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AGROBACTERIUM MEDIATED TRANSFORMATION TO BUILD RESISTANCE AGAINST BACTERIAL BLIGHT IN RICE

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Abstract

Agrobacterium mediated transformation and regeneration efficiency of rice cultivar IR-6 was studied. Various concentrations and combinations of growth regulators were used to establish efficient culture environment for enhanced regeneration efficiency. Maximum (70.96%) callus induction was achieved on MS medium containing 2,4-Dichlorophenoxyacetic acid with concentration of 2 mg/l. Maximum regeneration frequency (80%) was observed on regeneration media containing NAA 1.0 mg/l and BAP 5.0 mg/l. Calli of more than 5 mm size were infected with *Agrobacterium* containing the binary vector pTCL5. Age of the calli was also found to be a limiting factor in transformation efficiency. Older calli of over four weeks were less efficient in transformation whereas 22-25 days old calli were found to be highly efficient in transformation. Selection of the calli was carried out with Hygromycin (50 mg/l) in addition to Cefatoxime (500 mg/l) in combination with Carbenicillin (500 mg/l). Transgenic plants regeneration frequency was observed as 2.26%.

Introduction

In addition to basmati rice, coarse varieties are also grown in Pakistan. IR-6 is one of the coarse varieties, important because of its high yield and nutritional value. IR-6 was officially released for cultivation in 1971 with the name IR-6 in Punjab and Mehran-69 in Sindh. This variety is popular among the growers due to its high yield (Salim *et al.*, 2003). Unfortunately the yield of IR-6 is adversely affected by various diseases. Bacterial blight, caused by *Xanthomonas oryzae pathovar Oryzae (Xoo),* is one of the most serious diseases of rice in most of the rice growing countries due to its high epidemic potential and its destructiveness. The disease has plagued rice farmers since the beginning of recorded rice cultivation (Ou, 1985) and can cause yield loss as high as 50% in some areas of Asia including Pakistan.

The severity and significance of damage caused by bacterial blight have necessitated the development of strategies to control and manage it to reduce crop loss and to avert an epidemic. Today, the exploitation of host resistance appears to be the only reliable method of disease management. Fortunately, modern tools such as transgenic production for its manifold advantages can be effectively used to complement conventional breeding for the development of built-in resistance in rice cultivars. Most rice varieties belonging to different groups, classified on the basis of isozyme analysis, are generally recalcitrant to tissue culture and as well as transformation (Glaszmann, 1987). To achieve resistance for bacterial blight in rice cultivar IR-6, it was transformed with the cloned bacterial blight resistance gene *Xa21*, as it is known to confer resistance to all known races of *Xoo* (Khush *et al*., 1990; Ikeda *et al*., 1990).

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The objective of this study was to develop regeneration system and *Agrobacterium* mediated transformation in IR-6 which is not previously studied and also to optimize certain parameters affecting *Agrobacterium* mediated gene transformation according to environmental conditions of our country.

Materials and Methods

Mature seeds of IR-6 were manually dehusked and were washed by sterile distilled water for 10 minutes. First surface sterilized with 45% v/v Sodium hypochlorite with constant stirring for 20 minutes, then the explants were washed three times with autoclaved distilled water at a regular interval of 5 minutes. The N6 medium (Chu *et al.,* 1975) and MS medium (Murashige & Skoog, 1962) with different concentrations of 2,4-D, 3% sugar and 6 g/l agar were used for callus induction from scutellum of mature seeds (Fig. 1). For regeneration of plants from calli, MS medium with different concentrations and combinations of Naphthalene acetic acid (NAA), Benzyl amino purine (BAP) and amino acids, 3% sucrose, 3% sorbitol, 2 g/l Casine hydrolysate and 4 g/l gelrite were used.

Starting material for transformation. Three weeks old embryogenic and compact calli of IR-6 were selected for transformation (Fig. 2) as described by Rashid e*t al*., (1996).

Hygromycin selection: To check the lethal dose of Hygromycin, three weeks old scutellum derived calli of IR-6 were subjected to Hygromycin selection media. This media contained N6 salts and vitamins supplemented with 2 mg/l 2,4-D, 30 g/l sucrose, 4 g/l gelrite as solidifying agent and various doses of Hygromycin i.e. 25, 50 and 75 mg/l. The results were noted after two weeks.

Bacterial strain and plasmid: Transformation of rice calli were carried out by using *Agrobacterium tumefaciens* strain EHA101 containing pTCL5 plasmid. It is a binary vector containing Xa21 gene, resistant gene for hygromycin and GUS gene in T-DNA region. The gene for GUS has intron in the middle of the coding region and is driven by 35S promoter of cauliflower mosaic virus. This intron-GUS reporter gene expresses GUS in plant cells but not in the cells of *A. tumefaciens*.

***Agrobacterium* culture and co-cultivation:** *A. tumefaciens* strain EHA101 containing the binary vector pTCL5 (3-5µl) was grown overnight at 28˚C in 50 ml of liquid YEP medium (An *et al.,* 1988) containing 50 mg/l kanamycin, 50 mg/l hygromycin in a flask and shaken @ 100-110 rpm. Bacterial culture was centrifuge at 3000 rpm for 15 minutes, supernatant was discarded and pellet was resuspended in CI1 + As media and mixed on vortex. Embryogenic compact calli were drenched in bacterial suspension for 1-2 min. To remove excess of bacteria these soaked calli were blotted dry with sterile blotting paper. Then calli were transferred to piece of filter paper placed on the CI2 + As (Table 4). The co-cultivation plates were prepared by spreading 1-2 ml of liquid AA + As (Table 4) media on the filter paper placed over the top of CI2 + As (Table 4) medium. Then calli were transferred to the filter paper placed on the top of CI2 + As (Table 4) and the plates were sealed with parafilm (Fig. 3). The co-cultures were placed in the incubator at 28˚C for 2-3 days.

Selection: After co-cultivation, the infected calli were washed with CI1 + Cefotaxime media and calli were transferred to CI2 + Hyg + Cefotaxime. Selection was done for two weeks (Fig. 4).

Regeneration: After two weeks of selection period, transformed calli were transferred to regeneration medium that is RM5 + Hyg + Cf, containing NAA (1.0 mg/l) + BAP (5.0 mg/l) for full plant development with extensive root system (Fig. 5 and Fig. 6).

Assay of ß-glucuronidase (Gus) activity: Histochemical GUS assay was carried out essentially as described by Jefferson (1987). Calli were incubated in X-Gluc solution containing 1 mg/l 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5% triton X-100, 20% Methanol and 50 mM Sodium phosphate buffer (pH 7.0). The reaction mixture was incubated at 37˚C for 2-3 days and calli were examined under microscope.

Results and Discussion

Effect of different media on callus induction and growth: Both N6 and MS media containing 2,4-D 2 mg/l were compared for callus induction and callus growth frequency. Callus formation was observed from scutellum of mature seeds within two weeks of inoculation (Fig. 1) from both media. There was no clear cut difference observed with both the media, but MS media comparatively responded well. Maximum callus induction frequency was 77.7 % on MS and 75.0% on N6 media. Callus induction was observed only when 2,4-D was used. These results were in confirmatory with Rashid *et al.,* (1996; 2001 and 2003) and Noor *et al.,* (2005).

Factors affecting regeneration

i. Effect of different combinations and concentrations of hormones: MS media with 3% sucrose, 3% sorbitol, 2.0 gm/l Casine hydrolysate and different combinations and concentrations of NAA, BAP and amino acids were tested for the regeneration of IR-6 (Table 1). In this experiment the concentration of NAA used was 1.0 mg/l in combination of BAP with 0.5, 1.0, 2.0, 2.5 and 5.0 mg/l. Callus regenerated on all the concentrations and combinations tested, the highest frequency i.e. 80% (Fig. 5) was observed on RM5 (NAA 1 mg/l, and BAP 5 mg/l) followed by 70% on RM4 (NAA 1 mg/l and BAP 2.5 mg/l). It means that enhanced concentration of BAP has promoted regeneration from calli of IR-6. Earlier Lee *et al.,* (1989) found that addition of BAP in regeneration medium had a positive effect on regeneration frequency and plant production. These results were in confirmatory with Jiang *et al.,* (2000) and Cho *et al.,* (2004). They reported that N6 media supplemented with 2-6.0 mg/l 2,4-D, for embryogenic callus and 0.5-10 mg/l BAP combined with 0.02-1 mg/l for shoot regeneration have been selectively used depending on rice cultivars. It was also reported that high osmolarity also stimulate the regeneration frequency (Higuchi & Maeda, 1991). For this purpose high concentration of Sorbitol was used. Results showed that media with osmoticum and Casine hydrolysate showed high frequency of regeneration. We can conclude from the present study that now it is possible to obtain high regeneration frequency i.e., upto 80% from IR-6.

ii. Age of calli: Age of calli was found to be critical for regeneration. For this purpose, calli of different ages were used for regeneration. Scutellum derived calli of different ages i.e., 15, 21, 23, 28, 33 and 40 days were placed on RM5 (NAA 1.0 mg/l + BAP 5.0 mg/l) where 21 days old calli showed the highest plantlet formation i.e. 85%. These results are in confirmatory to the results of Rashid *et al.,* (1996).

Factors influencing transformation

i. Selecting lethal dose of Hygromycin: Four different concentrations of Hygromycin (25, 35, 50 and 75 mg/l) were tested to determine the lethal dose in transformation of rice (IR-6). At 25 mg/l only 20% calli turned brown and 70% calli exhibited growth. At 35 mg/l 50% showed growth and 40% calli turned brown, while at 50 mg/l 13% calli turned brown and no callus growth was observed**.** With the increase of hygromycin concentration to 75 mg/l the calli became black and showed no growth, which mean that at this dose calli started to die. In case of 75 mg/l Hygromycin necrosis was observed in 5 calli (Table 2). In these experiments 50 mg/l of Hygromycin was found to be lethal dose. Hygromycin is extensively used for rice transformation (Hauptmann *et al.,* 1987 and Ortiz *et al.,* 1996). Hygromycin differentiate between transformed and non transformed calli. It has already been reported that 50 mg/l hygromycin was lethal dose for Basmati 370, Basmati 385 and Basmati 6129 (Rashid *et al.,* 1996) and for Super Basmati (Rashid *et al.,* 2001).

ii. Age of calli: Scutellum derived calli of different ages (15, 21, 23, 25, 28, 33, and 40 days) were co-cultivated for two days with *A. tumefaciens* strain EHA101 containing pTCL5 keeping other conditions constant that is pH of co-culture medium at 5.8 and inclusion of Acetosyringone 50mM. About 40% of co-cultured calli were incubated with GUS substrate after each washing and the remaining were transferred to selection media (CI2 + 50 mg/l Hyg + 1000 mg/l Cf) (Table 4). The highest percentage (70%) of GUS expression was observed from 21 days old calli followed by 23 days old calli (60%). GUS expression was also observed from 25 days (40%), 15 days (30%) and 33 days (20%) old calli. No GUS expression was observed by 40 days old calli. Hashizume *et al.,* (1999) obtained high frequency of transformation i.e. 54% and 57% from 19 and 27 days old calli respectively. Rashid *et al.,* (1996) used 28 days old scutellum derived calli and reported 22% transformation efficiency. From these results it can be concluded that relatively younger and actively dividing cells and tissues can be used more efficiently as compared to older explants or cells in culture for transformation studies in rice.

iii. Effect of co-cultivation period on transformation efficiency: Twenty one days old scutellum derived calli (Fig. 2) were co-cultured with *Agrobacterium tumefaciens* strain EHA101 (Fig. 3) for varying co-cultivation period that is for 1, 2, 3 and 4 days keeping other factors constant i.e., pH 5.8 and acetosyringone concentration 50 mM. Fifty percent of the calli were used for GUS assay after co-culture. The highest percentage of GUS expression (76%) was observed when co-cultivation time period was two days and bacterial growth was observed normal. GUS expression was decreased as the time period was increased and excessive bacterial growth was observed. The lowest obtained percentage of GUS (8.0%) was observed when co-cultivation time period was kept for four days and excessive bacterial growth was observed. 44% GUS expression was observed when co-cultivation period was kept for three days. These results were in line to the study of Rashid *et al.,* (1996). Our results were in contrast to the results obtained by Hiei *et al.,* (1994) and Mohanty, (1999) in which 4 days of co-cultivation period showed highest GUS activity. The reason for this difference might be due to the difference in the varieties and genotype of rice tested.

Transgenic plants production: After two weeks of selection (Fig. 4), calli were transferred to regeneration media. For this purpose RM5 was used containing Hygromycin 50 mg/l + cefatoxime 1000 mg/l (Table 4). Out of 170 calli, 67 were selected on Hygromycin and only 5-6 calli showed regeneration (Fig. 5 and Fig. 6). Regeneration frequency was 2-3%. After regeneration different parts of transgenic plants were histochemical tested for GUS assay. Different part e.g. roots and leaves showed GUS positive result but Gus expression was clearer in leaves region. Under the microscope different parts of transgenic IR-6 showed blue color (Table 3). Our results were similar with Tang *et al.,* (2001) which showed transformation frequency upto 17%. The reason for less transformation efficiency might be the response of the rice genotype to *Agrobacterium* mediated transformation. However study is in progress to increase the transformation efficiency of this variety.

Table 1. Effect of different combinations and concentrations of hormones on plant regeneration from scutellum derived calli in rice (*Oryza sativa* L. cv. IR-6).

Media used	No. of calli	No. of calli showing browning	Calli showing differentiation	Calli showing green spots	No. of plants regenerated	Regeneration frequency (%)
RM1	10	03	07	00	00	00
RM2	10	02	08	06	02	20
RM3	10	01	06	08	05	50
RM4	10	02	08	08	07	70
RM5	10	03	07	08	08	80
RM6	10	05	05	05	06	60
RM7	10	02	05	05	03	30
RM8	10	04	04	04	04	40
RM9	10	05	03	04	05	50
RM10	10	06	04	03	04	40
RM11	10	05	03	05	04	40
RM12	10	03	05	04	03	30
RM13	10	04	04	05	02	20
RM14	10	06	06	05	03	30
RM15	10	04	04	04	01	10
RM16	10	04	03	03	02	20
RM17	10	05	05	02	03	30

Table 4. Media used for transformation protocol and regeneration of transformed calli of rice (*Oryza sativa* L. cv. IR-6).

Stage	Media	Composition
Co-cultivation	CI1 + As	N6 salts and vitamins + 2.0 mg/l 2,4-D + 100 µM acetosyringone, pH 5.7-5.8.
	AA + As	AA media + 2.0 mg/l 2,4-D+100 µM acetosyringone, pH 5.7-5.8.
	CI2 + As	CI1 + 50mM acetosyringone + 4.0 g/l gelrite, pH 5.7-5.8.
Selection	CI1 + Hyg + Cf	N6 salts and vitamins +2 mg/l 2,4-D + 1000 mg/l Cf, pH 5.7-5.8.
	CI2 + Hyg + Cf	N6 salts and vitamins + 2.0 mg/l 2,4-D + 50 mg/l Hyg + 1000 mg/l Cf + 4.0 g/l gelrite, pH 5.7-5.8.
Regeneration	RM5 + Hyg +Cf	MS salts and vitamins + 3% sucrose + 3% sorbitol + 2.0 g/l casine hydrolysate + 1.0 mg/l NAA + 5.0 mg/l BAP + 4.0 g/l gelrite+50 mg/l Hyg+1000 mg/l Cf, pH 5.7-5.8.

Table 2. Response of calli to different concentrations of hygromycin in rice (*Oryza sativa* L. cv. IR-6).

Concentration of Hygromycin (mg/l)	Total calli cultured	No. of calli showing browning	No. of calli showing necrosis	No. of calli showing growth
00	20	02	00	18
25	20	04	02	14
35	20	04	06	10
50	20	13	07	00
75	20	15	05	00

Table 3. Regeneration frequency of transgenic IR-6 on MS media with 1.0 mg/l NAA and 5.0 mg/l BAP + 1000 mg/l Cf and 50 mg/l Hygromycin.

S. No	No. of calli co-cultivated	Selected calli on Hygromycin	Proliferation and differentiation	Green spot formation	Plantlet formation on Hygromycin	Gus analysis	Transformation frequency (%)
Exp. 1	130	65	36	25	04	02	1.54
Exp. 2	160	55	38	29	05	04	2.50
Exp. 3	220	81	41	36	07	06	2.73
Average	170	67	38	30	5.33	04	2.26

Fig. 1. Seeds inoculated on MS medium showing callus induction.

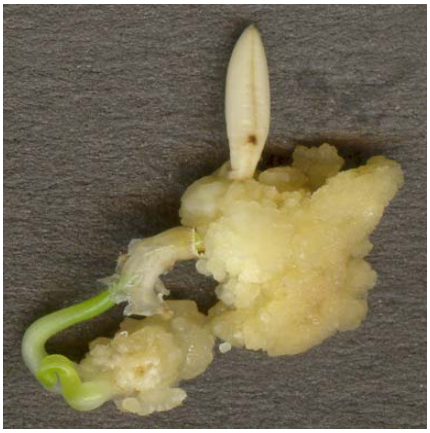

Fig. 2. 21 days old callus.

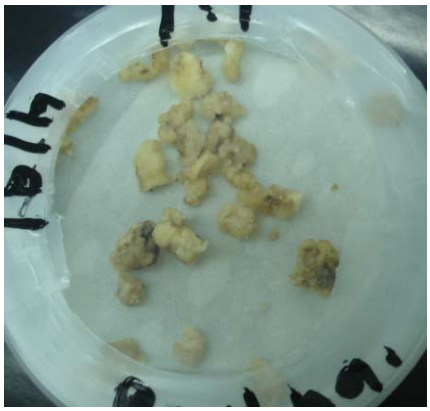

Fig. 3. Co-cultivation.

Fig. 4. Selection.

Fig. 5. Callus becoming green on regeneration medium.

Fig. 6. Regeneration of transgenic calli.

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