Electronic Journal of Plant Breeding, 1(4): 1244-1248 (July 2010)

Research Article

Agrobacterium – mediated transformation of *indica* rice under Acetosyringone –free conditions

N.Aananthi , C.R.Anandakumar[2] R.Ushakumari[3] and P.Shanthi[4]

Abstract
The possibility of developing transgenic *indica* rices through *Agrobacterium* –mediated transformation in the absence of Acetosyringone at bacterial preinduction or cocultivation or both stages was assessed. Six weeks old, scutellum derived calluses of *indica* rice viz., ASD 16, White Ponni, Pusa Basmati, Pusa Sugandh 4, Pusa Sugandh 5 were cocultivated with *A.tumifaciens* strain EHA 105, harbouring the binary vector pCAMBIA 1305.1 with the β-glucouronidase (GUS) and hygromycin phospho transferase genes in the T-DNA region. Addition of Acetosyringone (AS) to the pre induction medium and cocultivation medium induced higher levels of transient GUS expression than that obtained with the addition of AS to either of the stages. Adddition of sucrose to both the media revealed that the transient expression levels were similar to those obtained by the addition of AS. The resultant fertile plants were stable ttransformants as revealed by GUS histochemical assay and PCR analysis for the GUS and HPT genes. The results indicated that the addition of phenolics like AS may not be essential for the induction of *vir* genes and development of transgenic *indica* rices are also possible under AS free conditions.

Key words: *indica* rice, *Agrobacterium* –mediated transformation, Acetosyringone (AS)

Introduction

Rice is considered as a model crop for studying gene regulation and crop improvements in monocots, similar to tobacco in dicots. Plant transformation mediated by *Agrobacterium tumifaciens*, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. Two, transformation protocols are generally available for rice, namely *Agrobacterium* -mediated and biolistics. Of these the former method is most widely used one for the genetic improvement of several crop species including rice. The process of T-DNA transfer from *Agrobacterium* is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer for its own integration and stability. The virulence region is a regulon organized in six operons that are essential for the T-DNA transfer and also for increasing the transfer efficiency.

Different chromosomal –determined genetic elements have shown the functional role in the attachment of *A.tumifaciens* to the plant cell. All the successful reports on *Agrobacterium* –mediated transformation of rice have been based on *Agrobacterium* pre induction or co cultivation in the presence of AS (Hi ei *et al.*1994, Aldemita *et al.*1996, Rashid *et al.*1996, Khanna *et al.*1999, Datta *et al.*2000) or based on cocultivation in presence of suspension culture of potato cells, a rich source of phenolic compounds (Chan *et al.*1993). No transient expression of the GUS gene was observed in the absence of AS (Ramana Rao and Narasimha Rao 2007). Studies on the influence of AS at different steps of *Agrobacterium*-mediated transformation of a *indica*

Assistant Professor, Rice Research Station, Ambasamudram-627401

rices revealed that possibility of transformation of recalcitrant *indica* genotype with simple sugars like sucrose in the absence of AS.

Materials and Methods

Plant material and callus induction for *Agrobacterium*-mediated transformation

The matured seed derived embryogenic calli and immature embryos obtained from five *indica* rice genotypes namely ASD 16, White Ponni, Pusa Basmati, Pusa Sugandh 4, Pusa Sugandh 5 were used as explants in transformation. Media used for culture and transformation are listed in (Table1) Immature seeds of ASD 16, White Ponni, Pusa Basmati1, Pusa Sugandh 4, Pusa Sugandh 5, were collected 12 to 14 days after pollination. After the removal of glumes, seed, were surface sterilized first with 70 per cent ethanol for three minutes and 0.1 per cent $Hgcl_2$ for 5 min followed by three washes with sterile distilled water. Embryos were excised aseptically under a microscope. The embryos were incubated with their scutellar region facing upwards and precultured for one day on pre culture medium (MS +1mg l^{-1} kinetin+ 30 g l^{-1} maltose+ casein hydrolysate 1.0 g l^{-1} and pH adjusted to 5.8,gelled with 8.0g l^{-1} a gar).
Mature seeds of rice genotypes were sterilized as that of immature embryo and incubated on callus induction medium MS +2,4D 2.0 mg l^{-1}+ maltose 30 g l^{-1}, casein hydrolysate 1.0 g l^{-1} gelled with agar 8.0g $l^{-1}$for six weeks at 25±2ºc in the dark for callus development. After six weeks, only embryogenic calluses were selected and cut into 2-4mm diameter and inoculated onto pre induction medium for 4 days in dark prior to cocultivation.

The selected embryogenic calluses were transformed with a super virulent *A.tumifaciens* strain EHA 105 (pCAMBIA 1305.1). This vector containing GUS and HPT genes in the T-DNA region. One loopful of bacterial culture was streaked on AB minimal medium (Chilton *et al*.1974) supplemented with 0.5% glucose, rifampicin (10 mg l^{-1}) and kanamycin (50 mg l^{-1}) and grown at 28ºC in the dark and were cultured for 2 days. From this culture, 2-4 single colonies of the bacterium were transferred to 30ml of AB liquid medium containing rifampicin (10 mg l^{-1}) and kanamycin (50 mg l^{-1}). The culture was grown overnight and bacteria were collected by certrifugation at 3000 rpm for 10 min. The supernatant was removed and the pellet was re suspended in 30 ml of MS broth or AAM medium supplemented with 100 µM AS. The bacterial cell suspension adjusted to 1.0 O.D (A $_{600}$) with sterile sucrose was directly used for infection. The experiments were aimed to evaluate the influence of AS on transformation frequency (Table2) and 100 µM AS was added as optimum concentration to the bacterial culture 4 hours before cocultivation .

Co cultivation was performed according to Ramana Rao and Narasimaha Rao (2007) with minor modifications. Six week old calluses were inoculated with *Agrobacterium* by immersing them in the bacterial cell suspension dispensed in petriplates which were agitated for 15-30 minutes. The calluses were then blotted on sterile filter paper to remove excess bacterial cells and were placed on cocultivation medium with or without 100 µM AS at 25^0C in dark for three days for cocultivation. After completion of incubation the calluses were washed with sterile distilled water containing cefotaxime (100 mg l^{-1}) and carbenicillin (250 mg l^{-1}), dried on sterile filter paper and transferred to selection medium .The calluses were passed through four selection cycles of 15 days each. All growing calluses in the fourth selection cycle (Hygromycin resistant) were transferred to regeneration medium. Regenerated green buds thus obtained were transferred to rooting medium for root induction and development.

Results and Discussion

Transient GUS expression analysis was performed using histochemical analysis. Matured seed derived calli, 48 hour after co cultivation was incubated in X-Gluc staining solution at 37 C overnight. The frequency of transient transformation is expressed as the number of explants showing blue foci and the total number of explants inoculated. For qualitative assay, the area of GUS expression and the intensity of blue colour at each spot were considered to give the effect of various treatments tested. The distinction could be made visible as small spots (<0.5 mm in diameter) representing one or few GUS expressing cells, and large spots (≥ 1 mm in diameter), representing a complete cell cluster expressing the GUS gene (De Clercq *et al*.2002 and Ramana Rao and Narasimha Rao 2007). Based on these assays, the efficacy of AS was assessed.

Stable integration of GUS gene in rice genome was confirmed using polymerase chain reaction (PCR) analyses. Genomic DNA was extracted from the leaves of putative transgenic plants (T0) and non transformed plants by CTAB method. The primer sequences used for amplification of *gus* gene were,

G1(5'GTGGGAAAGCGCGTTACAAG3'), G2 (5' GTTTACGCGTTGCTTCCGCCA3'). The *gus* sequence in total DNA was amplified in a PTC-100 minicycler (MJ Research, USA) with following temperature conditions; pre-incubation period at 94C for 3 min, leading to 35 cycles of denaturation at 55C for 1 min, and synthesis at 72C for 1 min, followed by extension at 72C for 5 min. Amplified PCR product (10 l) was subjected to electrophoresis on a 1% agarose gel and visualised under UV light.

The study was aimed to assess the need for acetosyringone at preindution medium (*Agrobacterium* culture) or at co cultivation or both the stages for *Agrobacterium* –mediated transformation in rice. Addition of AS for both stages induced high levels of GUS expression zones consistently in contrast to low levels of expression in the presence of AS either only at pre induction medium or only at co cultivation medium stages. Similar levels of GUS expression zones were also obtained in sucrose supplemented media devoid of AS in pre induction medium or co cultivation medium or both (Table 3). The results are in agreement with the reports of Ramana Rao and Narasimha Rao (2007).

In addition to high transient GUS expression under AS-free conditions, stable transformation was achieved with both AS and AS-free plus sucrose treatments when immature embryo was used as explants. After four selection cycle, the proliferated shoots on selection medium showed stable GUS expression and the Gus positive plants produced normal green shoots in the regeneration medium (Table 4). The results are similar to the earlier report of Ramana Rao and Narasimha Rao (2007). The variety Pusa Basmati 1 using mature seed derived calli as explant exhibited a maximum transformation frequency with or without AS. These results showed that acetosyringone may not be essential for the induction of *vir* genes of *Agrobacterium* during coculture for transformation of recalcitrant genotypes like *indica* rices.

References

Aldemita, R. R., and T. K. Hodges. 1996. *Agrobacterium tumefaciens*- mediated transformation of *japonica* and *indica* rice varieties. Plants, 199: 612-617.

Chan, M. T., H. H. Chang, S. L. Ho, W. F. Tong and S. M. Yu. 1993. Agrobacterium mediated production of transgenic rice plants expressing a chimeric -amylase promoter / -glucouronidase gene. Plant Mol. Biol., 22: 491-506.

Chilton .M.D., T.C. Currier, S.K. Farrand, N. Baisakh, N. Oliva and S.K. Datta. 1974. Agrobacterium tumefaciens DNA and PS8 bacteriophage not detected in crown gall tumors.Proc.Natl Acad Sci USA 71: 3672-3676.

Datta, K., Z. Koukolikova Nicola, N. Baisakh, N. Oliva and S. K. Datta. 2000. *Agrobacterium* – mediated engineering for sheath blight resistance of *indica* rice cultivars from different ecosystems. Theor. Appl. Genet., 100: 832-839.

De Clereq, J., M. Zambre, M. Van Montagu, W. Dillen and G. Angenon. 2002. An optimized *Agrobacterium*-mediated transformation procedure for *phaseolus acutifolius* A. Gray. Plant Cell Rep., 21: 333-340.

Hi ei, Y., S. Ohta, T. Komari and T.Kumashiro. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J., 6: 271-282.

Khanna, H. K. and S. K. Raina. 1999. *Agrobacterium* – mediated transformation of *indica* rice cultivars using binary and superbinary vector. Aust. J. Plant Physiol., 26: 311-324.

Rashid, H., S. Yokoi, K. Toriyama and K. Hinata. 1996. Transgenic plant production mediated by *Agrobacterium* in *indica* rice. Plant Cell Rep., 15: 727-730.

Ramana Rao, M. V. and G. J. Narashimha Rao. 2007. *Agrobacterium* – mediated transformation of *indica* rice under acetosyringone-free conditions. Transgenic Note., 24: 507-511.

Table 1. Media used for tissue culture and transformation

Callus induction medium	MS +30g l^{-1} maltose , 2,4-D (1.5 mgl^{-1} - ASD 16; 2.0 mg l^{-1} - White Ponni; IAA 2.5 mg l^{-1} - Pusa Basmati 1 Pusa Sugandh 4, Pusa Sugandh 5) with 0.5 mg l^{-1} kinetin and 1.0 g l^{-1} C.H , Coconut Milk 50 m$l l^{-1}$, 8 g l^{-1} Agar, pH 5.8
Co cultivation medium	MS +30g l^{-1} maltose , 10 mg l^{-1}glucose, 2,4-D (1.5 mgl^{-1} ASD 16; 2.0 mg l^{-1} White Ponni; IAA 2.5 mg l^{-1} Pusa Basmati 1 Pusa Sugandh 4, Pusa Sugandh 5) with 0.5 mg l^{-1} kinetin and 1.0 g l^{-1} C.H , Coconut Milk 50 m$l l^{-1}$,8 g l^{-1} Agar, pH 5.8, with or without 100 µM AS
Selection medium	MS +30g l^{-1} maltose , 2,4-D (1.5 mgl^{-1} ASD 16; 2.0 mg l^{-1} White Ponni; IAA 2.5 mg l^{-1} Pusa Basmati 1 Pusa Sugandh 4, Pusa Sugandh 5) with 0.5 mg l^{-1} kinetin and 1.0 g l^{-1} C.H , Coconut Milk 50 m$l l^{-1}$, 8g l^{-1} Agar, 100 mg l^{1} cefotaxime + 250 mg l^{-1} carbenicillion + 30 mg l^{-1} hygromycin B (Boehringer Mannheim, GmbH, Germany), pH 5.8
Regeneration medium	MS +1.0 g l^{-1}tryptophan, 30 mg l^{-1}maltose, 2.5 mg l^{-1}BAP, 0.5 mg l^{-1} NAA and 1.0 mg l^{-1}kinetin, 1.0 g l^{-1} C.H , Coconut Milk 50 m$l l^{-1}$, 8 g l^{-1} Agar, pH 5.8,
Pre induction medium	AB buffer and salts, 5 g l^{-1} sucrose

Table 2.The effect of AS and sucrose on transient transformation was assessed using following treatment combinations.

A - Pre induction of *Agrobacterium* in the presence of AS and sucrose followed by cocultivation in the absence of AS

B - Pre induction of *Agrobacterium* in the absence of AS and presence of sucrose followed by cocultivation in the presence of AS and sucrose

C - Pre induction of *Agrobacterium* in presence of both AS and sucrose followed by co cultivation both in the presence of AS and sucrose.

D - Pre induction of A*grobacterium* in absence of AS followed by co cultivation in the absence of AS.

Table 3. Effect of acetosyringone and sucrose on transient transformation efficiency in rice variety Pusa Basmati1 using seed callus.

Treatment	Pre induction medium		Cocultivation medium		GUS expression zones* Mean ±SD	
	AS	Sucrose	AS	Sucrose	Large	Small
A	+	+	-	+	21.6 ± 0.93	35.0 ± 1.3
B	-	+	+	+	27.4 ± 0.18	35.2 ± 1.2
C	+	+	+	+	35.40 ± 1.36	45.2 ± 1.65
D	-	+	-	+	12.4 ± 0.93	46.6 ± 1.4

* pooled data of two experiments with fifty explants each

A preinduction of *Agrobacterium* in presence of AS and sucrose followed by cocultivation in the absence of AS

B pre induction of *Agrobacterium* in absence of AS and presence of sucrose followed by cocultivation in the presence of AS and sucrose

C pre induction of *Agrobacterium* in presence of both AS and sucrose followed by cocultivation both in the presence of AS and sucrose.

D pre induction *Agrobacterium* in the absence of AS followed by cocultivation in the absence of AS in the presence of sucrose at both stages

Table 4. Effect of AS and sucrose on rice transformation in *indica* rice variety Pusa Basmati1 using immature embryo explant.

Cocultivation	Number of plants producing				
	Number of embryo inoculated (x)	No. of hyg ^{R} producing	No.of Hyg ^{R} and GUS +	No. of Hyg^R + GUS+ Pht regenerated (y)	y/x Transformation frequency (%)
With AS	50	22	12	11	22.0
Without	50	15	5	5	10.0