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IMPROVEMENT OF BASMATI RICE AGAINST FUNGAL INFECTION THROUGH GENE TRANSFER TECHNOLOGY

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Abstract

In this study, gene for fungal resistance (RCC2) has been introduced into Basmati 385 by *Agrobacterium* mediated transformation. Maximum callus induction (88%) was achieved on MS medium with 2.0 mg/l 2,4-D. Different combinations and concentration of growth regulators (NAA and BAP) were used to develop an efficient culture environment for higher regeneration frequencies. Maximum plant regeneration (80%) from calli was achieved on RM5 (NAA 1.0 mg/l+ BAP 5.0 mg/l). Hygromycin was used as selectable agent and at concentration of 50 mg/l proved to be lethal for scutellum derived calli. Calli of more than 5 mm in size were infected with *Agrobacterium* strain EHA101. Rice chitinase gene-RCC2 with vector pB1333-EN4 was introduced under the control of enhanced CaMV 35S promoter. Transformation efficiency proved to be highest when 21-24 days old calli were used with co-cultivation period of 2-3 days. Selection of the calli was carried out with hygromycin (50 mg/l) in addition to cefotaxime (1000 mg/l). After two weeks of selection, calli were transferred to RM5 containing hygromycin 50 mg/l + cefatoxime 1000 mg/l. A significant regeneration frequency of transformed plants was attained which was 10-11%.

Introduction

Rice is one of the most important cash crops in the world and it plays a very significant role in Pakistan economy. Unfortunately it is subjected to more than 40 diseases causing low yields of rice in the world including Pakistan. Rice blast caused by *Pyricularia oryzae* is one of the most destructive and widespread diseases of rice (Jia *et al.,* 2000). It is well documented that each year the disease destroys rice enough to feed 60 million people and cause farmers a loss of $ 5 billion. Conventional breeding methods may be employed to create resistance varieties, however it will be a time consuming and labour intensive task. On the contrary biotechnological approaches are valuable in introducing genes which provide resistance against *Pyricularia oryzae* (Lin *et al*., 1995). In this study cloned blast resistance gene RCC2 was introduced in rice cultivar Basmati 385 to develop disease resistance against fungal stress through *Agrobacterium* mediated transformation. RCC2, in a previous study (Lin *et al*., 1995; Nishizawa *et al*., 1999) has shown enhanced resistance against blast.

Materials and Methods

Callus induction and regeneration: Mature seeds of Basmati 385 were manually dehusked, washed by sterile distilled water and surface sterilized with 45% v/v Sodium hypochlorite with constant shaking for 20 minutes. The explants were then washed three times with autoclaved distilled water at a regular interval of 5 minutes. These seeds were aseptically inoculated on MS medium (Murashige & Skoog, 1962) with 2 mg/l 2, 4-D, 3% sugar and 6 g/l agar were used for callus induction from scutellum of mature seeds.

MS medium with different combination and concentrations of Naphthalene acetic acid (NAA), Benzyl amino purine (BAP), 3% sucrose, 3% sorbitol, 2 g/l Casine hydrolysate and 4 g/l gelrite were used for regeneration of plants from calli.

Bacterial strain and plasmid: Transformation of the calli was carried out by using *Agrobacterium tumefaciens* strain EHA101 containing vector pB1333-EN4 (Fig. 2). It is a binary vector containing RCC2 gene and Hygromycin resistance gene in the T-DNA region.

Transformation procedure: *A. tumefaciens* strain EHA101 containing binary vector pB1333-EN4 (Fig. 2) (3-5µl) was grown overnight at 28˚C in 50 ml of liquid YEP medium (An *et al*., 1988) containing 50mg/l kanamycin, 50mg/l hygromycin and shaken at the rate of 100-110 rpm. Bacterial culture was centrifuged at 3000 rpm for 15 min., and the pellet was resuspended in Amino Acid medium + Acetosyringone. Embryogenic compact calli were drenched in bacterial suspension for 1-2 min., dried with sterile blotting paper and transferred to filter paper placed on the co-cultivation plates which was prepared by spreading 1-2ml of liquid AA + As media on the filter paper, placed over the top of CI2 + As medium i.e. MS salts and vitamins + 50µM acetosyringone + 4.0 g/l gelrite + 2.0 mg/l 2,4-D). These plates were sealed with Parafilm and incubated at 28˚C for 2-3 days. After co-cultivation, the infected calli were washed with CI1 + Cefotoxime media (MS salts and vitamins + 2 mg/l 2,4-D + 1000mg/l Cefotoxime), and were transferred to CI2 + Hygromycin + Cefotaxime. Selection was done for two weeks.

Regeneration: Transformed calli after two weeks of selection period were transferred to regeneration media i.e., RM5 + Hygromycin + Cefotaxime, (MS salts and vitamins + 3% sucrose + 3% sorbitol + 2.0 g/l Casine hydrolysate + NAA 1.0 mg/l + BAP 5.0 mg/l) for full plant development with extensive root system.

Results and Discussion

Factors affecting regeneration

i. Effect of different combinations and concentrations of hormones: Regeneration medium containing different concentrations of NAA and BAP i.e., concentration of NAA 1.0 mg/l in combination of BAP with 0, 1, 2, 3, 4 and 5 mg/l. Highest frequency i.e. 80% was observed on NAA 1mg/l, and BAP 5 mg/l followed by 75% on RM3 (NAA 1mg/l and BAP 3 mg/l (Table 1) as reported by Noor *et al.,* (2005). Earlier wat is proved that addition of BAP in regeneration medium had a positive effect on regeneration frequency (Lee *et al.,* 1989). Enhanced concentration of BAP has promoted regeneration of calli (Jiang *et al*., 2000; Cho *et al*., 2004).

Factors affecting transformation

i. Age of calli: Scutellum derived calli of different ages (15, 18, 21, 24, 27 and 30 days) were co-cultivated for two days with inclusion of Acetosyringone 50 µM. After washing with CI1 + Cf was transferred to selection media (CI2 + 50mg/l hyg + 1000mg/l Cf). The highest percentage of transformation efficiency (25%) was observed in 21 days old calli followed by 24 days old calli (20%). It was also observed that with the increase of age, the frequency of gene transformation decreased as reported before (Hiei *et al*., 1994;

Hashizume *et al*., 1999). Thirty days old calli did not survive during the selection period (Table 2). Relatively younger and actively dividing cells and tissues can be used more efficiently as compared to older explants, for transformation in rice.

Table 1. Effect of different combinations and concentrations of hormones on plant regeneration from scutellum derived calli in rice cv. Bas-385.

Media used	No. of calli	No. of calli showing browning	Calli showing differentiation	Calli showing green spots	No. of plants regenerated	Regeneration frequency (%)
RM0	20	05	08	00	00	00
RM1	20	05	15	13	4	20
RM2	20	03	13	16	11	55
RM3	20	04	12	16	15	75
RM4	20	10	9	10	12	60
RM5	20	06	14	18	16	80

Table 2. Effect of age of calli on transformation efficiency after two days of co-cultivation with *Agrobacterium tumefaciens* strain EHA101 (EN4-pB1333) in rice cv. Bas.385.

Age of calli (Days)	Total calli cultured	Calli survived on hygromycin	Transformation efficiency (%)
15	20	01	5
18	20	02	10
21	20	05	25
24	20	04	20
27	20	01	5
30	20	00	00

ii. Effect of co-cultivation period on transformation efficiency: Co-cultivation period was varied i.e., for 1, 2, 3 and 4 days. The highest transformation percentage (66.66%) was observed when co-cultivation time period was kept for two days and normal bacterial growth was observed (Table 3). The lowest obtained percentage (8.33 %) was observed when co-cultivation time period was kept for four days and excessive bacterial growth was observed which is consistent to earlier findings (Rashid *et al.,* 1996). Transformation efficiency decreases as the time period increased and excessive bacterial growth was observed.

iii. Use of antibiotics: To control the bacterial growth, different levels of Cefotaxime were tested. At 1000 mg/l Cefotaxime, bacterial growth was negligible and callus growth was significantly enhanced to 60%.

iv. Regeneration frequency of transgenic plants: After two weeks of selection calli were transferred to RM5 containing Hygromycin 50 mg/l + Cefotaxime 1000mg/l. Transgenic plant production efficiency was 10-11 % (Table 4). The reason for less transformation efficiency might be the response of rice genotype to *Agrobacterium* mediated transformation. However further studies are being carried out to enhance the transformation efficiency of this variety.

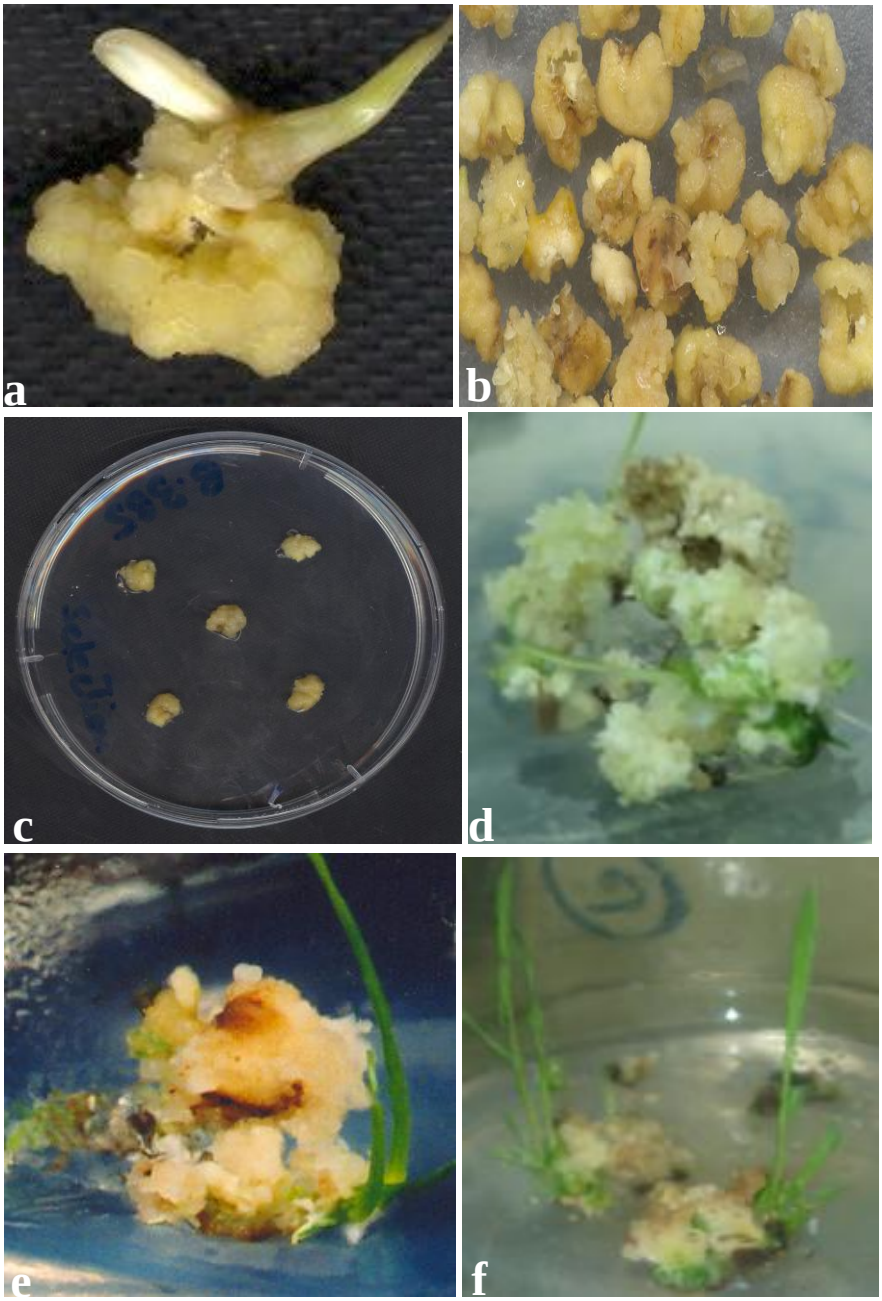

Fig. 1. Photographic presentation of *Agrobacterium* mediated transformation in cv. Bas-385: (a) 21 days old callus, (b) Co-cultivation (c) Selection, (d) Callus becoming green on regeneration media, (e) Arising of shoot from transgenic calli, (f) Regeneration of transgenic calli.

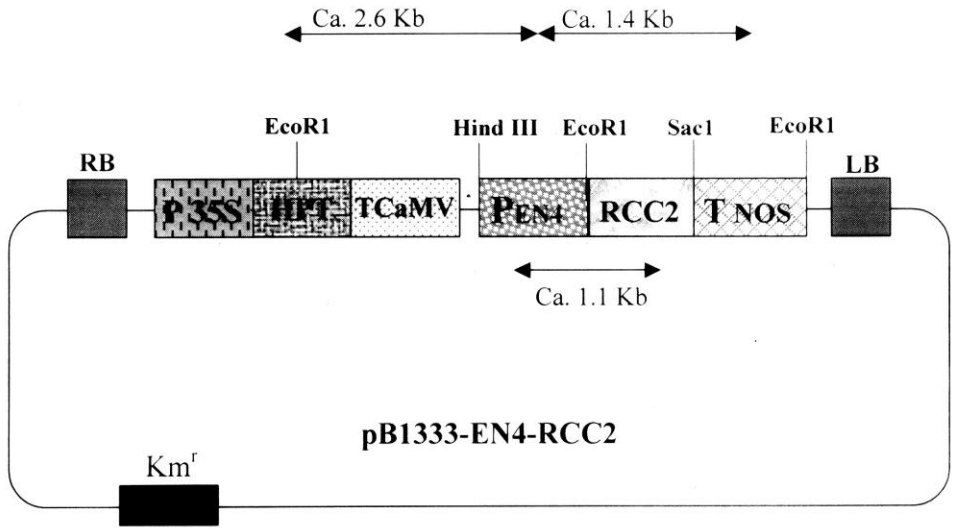

Fig. 2. Partial diagram of binary vector pB1333-EN4

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