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EFFECT OF ACETOSYRINGONE AND CALLUS AGE ON TRANSFORMATION FOR SCUTELLUM –DERIVED CALLUS OF RICE

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ABSTRACT

An efficient and reproducible protocol for Agrobacterium- mediated transformation of an agronomically useful *Oryza sativa* variety, Pusa Basmati- 1 (IET- 10364) has been standardized. Although several reporters have been claimed the Agrobacterium- mediated transformation but here we studied the effect of acetosyringone and age of callus on transformation frequency for scutellum-derived callus of Pusa basmati- 1. By optimizing these two parameters for Agrobaterium- mediated transformation, we can produce an efficient and reproducible protocol. The callus derived from mature rice seeds were used as explants for transformation studies. Callus were co-cultured with *Agrobacterium tumefaciens EHA 105* harboring a binary vector p35SGUSINT that carried Beta-glucuronidase (*uid A*) and It contains gene *npt II*, determining resistance to kanamycin, under the control of the constitutive (*nos*) promoter. Maximum Agrobacterium- mediated transformation frequency was observed at 350μM/l acetosyringone with 35 days old callus.

KEYWORDS

Pusa Basmati – 1, Beta- glucuronidase, Transformation, *Agrobacterium tumefaciens EHA105*, p35SGUSINT and Callus transformation.

INTRODUCTION

Rice is one of the most versatile and important cereal crops after wheat and maize because more then half of the worlds population depends on rice as a primary staple food. More than 200 million tons of world production of rice is lost every year due to abiotic and biotic factors.[1] Rice plants are more susceptible to attack from many pest and pathogens.[2] The constructions of genetically improved transgenic plants are the solution of above mentioned drawback in the traditional breeding strategies.[3] An efficient and reproducible protocol for Agrobacterium mediated transformation is required for genetic improvement of cereal crops. Genetic transformation of rice by using Agrobacterium is a most favorable method because it will transfer of DNA with defined ends, minimal rearrangement and integration of a small number of copies of the gene and more importantly, the possibility that even large segments of DNA can be efficiently transferred.[4] Strategies to improve plant regeneration frequency in cereals, including rice have been steadily evolving during the last decade.[5,6,7]

Two type of gene delivery method have been generally used by previous reporters in

rice, namely Agrobacterium mediated & biolistics.[8] Agrobacterium transformation has several advantages over biolistics, such as higher transformation efficiency, large pieces of DNA can be transfer, minimal re- arrangement of transformed DNA, integration in low copy cost etc[8]. The most commonly used reporter gene is Beta-glucoronidase.[9] The GUS gene was originally isolated from *E.coli.*[10] Some reporters were observed intrinsic GUS like activity in rice.[11] It was controlled by treating tissues at alkaline pH 8.0 or by including 20% methanol in the assay.[12] Transformation screening had increased by selectable marker such as antibiotic resistance. The neomycin phosphotransferace (*npt II*) confers resistance to kanamycin[13] and hygromycin phosphotransferase (*hpt* also called *hph*) to hygromycin antibiotic.[14] *Agrabacterium tumefaciens* strain EHA 105 containing p35SGUSINT.[15] This binary vector carried intron containing GUS and in the T-DNA region.

MATERIALS & METHODS

Plant materials/ media

Mature seeds of *Oryza sativa L.* variety Pusa Basmati- 1 (scientific name of this varity is *IET - 10364*) were obtained from National Seed Corporation, New Delhi, India. Plant growth regulators, MS and LB media were supplied by Titan Biotech Limited, India. All chemicals were purchased from Mark Limited, India.

Binary vector and Agrobacterium strain

Super virulent strain of *Agrobacterium tumefaciens EHA 105* having a binary vector p35S GUSINT[15] was used for transformation. It contains gene *npt II*, determing resistance to kanamycin, under the control of the constitutive (*nos*) promoter and the enzyme Beta-glucuronidase, popularly described as GUS, has become the most frequently used reporter gene for the analysis of plant gene expression. The GUS gene was originally isolated from *E.coli.*[10] *UidA* gene encodes a soluble Beta-glucuronidase enzyme breaks down glucuronide substrate to give a colored reaction so that its presence can be detected insitu, i.e. inside the plant tissue; several glucuronides, which can be used as substrate, inside p-nitro phenyl glucuronide (PNPG), 5-bromo,4-chloro, 3-indolyl-β-D-glucuronide (X-glu).

The GUS gene gets expressed in bacterial cell even if it is driven by an eukaryotic promoter which makes the detection of early plant transformation events difficult. Various strategies have been employed to reduced or eliminate the expression of this gene in bacteria. Initial attempt to reduce the expression involved the *mas-uidA* fusion.[16] The mannopine synthase promoter (*mas*) generally inactive in bacteria could reduce the expression of GUS in bacteria. A modified construct pKIW110 made with containing the GUS gene in which the bacterial ribosome binding site was replaced by an eukaryotic translation initiator.[17] Therefore the GUS expression in Agrobacterium is negligible by above modification.

However a third kind of modification involved the introduction of a typical plant intron into the coding region of the GUS gene which resulted in selective expression in plant cells, since bacteria lack the splicing machinery.[15] The construction p35SGUSINT contained a 189bp away from the N-terminus of the GUS polypeptide. This modification construct allows transient and stable expression of GUS to be studied in plant cells without any interference even in the presence of bacteria.

Media

Vitamins, growth regulators, and antibiotics were filter- sterilized and added to the media after autoclaving. Growth media were autoclaved at 121^0C for 20 minute. The pH of the all media was adjusted to 5.7 except Luria- Bertani (LB) media for Agrobacterium which was adjusted to pH 7.0. Media used for culture and transformation are listed in table 1.

Table 1
Composition of different media used in the transformation and regeneration of plantlet

S.No.	Medium	Compositions
1.	CIM	3.42 g/l MS salt, 440 mg/l $CaCl_2 \cdot 2H_2O$, 2.5 mg/l 2,4-D, 500 mg/l Casein Hydrolysate, 300 mg/l proline, 30 g/l Sucrose, 2.5g/l phyta – gel, pH – 7
2.	CCM	CIM + 50-500μM Acetosyringone (without phyta – gel) pH- 5.7
3.	CIM – Sel	CIM + 25mg/l Kanamycin + 200 mg/l Cefotaxime, pH – 5.7
4.	SIM	3.42 g/l MS salt, 440 mg/l $CaCl_2 \cdot 2H_2O$, 2.0mg/l BAP, 0.5mg/l NAA, 0.5mg/l Kinetin, 30g/l sucrose, 50mg/l Kanamycin, 250mg/l Cefotaxime, 2.5g/l phyta-gel, pH-5.7
5.	RIM	3.42 g/l MS salt, 440 mg/l $CaCl_2 \cdot 2H_2O$, 60 mg/l FeSo. 7HO, 40mg/l Na_2 EDTA, 10mg/l Kanamycin, 2.5 g/l phyta-gel, pH- 5.7
6.	GUS assay buffer	0.1M EDTA, 0.1M Phosphate Buffer, 1% Triton x 100, 50mm K-jerrocynate, Methanol, 100MM x-Glue, dH_2O

Callus induction

Mature seeds were dehusked manually and surface sterilized with 70% ethanol for 2 min inside the laminar hood. Subsequently they were incubated with 0.1% $HgCl_2$ for 5-6 min. Then seeds were washed thrice with sterile distilled water. These seeds were blot dried on sterile filter paper and subsequently sterilized seeds were cultured on CIM medium (table -1) for callus induction. The pH of the medium was adjusted to 5.7 with KOH and phyta-gel was added prior to autoclaving at 121^0C for 20 min. The calli which formed in the scutellar region of the seeds were removed and transferred onto fresh callus induction medium and incubated in the dark condition at 28 ± 1^0C. After 35 days a healthy, nodular, creamish white embryogenic callus were obtained and used for transformation study.

Callus transformation

The co-cultivation of callus with *Agrobacterium tumefaciens EHA 105* and regeneration to be done essentially according to the procedure described earlier[18] with some modification on the basis of results of the experiments described below. A supervirulent *Agrobacterium tumefaciens EHA 105* (p35SGUSINT) was grow in liquid L.B. medium containing rifampicin and kanamycin (50mg/l) and grow on an orbital shaker at 28^0C and 250rpm. The bacterial cell suspension adjusted to optical density at 0.3 – 0.6. afterwards, the culture was centrifuged at 3500rpm for 30 – 40 minute and the pellet was resuspended in CCM medium (Table- 1) a density at 10^9 ml^{-1} (OD 600 -1.5 to 2). The co-cultivation medium used here with various concentration of acetosyringone ranges from 100 to 500μM/l (table -1). The callus were immersed in the above bacterial suspension for 15- 20 minute. The callus were then blotted dry on a sterile filter paper to remove the excess bacterial suspension and transferred to CIM – Sel medium (table -1) for 25 days at 28^0C in dark. Sub culturing was done on fresh CIM - Sel medium (table -1) after every 5 days.

Plantlets regeneration

After the selection of transformed callus were transferred to SIM medium (table -1) for regeneration of shoot. The pH of the medium was adjusted to 5.7 with KOH and phyta-gel was added prior to autoclaving at 121^{0}C for 20 min. Twenty callus cluster were placed on plastic petri-dish containing 25 ml of the regeneration medium. Plates were incubated under the regime of 16hr-light at 28^{0}C and 8hr- dark at 25^{0}C. Following regeneration, approximately 3-4cm plantlets were transferred to box containing regeneration medium for rooting to RIM medium (table -1).

GUS assay

The GUS activity in plantlets was determined by a histo-chemical assay according to Jefferson.[10] The tissues to be assayed for GUS were transferred to GUS assay buffer (table -1) in a test tube covered with aluminium foil or black paper (to maintain perfectly dark condition). The test tube containing buffer and sample was incubated at 37^{0}C over night (16hrs). Finally the sample was taken out of test tube and washed twice with 70% ethanol.

RESULT AND DISCUSSION

This study was performed with the objective of maximizing transformation frequency using Agrobacterium mediated transformation. The plasmid p35SGUSINT was used in this study which contains the selectable marker gene *npt II* which imparts kanamycin resistance. In addition it also contains GUS gene with an intron.

Effect of callus age on transformation

Most workers used 3 weeks old calli as ex-plant[18,19] and some reporters have used 8 weeks old callus as ex-plant.[20] Himani Tyagi suggested that 45 days old callus were optimum.[8] In the present study, we have optimized the age of callus to achieve high transformation frequency. The age of callus is a major parameter to obtain the high transformation frequency because every age of callus can not able to withstand infection. We have used 25-45 days old callus for Agrobacterium mediated transformation study and found that 35 days old calli is optimum for Agrobacterium-mediated transformation (fig-3).

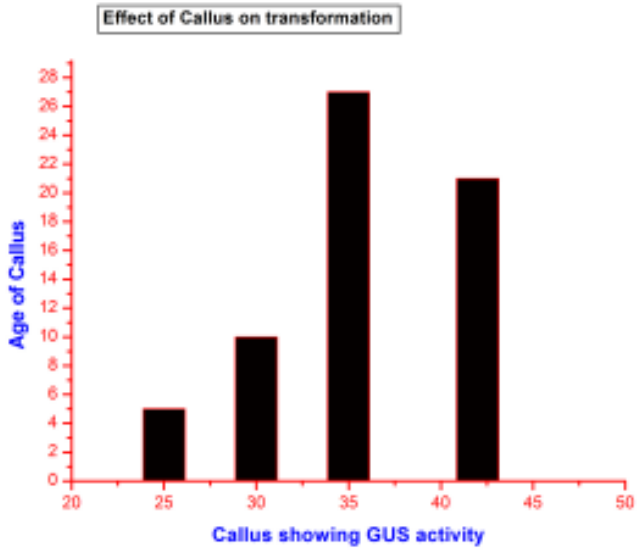

Figure 3
Effect of age of callus on transformation frequency

Effect of acetosyringone on transformation

The concentration of acetosyringone play an important role in the transformation. The previous reporters used 50μM/l acetosyringone to obtained better transformation results[21] and 100μM/l reported by Himani Tyagi.[8]

In the present study, we have optimized the concentration of acetosyringone to find the high transformation frequency for scutellum – derived callus of Pusa Basmati-1 (IET – 10364). We have transferred 35 days old callus in CCM medium (table -1) with various concentration of acetosyringone ranges from 50 to 500μM/l in CCM medium (table -1) and observed that 350μM/l concentration of acetosyringone supported to achieve high transformation frequency (fig-3). Transformation frequency was measure here by means of GUS assay analysis (fig-2).

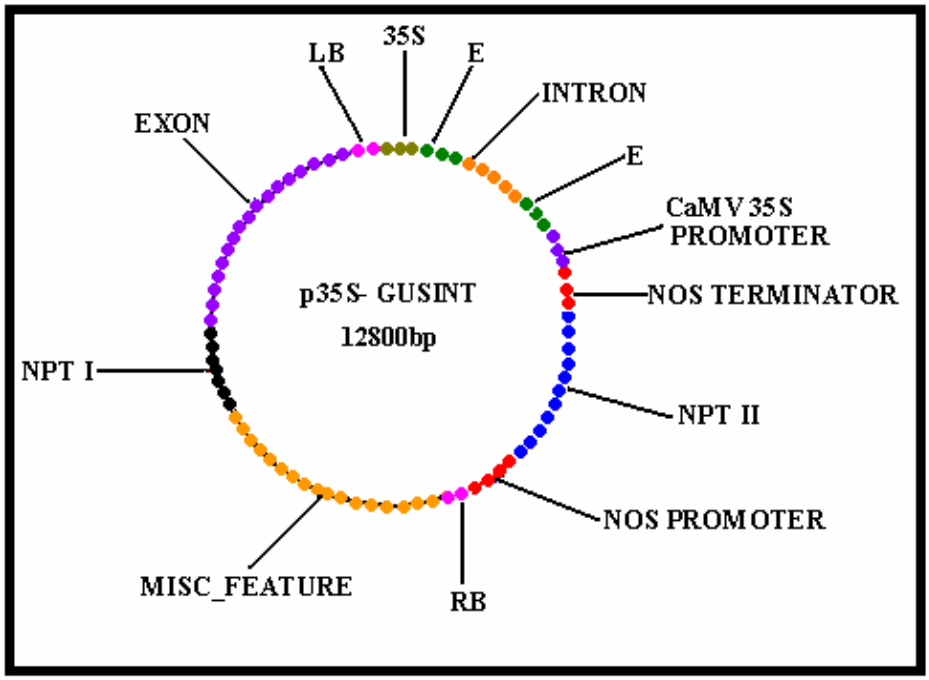

Figure 1
Diagrammatical representation of a binary vector p35SGUSINT

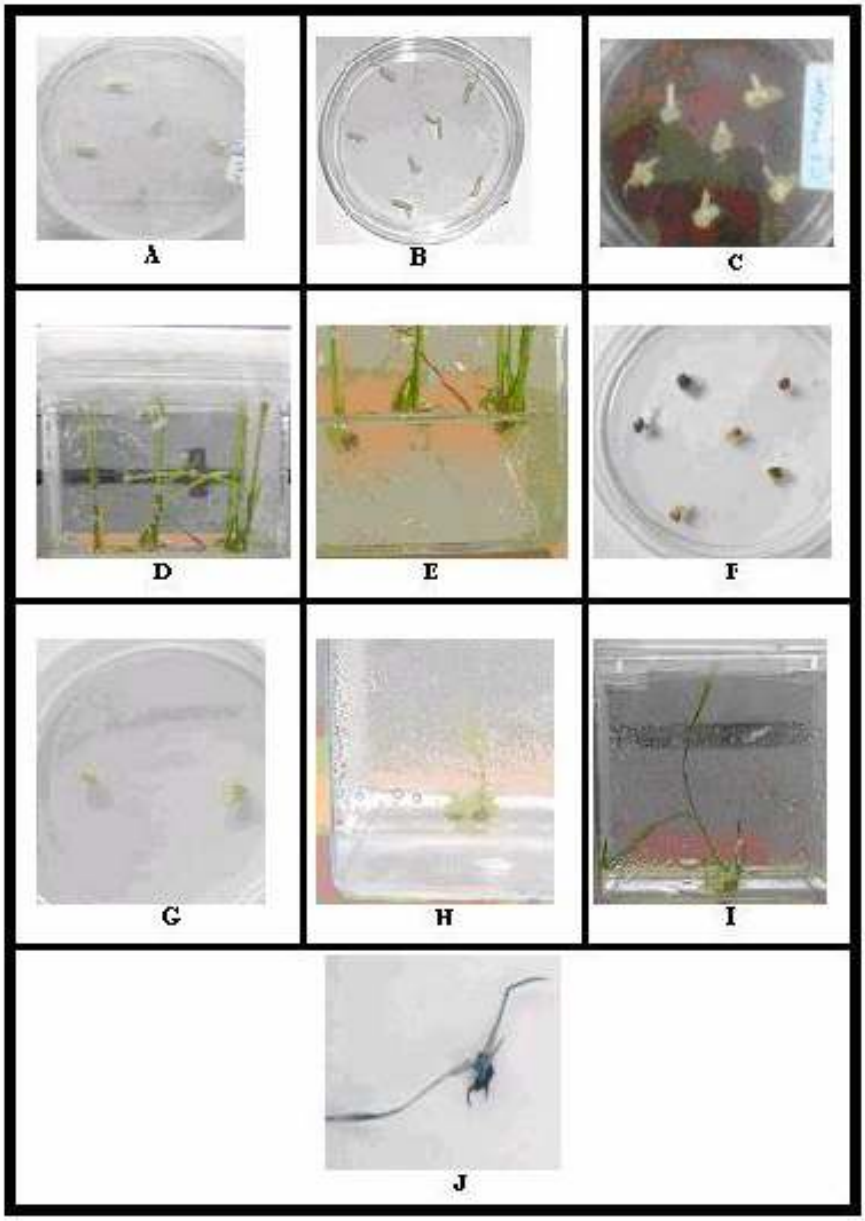

Figure 2

Regeneration and Agrobacterium-mediated transformation in Pusa Basmati-1:
(A) Mature seed of Pusa Basmati-1 on callus induction medium, (B) Germination from rice seeed, (C) Callus formatiom, (D) Shoot induction, (E) Root induction (F) Agrobacterium infected callus and two proliferating callus at the end of second round of selection on Kanamycin, (G) Actively growing callus were transferred on shoot induction medium, (H & I) Shoot regeneration from transformed callus, (J) The regenerated plants were assayed for GUS activity and plant showing GUS activity.

Regeneration and characterization of transgenic plants

Callus were transferred on CIM-Sel medium (table-1) after co-cultivation with *Agrobacterium tumefaciens EHA105* containing p35SGUSINT. The callus which survived in selection medium was subsequently transferred to SIM medium (table-1) for shoot regeneration (fig-2) and kept in dark for one week following which the callus were kept under 16hrs light and 8hrs dark photo period. Shoots were obtained (fig-2). These shoots were transferred to RIM medium (table-1) for root induction. The regenerated plants were assayed for GUS activity and some plants were showing GUS activity (fig-2).

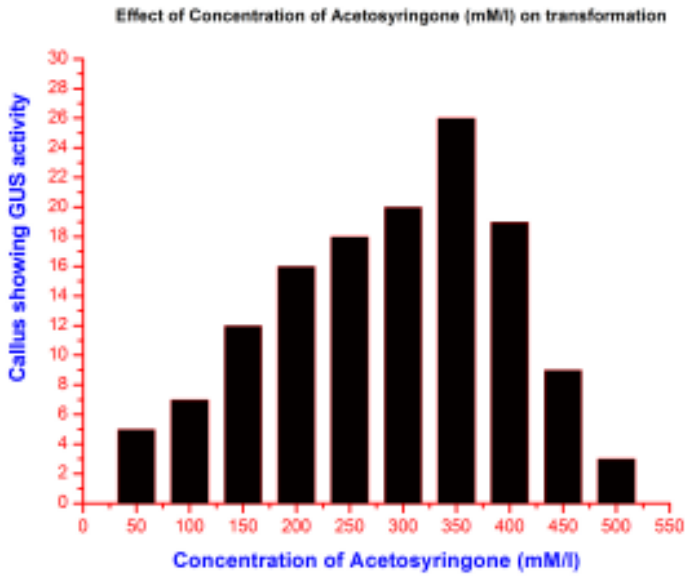

Figure 4
Effect of acetosyringone on transformation frequency

CONCLUSION

In this present study we have optimized the acetosyringone concentration and callus age for transformation for scutellum derived callus of Pusa Basmati -1 (IET - 10364). After the analysis of our results, we have concluded that 350μM/l acetosyringone and 35 days old calli was supported to obtain the high transformation frequency.The above standardized concentration of acetosyringone and age of callus for Agrobacterium-mediated transformation can be used as an effective protocol to introduce agronomically desired gene in *Oryza sativa* variety Pusa Basmati- 1 (IET- 10364) for its genetic improvement.

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