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Genetic engineering protocol using *Agrobacterium tumifaciensin* HYV rice (*Oryza sativa L.*) through embryogenic callus

Abstract

Genetic engineering is a technique of which usually requires aseptic condition and a long process to regenerate leaves, stem and roots from the transformed tissues. This method contains two part that is one is tissue culture and another cloning of foreign gene. Recently plant tissue culture, biotechnology & genetic engineering have made possible to produce engineered crops that is resistant to a variety of biotic and abiotic stress. *Agrobacterium*-mediated gene transfer, which is one of the most common rice transformation methods, has been extensively used for developing transgenic rice to study gene function and gain many traits, such as tolerance to drought submergence and salt and higher quality and yield. The objective of this research work was outlined to optimize for this genetic transformation protocol for indica rice through embryogenic callus induction. Mature rice grain embryos were used as explants for callus induction with different concentrations of 2,4-D (1.5, 2.0, 2.5 and 3.0 mgL^{-1}) and kinetin (0.3, mL^{-1}) for callus induction in MS medium. HYV rice varieties were used for callus induction such as Binadhan-4, Binadhan-5, Binadhan-7, Binadhan-14, Binadhan-16, Binadhan-17, BRRI dhan28, BRRI dhan29. Among the varieties, the better callus induction ability was observed in Binadhan-14 and Binadhan-16 with the combination of 3.0 mg/l 2,4-D + 0.3 mg/l kinetin than other varieties. For regeneration ability was performed highly with the combination of BAP (2.0 mg/l) and NAA (0.1 mg/l) for Binadhan-14 and Binadhan-16. We have used embryogenic callus for transformation of BINAdhan-14 & BINAdhan-16 and as a result BINA dhan-14 showed better transformation efficiency than BINA dhan-16.

Keywords: *agrobacterium*, embryogenic callus, co cultivation, transformation

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Abbreviations: 2,4-D, 2, 4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine, benzyl adenine; NAA, naphthalene acetic acid; HYV, high yielding variety; DNA, deoxyribonucleic acid; MS, Murashige and Skoog; BINA, Bangladesh institute of nuclear agriculture; OD, optical density; GMOs, genetically modified organisms; BRRI, Bangladesh rice research institute; pH, negative logarithm of hydrogen ion concentration (-Log[H+I); T-DNA, Transfer DNA; LB, Luria Broth; GUS, β-1,4-glucuronidase

Introduction

Even though Conventional breeding program has been used for increasing crop yield in Bangladesh but recently genetic engineering technologies is very essential to introduce foreign gene into another plants for producing transgenic product. This method contains two part that is one is tissue culture and another cloning of foreign gene. Transgenic product of rice has been successfully developed by using many methods such as electroporation, polyethylene glycol treatment, particle bombardment and *Agrobacterium*- mediated method since the late 1980s.[1] *Agrobacterium*-mediated gene transfer is the most popular technique in rice which has been expansively used for developing transgenic product against pathogen, pest insect, tolerance to submergence and greater yield and quality contributing traits. Generally, the regeneration from cell and tissue is a key piece of genetic engineering that is mandatory for transformation.[2] High regeneration ability of crop parts from in vitro cultured tissues and cells are a pre-requisite of successful genetic engineering technologies for crop improvement.[3] Plant cell tissue culture and biotechnology have made possible to produce engineered crops that is resistant to a variety of biotic and abiotic stress, and that can help to substantially reduce or even eliminate the huge crop losses.[4] *Agrobacterium*-mediated transformation is now the ideal method of gene transfer into rice to direct DNA uptake procedures.[5,6] These methods usually result in the insertion of unarranged segments of DNA into recipient plant genomes, often at low copy number.[7] Additionally, relatively large DNA fragments with defined ends (i.e. left and right T-DNA borders) can be integrated at high frequency into recipient plant genomes.[8] Many reported successful transformation of japonica and easily amenable indica rice genotypes by using mature seed-derived callus as explants and found that 3-week-old mature seed-derived callus are the most suitable explants for this method.[9,10] There are several reports on transformation of recalcitrant indica rice cultivars, for example, IR64 and IR72, through *Agrobacterium* referred[11–17] with a relatively low frequency of transformation. We report here an improved transformation protocol for indica rice cultivars as BINAdhan-14 and BINAdhan-16 by use of mature seed-derived callus as explants. The objective of this research work was outlined to optimize for this genetic transformation protocol for indica rice through embryogenic callus induction. Here, the aim of this study also reported here the achievement protocol of transgenic rice production using an *Agrobacterium*-mediated method of which can be used to study for future biotechnological and genetic engineering subject.

Materials and methods

Embryogenic callus induction

Experimental materials were Binadhan-4, Binadhan-5, Binadhan-7, Binadhan-14, Binadhan-16, Binadhan-17, BRRI dhan28, BRRI dhan29 at the Biotechnology lab., Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh. The mature embryos of three aromatic rice varieties were cultured on MS medium supplemented with five concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 and 3.0mg/l) in combination with a single concentration of BAP (0.5 mg/l).[2] Mature rice grain embryos were used as explants for callus induction with concentrations of 2,4-D (1.5, 2.0, 2.5 and 3.0mgL^{-1}) and kinetin (0.3mgL^{-1}) for callus induction in MS medium (Table 1). Furthermore, for regeneration from callus it was supplemented on MS medium with combinations of BAP (1.0 and 2.0mg/l) and NAA (0, 0.1, 0.2, 0.3, 0.4mg/l) in Table 2. Mature rice grains attached to endosperm were the main source of explants for embryo culture. Mature seeds were dehucked manually with a special thoroughly in running tap water. The floating dehusked seed was carried discarded and others were sterilized with 70% ethanol for 1min (3 times) and wash with 3 times washes in sterile distilled water and then with 50% cloras for 20min with shaking, followed by 4-5 washes in sterile distilled water and air dry for 20min. Sterilized seeds were cultured on callus induction medium MS media[18] containing 30g/L sucrose, 3.0 mg/L 2,4-D, 0.3mg/L kinetin, 4.0g/L phytagel, and 2.0g/L agar at 26°C in the dark. Para film used to seal the petri dish. After 21-25 days, old callus (without breaking) were sub cultured to freshly prepared MS medium supplemented with same concentration of medium for convenient size of the callus (Figure 1) and cultured for another 21-25 days. When white hard embryogenic callus appeared and diameterin 5-6 mm then it was subcultured onto the same medium 5d before infection with *Agrobacterium*.

Table 1 Callus induction from different concentrations of 2, 4-D & Kinetin in MS medium from mature embryos of eight rice varieties

Variety	Treatments		Callus induction/ explant	Callus induction %	Days required for callus induction
	2,4-D (mg/l)	Kinetin(mg/l)			
	1.5		2	10	
Binadha-4	2.0	0.3	5	25	
	2.5		9	45	
	3.0		12	60	
	1.5		5	25	
Binadhan-5	2.0	0.3	7	35	
	2.5		10	50	
	3.0		14	70	
	1.5		6	30	
Binadhan-7	2.0	0.3	5	25	
	2.5		14	70	
	3.0		13	65	
	1.5		15	75	
Binadhan-14	2.0	0.3	12	60	
	2.5		15	75	21-25 days
	3.0		18	90	
	1.5		14	70	
Binadhan-16	2.0	0.3	12	60	
	2.5		16	80	
	3.0		17	85	
	1.5		10	50	

Table Continued

Variety	Treatments		Callus induction/ explant	Callus induction %	Days required for callus induction
	2,4-D (mg/l)	Kinetin(mg/l)			
Binadhan-17	2.0	0.3	12	60	
	2.5		15	75	
	3.0		14	70	
	1.5		13	65	
BRRI dhan28	2.0	0.3	12	60	
	2.5		16	80	
	3.0		13	65	
	1.5		12	60	
BRRI dhan29	2.0	0.3	14	70	
	2.5		13	65	
	3.0		16	80	

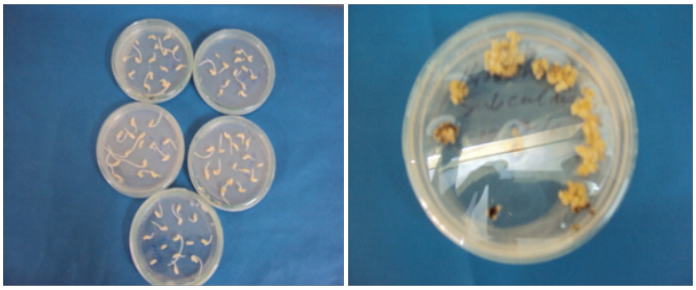

Figure 1 Embryogenic callus induction from mature rice grains for transformation (Binadhan-14 & Binadhan-16).

Co cultivation procedure

A super-virulent *Agrobacterium* tumefaciens GV3101 strain harbouring plant expression vector pB2WG7 containing Calmodulin construct (OsCAL) from glycerol stock at -80°C(100µl) was inoculated in liquid 10ml LB medium (Luria-Bertani medium) + 10ml kanamycin (50 mg/ml stock + 10 ml rifampicin (10 mg/ml stock). A super-virulent *Agrobacterium* strain EHA105 was transformed with pCAMBIA1301 by triparental mating.[19] *Agrobacterium* EHA105 (pCAMBIAI301) was grown to an optical density (OD) of 1.0 in AB medium[20] containing 100 mg/L kanamycin and 10 mg/L rifampicin. The culture was grown in constant temperature incubator shaker at 30°C for overnight to grow optimal organism of *Agrobacterium* for co-cultivation with embryogenic callus. After that, this culture was centrifuged with the spin at 3220g for 10min, then removed upper supernatant and the pellet (Figure 2) was resuspended in equal volume of AA-AS medium[21] in Table 3. For suitable and sufficient infection with the explants, freshly excised callus was wounded and immersed in *Agrobacterium* suspension for 1minute gentle shaking and then transferred them on blotted dry with sterile filter papers for a short period of time to remove the excess bacterial suspension. All infected callus was transferred onto an MS cocultivation medium (Table 3) overlaid with Whatman no. 1 filter paper. Before transfer of callus the Whatman no. 1 filter paper was wetted with 1mL of AA-AS medium. Infected callus was incubated at 26°C 3d in the dark. After 3d, infected callus was washed 6-7 times with sterile distilled water and finally once with aqueous solution containing cefotaxime (50mg/ml stock) and streptomycin (50 mg/ml stock), blotted on sterile tissue paper, and again transferred to MS selection medium prepared from the information in the Table 3. For the excess bacterial after 5 days infected callus again was washed 5-6 times with same antibiotics on same MS selection medium and washed for another 7-8 days. By using of many times selection (maximum 4 times), vigorous callus was transferred to MS regeneration medium (Figure 3). Active callus again subcultured to increase root and shoots regeneration (Figure 4).

Figure 2 Pellet of *Agrobacterium tumefaciens* GV3101 strain harbouring plant expression vector pB2WG7 containing OsCAL.

Table 2 Regeneration from embryogenic callus with the different combinations of BAP and NAA

BAP (mg/l)	NAA (mg/l)	Sucrose (g/l)	Phytagel (g/l)	Regenerated plantlets/callus	
				Binadhan-14	Binadhan-16
	0	30	4	0	0
1	0.1	30	4	1	2
	0.2	30	4	3	2
	0.3	30	4	2	1
	0	30	4	0	0
	0.1	30	4	4	5
2	0.2	30	4	2	3
	0.3	30	4	1	2

Table 3 Composition of different media for transformation

SL No.	Name of different media	Stock	Composition	Volume
1	Embryogenic callus induction	-	MS basal +3.0mg 2, 4-D + 0.3mg Kinetin + pH 5.8	1000ml
2	LB media	-	1.0gm tryptone (1%) +0.5gm yeast extract (0.5%)+1.0gm Nacl(200mM) + pH 7.0	100ml
3	AA media	-	1.0 gm sucrose + 0.5gm glucose+0.05gm casamino acid + pH 5.6	50ml
4	AA-AS media	0.1962 gm/10ml(100mM) acetosyringone	AA+50µl As (acetosyringone)	50ml
5	MS co-cultivation	0.1962 gm/10ml(100mM) acetosyringone	MS basal, + 1.0ml B5 vitamin + 3.0gm maltose +1.0gm glucose+100 µl acetosyringone + pH 5.6	100ml
6	MS selection media	50mg/ml cefotaxime and 50mg/ml streptomycin	MS basal +24µl2, 4-D+ 6µl kinetin +1.00ml cefotaxime, +500 µl streptomycin+ pH 5.8	100ml

Results

Agrobacterium- mediated transformation is a dominant and vital tool in molecular biology that can be used in future breeding program and genetic study for rice improvement. It offers an alternative method for adding specific desirable trait from wild to existing cultivars through molecular gene transfer. But an effective transformation protocol is required to produce GMO of rice successfully. Besides, in primary study, investigations were made to generate transgenic rice and the condition was optimized for *Agrobacterium* -mediated genetic transformation in BINAdhan-14 and BINAdhan-16 varieties. Using mature embryo direct somatic embryogenesis has been proved to be a more reliable method of obtaining plant of true to type. So, firstly callus initiation and regeneration performance from mature embryos of eight HYV rice varieties such as Binadhan-4, Binadhan-5, Binadhan-7, Binadhan-14, Binadhan-16, Binadhan-17, BRRI dhan28, BRRI dhan29 were evaluated in this study. To meet this purpose, callus induction potentiality of mature embryo on MS media supplemented with different combinations of hormone and growth regulators were investigated. Embryo of mature seeds of seven varieties of rice used as explants were cultured on MS medium supplemented with the concentrations of 2,4-D (1.5, 2.0, 2.5 and 3.0mgL^{-1}) and kinetin (0.3 mgL^{-1}) for callus induction. Among the eight varieties, the highest callus induction ability (90% & 85%) was observed in Binadhan-14 & Binadhan-16 with combination of 3.0mg/l 2, 4-D + 0.3mg/l kinetin than other varieties (Figure 1) and all genotypes has been taken 21-25days (Table 1). Moreover, for regeneration from callus it was supplemented on MS medium with combinations of BAP (1.0 and 2.0mg/l) and NAA (0, 0.1,0.2,0.3,0.4mg/l) and highest performance was the combination of BAP (2.0mg/l) and NAA (0.1mg/l) for Binadhan-14 and Binadhan-16 (Table 2). So, we used embryogenic callus for transformation of BINAdhan-14 and BINAdhan-16.

Age of embryogenic callus, duration of infection time and co-cultivation periods were found an important factor to influence genetic transformation. For the co-cultivation periods viz. 2, 3, and 4 days it was followed using *Agrobacterium* suspension having constant optical density (OD of 0.5). Here, we used 5-week old embryogenic callus for co cultivation (Figure 5). In the present study, we already observed that the use of a lower concentration (OD of 0.5) of *Agrobacterium* suspension in AA-AS medium and co-cultivation media overlaid with Whatman no. 1 filter paper reduced the browning of callus after co cultivation probably because of reduced damage to embryogenic callus during *Agrobacterium* infection, which resulted in less phenolic production and better recovery of callus during selection. When we used higher concentration of *Agrobacterium* suspension in AA-AS medium, it was observed that excess bacterial growth and ultimately callus was died. After 3d, infected callus was washed by the distilled water with 5-6 times and last once with aqueous solution containing cefotaxime (50mg/ml stock) and streptomycin (50mg/ml stock), blotted on sterile tissue paper, and transferred to MS selection medium (Figure 6). During the second selection, the callus was kept for a minimum period of 25 days on media to determine the transformed

from the non-transformed cells. In our opinion, any callus that turned into brown within 10 days and new callus growth appearing after 20 days during the third selection were found to be untransformed. After 3 rounds of selection, callus continued on MS regeneration medium for 2 wk in the dark to supported healthy growth of embryogenic callus (Figure 5). After incubation and co-cultivation with *Agrobacterium*, capacity of transformation was observed by histochemical assay of GUS reporter gene in callus tissue. Transient GUS assay was done at the end of co-cultivation and it was randomly selected by 20% inoculated callus tissue. Following GUS histochemical assay, it was found that both the two varieties namely BINA dhan-14 and BINA dhan-16 exhibited positive responses towards transformation. In the present study, between the varieties, we obtained BINAdhan-14 showed better transformation efficiency than BINAdhan-16.

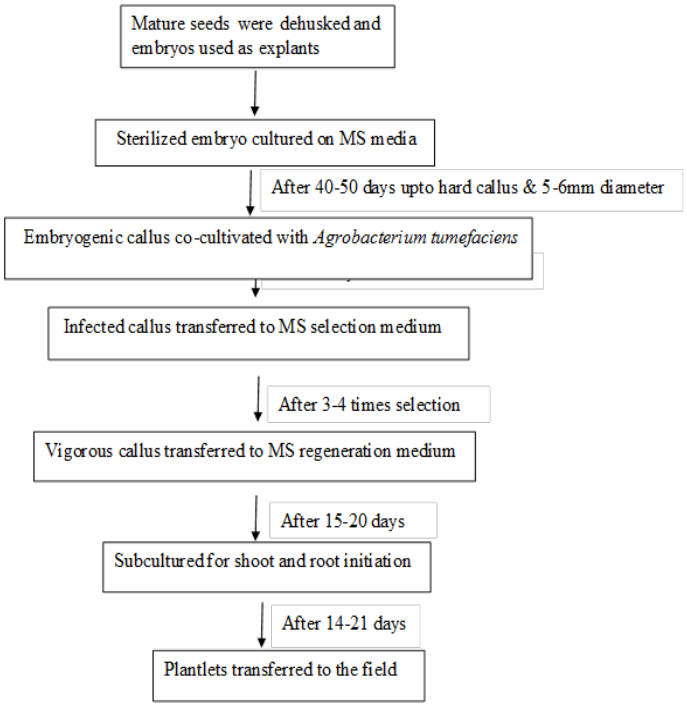

Figure 3 Technical route for the development transgenic plants.

Figure 4 Actively growing pieces of callus were transferred to MS regeneration medium to proliferate and differentiated into green shoots of Binadhan-14.

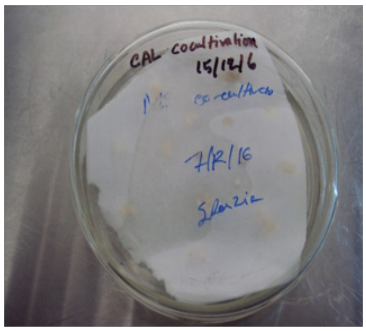

Figure 5 Embryogenic callus co-cultivated with Agrobacterium (OsCAL) on MS co-cultivation medium.

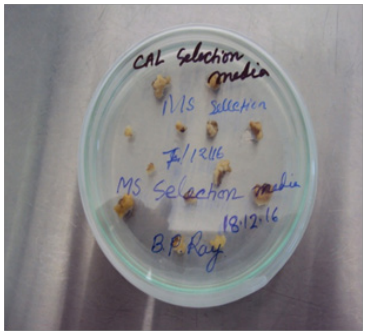

Figure 6 Co cultivated embryogenic callus transferred to MS selection medium.

Discussion

The efficiency of transformation from the embryogenic callus was varied in different plant species. Recently, the study of the abiotic stress such as submergence, cold, saline & drought reaction has forward-thinking significantly as referred.[22] A differential response of rice cultivar has also previously been reported for somatic tissue and anthers from different rice genotypes.[23–25] For induction of shoots from callus of eight rice varieties, Binadhan-14 and Binadhan-16 was better than others. Most of the rice verities exhibited the lowermost shoot regeneration percentage both in 0.5mg/l NAA+ 5.0mg/l Kn and 0.5mg/l NAA+ 13.0 mg/l Kn combinations.[2] It was reported that plant regeneration was influence by genotype.[26] It was also reported that prolonged infection time adversely affects the callus growth and subsequent regeneration.[27] In that case, MS medium containing maltose 30 g/l maltose, supplemented with 2 mg/l BAP, 0.1 mg/l NAA, 2 g/l casein hydrolysate were observed highly for the three genotypes and here after this regeneration combinations used for this study.[28] Two-month-old hard friable callus could withstand infection with *Agrobacterium* efficiently. Earlier workers used 3-week-old callus.[9,28] In the present study, between the varieties, we obtained BINAdhan-14 showed better transformation efficiency than BINAdhan-16. This result has supported with the findings[29] who reported influenced of varieties on transformation efficiency was found largely. The efficiency of anther transformation in *A. tumefaciens* strain EHA105 was compared to that of *A. tumefaciens* strain AGL1 using the floral-dip condition as described.[30] The inoculation medium without PH adjustment or without MS has reduced the transformation efficiency slightly as well as the substitution of 5% sucrose with 5% glucose. Deletion of sucrose and Tween-20 from the inoculation medium has reduced the transformation efficiency dramatically. Although substitution of sucrose with glucose in the inoculation medium could

increase the transformation efficiency of Arabidopsis[31,32] the results demonstrated that sucrose could not be efficiently substituted by glucose in the inoculation medium for the floral-dip transformation of rice. However, these results indicate that embryogenic transformation is a simple, active and efficient method for the development of transgenic product in rice.

Conclusion

Plant cell & tissue culture from explants for regeneration is a main component of biotechnology and genetic engineering that is required for the genetic transformation of rice. The objective of this research work was outlined to optimize for this genetic transformation protocol for indica rice through embryogenic callus induction. Among the varieties, the better callus induction ability was observed in Binadhan-14 and Binadhan-16 with combination of 3.0 mg/l 2,4-D + 0.3 mg/l kinetin than other varieties. For regeneration ability was performed highly with the combination of BAP (2.0 mg/l) and NAA (0.1 mg/l) for Binadhan-14 and Binadhan-16. We have used embryogenic callus for transformation of BINAdhan-14 & BINAdhan-16 and as a result BINA dhan-14 showed better transformation efficiency than BINA dhan-16.Further research is needed, however, before the use of such materials can be recommended in Bangladesh.

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Ethics approval and consent to participate

Not applicable

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Competing interests

The authors declare that they have no competing interests.

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