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Research Article

AN EFFICIENT AND REGENERATION PROTOCOL FOR AGROBACTERIUM MEDIATED TRANSFORMATION OF INDICA RICE

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Abstract

A simple and efficient protocol for regeneration and agro bacterium –mediated transformation of an agronomical useful indica rice has been standardized. Initiation of callusing and its sub-culture were best achieved in Nitsch medium with 300 mg/l casein enzyme hydrosylate.The best callusing and regeneration responses were observed at concentration of 20mg/l 2,4-D.The regenerated phase contains MDU5 and hormone was analyzed for Indica genotype. Callus induction was about 45 days in dark and further growth was arrested with ABA and 70% embryonic callus was transferred to regeneration media with green buds of 90%.the embryogenic calli derived were used for transformation with agro bacterium tumifaciens(lba 4404or Eha 101 strain).The binary vector pkhg4 contain cry1 Ac gene were used in the transformation studies. The indica rice genotype responded to the optimize media producing hygromycin resistance calli.

Key words: Indica rice, regeneration, transformation.

Abbreviations: 6-benzyl Aminopurine; 2, 4-dichlorophenoxy acetic Acid; NAA: naphthalene acetic acid.

Introduction

Rice (*Oryza sativa*) is likely the most important food crop in the world and almost half of the world population depends on rice as their staple food .one producer to increase rice productivity and farmer avenues is minimize the use

of pesticides through the production of transgenic rice that auto resist notorious pest, namely rice blast and rice stem borer which cause upto 50% yield loss in some areas. Rice genetic transformation (Rotino G. L and Gleddie S et al.,1990) has taken the rapid studies since the first transgenic rice plant was produced 15 years ago. Although the introduction of DNA into rice cells is easy, regeneration of fertile transgenic plant is difficult and limited to some indica cultivars.

Materials and Methods

Dehusked mature seeds of indica rice with milder detergent and surface sterilized by soaking in 70% ethanol for 20min and tween 20 ml for 60 min.after three washes in sterile water ,these seeds were cultured in nitch medium pH 5.7 containing 20 mg /l 2,4-dichloro phenoxy acetic acid and 100 mg/l casein hydrolysate ,30 g/l sucrose and 0.8% agar .The explants(Murashinge T and Skoog F .,1962) were kept in dark for 45 days then the callus were isolated and sub-cultured in 1mg/l aba .then the embryogenic calli were isolated.

Plant regeneration medium.

45 days callus were cultured on N6 medium with 20 mg/l 2,4-Dichloro phenoxy acetic acid and 100mg/l casein hydrolyase,their petridishes were kept at $25c^0$ under light condition for 25 days. Six weeks after culture ,the number of calli forming shoots and the number of shoots per callus (M.L.Christianson et al,1983) were counted to determine the optimum combination of IAA or NAA and BAP or kinetin for plant regeneration from seed derived calli.

Effects of antibiotics:

Surface sterilized seeds of rice japonica rice were induced to form calli on N6 medium (PH 5.7) containing 500 mg/l casein hydrolysate, 30 g/l sucrose and 0.8% agar. After 10 days, calli are transferred to the callus induction medium (Lane WD et al, 1979) supplemented with hygromycin and cefotaxine.

For determination the effect of antibiotics on callus induction, hygromycin and cefotaxine were added to the callus induction medium. The concentrations were tested with 50,100, 150 and 250 mg/l hygromycin and 250,300,350,400,450, and 500 mg/l for cefotaxine .All levels of the 2 antibiotics were added to the callus induction

medium after it was autoclaved. The calli were cultured at $25c^0$ less than 16 hrs photoperiod .Four weeks after culture, the effectiveness of antibiotics was evaluated.

Transformation: The plant transformation vector ,pkhg4 contain cry 1 Ac .This genes (cry 1 Ac) was mobilized into Agro bacterium host strain. LBA 4404 by electroporation(Chandra A and Pental D, 2003) and hygromycin resistant gene ,each of which was expressed under camv 35 s promoter.

Effect of co- cultivation condition on transformation efficiency.

Sterilized mature seeds, 40 days old seeds derived calli of rice were used as the explants for transformation (Jaiwal P. K et al, 1995) in this experiment. Agro bacterium tumefaciens strain lba 4404 pkhg4 vector was cultured over night at 28c in LB medium containing 50 mg/l kanamycin for 48 hrs until O.D -1.0 .the explants were soaked in the agro bacterium suspension for 10,20,30,40 and 50 min,then blotted dry on sterilized filter paper and transferred to the induction medium for 3 days .Five days after co- cultivation ,the optimal co cultivation time for transformation efficiency was determine.

Agro bacterium mediated transformation.

45 days old seed derive calli were immersed in the agro bacterium suspension for 30 min, then blotted dry on sterile filter paper .They were then co-cultivated on the callus induction medium for 3 days .After co cultivation, the calli were washed thoroughly in sterile distilled water containing cefotaxime .Explants co- cultivated with agro bacterium tumefaciens (Somers D .A et al, 2003) were transferred for selection producer to the same medium supplemented with same selective agents. After 60 days of culture, regenerated plants with well developed roots were potted grown in a green house.

Particle bombardment.

Prior to bombardment, the plasmids containing chimeric DNA (Porebski. S et al,1987) were precipitated and adsorbed to M_{17} tungsten particles following the procedure recommended by Sanford et al., 1993).40 days old seed derived calli of indica rice were used as the explants for bombardment. The calli were placed in the center of petri dishes containing N6 medium.

Tables: 1. Effect of growth regulators against shoot regeneration.

S.NO	Growth regulator BAP	ABA	NAA	No of explants	shoot response	Percentage of shoot response
1	1	1	0.5	12	Callus	-
2	1.5	1.5	1.0	12	Callus	-
3	2.0	2.0	1.5	12	Callus	-
4	3.0	3.0	2.0	12	Callus	-
5	4.0	4.0	2.5	12	Green spots	-
6	5.0	5.0	3.0	12	4-5	75
7	6.0	6.0	3.5	12	1-2	60

Tables: 2.Effect of NAA against root regeneration.

s.no	Growth regulator NAA	No of days	Root response	Percent of root response
1	0.5	21	-	-
2	1.0	21	-	-
3	1.5	21	Callus	-
4	2.0	21	Rooted	60
5	2.5	21	Rooted	70
6	3.0	21	Rooted	75
7	3.5	21	Rooted	85

Results

Callus induction medium.

The development of rice embryos was distinct at 2-3 days after being cultured on N6 medium containing 2,4-Dichlorophenoxy acetic acid and NAA.Scutellum derived callus was yellow and the callus had compact appearance under light condition(T.A.Thorpe et al ,1980) while the color of calli cultured in darkness was creamy white and callus had(Z.W.Shapple.2003) tradable character.N6 medium supplemented with 2,4-Dichloro phenoxy acetic acid and NAA in every concentration ,produced calli under light and dark conditions

Plant regeneration:

Dehydrated calli cultured on ms medium or without coconut water containing either green spots were transferred to n6 medium for 1 week. However, low frequencies of shoots derived from green spot calli were observed. The dehydrated calli cultured on n6 medium supplemented with hormone 20 mg/l 2, 4-Dichlorophenoxy acetic acid and 300mg/l casein hydrolase had the highest percentage of shoot regeneration (40%) (Orlikowska TK, and Der WE.1993) and the largest number of shoots per callus.

Effect of antibiotics:

Antibiotics used in the study strongly reduced callus induction of rice in presence of 50-250 mg/l hygromycin and cefotaxime ,a slight inhibition effect was observed .The highest dose of cefotaxime that yield surviving calli was 400 mg/l .

Transformation

Rice transformation via particle bombardment (Sambrook, J et al., 1989) at the distance of 9 cm from stopping screen to callus yielded, a higher percentage expression (100%) compared to using agro bacterium (61%) particle bombardment at the distance of 12 in (35.5%)

Discussion

The presence of plant growth regulator is generally essential to growth promotion .They are required either singly or more commonly in combination(Erdelsk K E. et al ,1987) ,induction of rice in tissue culture is 2,4-Dichlorophenoxy acetic acid at various concentrations depending on the explants source and genotype of rice. Although 2,4-D alone induced callus formation from rice embryos(Halamkov et al ,2004) ,some organic substance such as casein hydrolysate ,auxin (NAA,IAA) added to the callus induction medium containing 2,4-Dichlorophenoxy acetic acid could enhance the efficiency of callus formation.

Dehydration of calli for 5 days under light condition before transferring to regeneration medium promoted plant regeneration capacity. They were transferred to the regeneration medium and this consequently resulted in higher capacity of plant regeneration. Increasing the hormone (BAP, ABA, NAA) increase the shoot regeneration .A

selective agent is crucial for selection of transform ants (Dunwell J. M et al., 2000) in distinguishing medium. For selection hygromycin (20 mg/l) and cefotaxime (250 mg/l) were used.

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