation, lower copy number of inserted gene minimizing the risk of silencing, improved fertility of transformants etc. The ability of *Agrobacterium* to transform monocotyledonous has been a matter of debate for a long time, since these plants were not natural hosts to this bacterium. Since the first successful demonstration of integration of foreign DNA in rice chromosomes in a process mediated by *Agrobacterium tumefaciens* by Chan *et al.* (1993), subsequent improvements in the *Agrobacterium* transformation technology of monocots more especially rice have been remarkable (Hei *et al*., 1994; Li *et al*., 2002; Akutsu *et al*., 2004; Ramesh *et al*., 2004). Following a further attempt in this chain, a rice transformation protocol has been refined more precisely by the authors of this study with regard to use of wide- host range *Agrobacterium* agropine strain EHA-105.

The transfer of *T- DNA* from bacterium to plant cell is a tightly regulated process and multiple factors from both plant and bacterial cells are simultaneously required for the transformation process (Tzifra and Citovsky, 2002). At bacterial level, it is mediated by a set of virulence genes such as *VirA*, *VirG*, *VirE*, *VirD* and a complex of *VirB* (Park *et al*., 2000; Gelvin, 2000), with optimal

expression occurring at acidic pH (Turk *et al*., 1991), in the presence of phenolic inducers more, such as acetosyringone, that are released by wounded plant cells (Hei *et al*., 1999) and certain sugars (Cangelosi *et al*., 1990). A series of experiments based on an already established protocol was set up aiming at a better induction of the virulence genes by the modification of above one or more components.

An effective role of nutrition medium is very important in the activation of bacterial virulence genes that, subsequently, is related to the plant transformation efficiency. A hypothesis can be inferred from previous studies (Hei *et al*., 1994; Kumria *et al*., 2001; Li *et al*., 2002) regarding the selection and use of appropriate medium that nutritive constitution of medium might affect differentially the virulence induction efficiency of *Agrobacterium*. Kumria *et al*. (2001), preferred MS medium in their study, while Li *et al*. (2002) considered better AB medium for EHA-105. Of the three tested media in our experiments, the results revealed that although the medium composition affected the efficiency of *Agrobacterium* differentially, yet this effect was also variable with respect to genotype at the same time. So it is too important to keep in mind the genotypic factor while choosing an appropriate medium for better virulence induction.

The recalcitrance of monocots like rice to *Agrobacterium* transformation is due to absence or deficiency of phenolic inducer compounds like acetosyringone. In the virulence system of *Agrobacterium*, *VirA* acts as sensor of phenolic compounds like acetosyringone and subsequently acts as kinase of *VirG* phosphorylation which, in turn, regulates the activity of other Vir operons (Turk *et al*., 1991). However, the sensitivity of *VirA* regarding the acetosyringone concentration is variable with respect to plasmid type and bacterial strain (Aldemita and Hodges, 1996). In this study, although a range of acetosyringone concentrations (0-300 μM) was evaluated, 100-150 μM was the optimal concentration giving maximal transformation efficiency irrespective of rice genotypes tested. At reduced concentrations, the phenolic inducers in the explants of monocotyledonous may be deficient for increased bacterium virulence (Hei *et al*., 1999). The higher concentrations than optimal may too proved be lethal for both *Agrobacterium* and calli. The result is also in accord with those of Clercq *et al*. (2002) who found necrotic zones on calli at AS concentration (500 μM) more than an optimized one.

The bacterial density used for infection was also a critical factor as higher density (0.8-1.0) or prolonged time period (20 min) adversely affected the callus growth and subsequently transformation efficiency. Similar observations have been reported by some previous studies (Kumria *et al*., 2001; Clercq *et al*., 2002). Such circumstances also prevented the proper killing of the bacteria after co-cultivation and were detrimental for the calli, resulting in decreased explant survival 3 days after co-cultivation.

In addition, an acidic pH is needed for optimal expression of the virulence genes (Hei *et al*., 1999). Different *VirA* genes in different strains show differential pH sensitivity. Turk *et al*. (1991) found that a maximum *vir* gene expression at pH 5.3 and 5.8 for an octopine and nopaline strain, respectively. The agropine strain, EHA-105, in our experiment exhibited too differential sensitivity at various pH levels, while giving maximal virulence expression at pH 6.0 irrespective of genotypes. Furthermore, the role of MES was regarded as critical too in maintaining a stable pH level. A relatively higher GUS activity detected in a medium with than without MES, was in contrast with those of Becker *et al*. (1994) who observed that buffering with MES.

Inclusion of sugars in the virulence induction and co-cultivation media also has some definite role in the increased virulence gene expression. Certain sugars may act synergistically with the phenolic inducers (Cangelosi *et al*., 1990). But, the regulatory pathway of sugars is different from phenolic compounds that include VirA and chromosomally encoded protein *ChvE* instead of only *VirA* for phenolic compounds. Various sugars have been tried in the previous transformation experiments to find the best choice. Hei *et al*. (1994) preferred glucose in their studies while Kumria *et al*. (2001) suggested that the use of maltose had more positive effect than sucrose in rice transformation. The higher transformation efficiency with the media included with than without sugar proved definitely the synergistic role of sugars. However, the sucrose and glucose both generated a result remarkably different from maltose as opposed to Kumria *et al*. (2001).

The effect of light on transformation frequencies is not clear whether it is at plant or bacterial level (Zambre *et al*., 2003). Light may enhance the amount of coniferyl alcohol, a virulence gene inducer from the orchid *Dendrobium* (Nan *et al*., 1997), however, it is a normal and usual routine to carry out co-cultivation phase under dark conditions in rice transformation. Light culture may be beneficial during *Agrobacterium* transformation (Lee *et al*., 1999; Zambre *et al*., 2003). Clercq *et al*. (2002) worked out that light strongly promoted gene transfer from *Agrobacterium* to plant cells and co-cultivation in dark was deleterious for the calli in *Phaseolus acutifloius*. Nevertheless, when this practice was attempted in our experiments, we concluded that co-cultivation under

light conditions was not effective. Incubation in dark seems to improve the morphogenic capacity of calli (Compton, 1999), essentially by preserving endogenous light-sensitive hormones (Compton, 1999) or by preventing accumulation of phenolic compounds (Arezki *et al*., 2001). This was in contrast to those of results obtained by Clercq *et al*. (2002) and Zambre *et al*. (2003). These research workers observed that light could have promotive effect with *Agrobacterium* strains such as *octopine*, *agropine*, *succinamopine* types etc. Such an effect was too noted in our investigation while dealing with indica rice. Most probably, the difference between the results may be some plant genotypic factor that responded variably to light conditions through exhibiting the physiological changes such as plant hormone levels, cell proliferation and cell cycle stage. These physiological factors may, in turn, affect the plant cell competence for *Agrobacterium* attachment or *T-DNA* uptake. So, the beneficial effect of light during co-cultivation or other transformation steps should not be ruled out absolutely even in case of japonica sub-species. The rice genotypes belonging to both groups (japonica and indica) could be screened out that respond well to light conditions.

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