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Full Length Article

Development of Efficient Regeneration System in Different Recalcitrant Rice Cultivars and Expression Analysis of Putative Transgenic Plants

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Abstract

Establishment of an efficient and reproducible regeneration system in three different rice cultivars, IRRI-6, IRRI-9 and KSK-282 remained obscured. In the present study regeneration system was developed for all three recalcitrant cultivars and resultant putative transgenic plants were analyzed for expression by reverse transcriptase polymerase chain reaction (RT-PCR). Cultivars responded to a particular concentration of 2, 4-D ranging from 2 to 4mg $L^{-1}$for calli initiation and induction. Two types of calli with different frequencies were observed on both N6 and N6 supplemented with proline, glycine and MES. N6 media produced more number of type II calli while N6 supplemented favored type I calli in all three cultivars. Type I calli were found as yellowish, smaller in size and globular shape and better explants for transformation. *GUS* histochemical assay showed that 11, 13 and 14 days older type I calli of KSK-282, IRRI-6 and IRRI-9were suitable for transformation respectively. Fifteen minutes infection time and 2 days co-cultivation period showed the highest *GUS* positive expression in calli of all three cultivars. KSK-282 and IRRI-9 showed best regeneration response on 0.5 mg L^{-1} kinetin, 1 mg L^{-1}NAA and 3 mg L^{-1} BAP, while the IRRI-6 showed the highest regeneration at 0.5 mg L^{-1} kinetin, 1mg L^{-1} NAA and 2 mg L^{-1} BAP. Using optimized protocol for each cultivar *GUS* reporter gene has been successfully incorporated in the genome of all three cultivars. Transgene expression in resultant lines was confirmed at transcriptional level through reverse transcriptase polymerase chain reaction (RT-PCR). © 2014 Friends Science Publishers

Keywords: Recalcitrant rice cultivars; Efficient regeneration; RT-PCR analysis; *GUS* histochemical assay

Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world population and a model monocot grass plant for both genetic and functional genomics studies (Xiaojia *et al*., 2006). In Pakistan rice is cultivated on an area of 2.57 million hectares with annual production of 6.16 million tons and average yield of 2396 kg ha^{-1}. In Pakistan, rice occupies about 10% of the total cultivated area, accounts for 6.1% of value added in agriculture and 1.3% in gross domestic product (Government of Pakistan, 2012).

Current research mostly emphasizes on the development of an efficient and genotype independent regeneration system for *indica* rice. In this respect the role of different phytohormones, carbon source and adequate amount of chemicals such as proline, cysteine and MES has been recognized (Shahsavari *et al.,* 2010). Various growth regulators and amino acids have been investigated to recover better frequency of callogenesis, as a pre-requisite to identify the cells capable to carry the transgenes and subsequent recovery of plants with desire superior traits (Visarada and Sarma, 2002). Moreover, amino acid in culture media has enhanced the somatic embryogenesis. Asparagine, L-glutamine, glycine, L-proline and L-tryptophan were frequently used in culture media (Zaidi *et al*., 2006).

Agrobacterium mediated transformation has been affected by various factors like genotype, type and physiological status of explants, composition of media, inoculation time, type of vector, bacterial strain, selectable marker, *in vitro* conditions and the addition of phenolic compound (Hiei *et al.,* 1997; Khalida and Al-forkan, 2006; Tyagi *et al*., 2007). These factors may be altered to increase the frequency of transformation in economically important rice cultivars.

At present the IRRI-6, IRRI-9 and KSK-282 are the most important commercial rice cultivars in Pakistan. However, biotic and abiotic factors frequently affect its

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production. Establishment of an efficient tissue culture system and genetic transformation procedure is necessary to enhance resistance against the biotic and abiotic stresses (Sujatha and Sonti, 2005).

The objectives of the present study was to develop efficient and reproducible regeneration system in three different rice cultivars, IRRI-6, IRRI-9 and KSK-282 by investigating different parameters that affect transformation efficiency and to analyze the resultant putative transgenic plants for expression by reverse transcriptase polymerase chain reaction (RT-PCR).

Materials and Methods

The present study was conducted at National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC Islamabad during the years 2011-2012. Three different rice cultivars, IRRI-6, IRRI-9 and KSK-282 were taken from Rice program of NARC. Already available pCAMBIA 1301 vector at NIGAB was used in the present study. Different parameters were investigated that affect callogenesis and transformation efficiency. Three different levels of clorox 30, 40 and 60% were evaluated for contamination control and their affect was observed on callus induction frequency. Three different concentrations of 2, 4-D (2, 3 and 4mg L^{-1}) were selected to evaluate the days to calli initiation and calli induction in above three different cultivars. Effect of N6 simple and N6 supplemented media (Table1) was determined on type and quality of callus production. In order to identify the proper stage of calli for transformation, eleven, thirteen and fourteen days older calli of each cultivar were selected and subjected to *GUS* histochemical assay (Rueb and Hensgens, 1989). Three different infection times 5, 10 and 15 minutes were selected and then calli were incubated for 1 and 2 days on co-cultivation media to check the effect of infection times and co- cultivation periods on transformation efficiency. The calli of all three cultivars were transferred to different regeneration media RM I, RM II and RM III (Table 1) to determine the regeneration efficiency. Regenerated shoots were transferred to the rooting medium (Table 1). After vigorous root development, plants were transferred to sterile soil pots and kept at 25±2°C under 16 h photo periods for hardening. Plants were covered with polyethylene bags to retain humidity. After 7-10 days, envelopes were backed off to reduce humidity gradually until plants were acclimatized to ambient humidity and temperature. Genomic DNA was extracted from both putative transgenic and non-transgenic plantlets through CTAB method (Sambrook and Russel, 2001). The confirmations of transgenic plants were carried out through PCR by using 5' GTCGGCTTTCAGCTGTCTTT 3' and 5' GGTGGTGGCTAGCTTGTTTG 3' *GUS* specific primers. The PCR profile was optimized as denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 57°C for 40 sec, 68°C for 1 min and final extension of 68°C for 7 min. Total RNA was extracted from the leaves of all three cultivars through invitrogen RNA extraction kit. A total of 7ng RNA from each sample was used to generate first strand of cDNA in two steps RT-PCR by AMV Reverse transcriptase # EP0641. 3µL of reverse transcription reaction product of each sample was used to amplify the *GUS* gene in 50 µL PCR reaction.

Results

The results indicated that the average contamination frequency on 30% clorox was higher than 40 and 60% clorox respectively. Reference to Table 2, the 40 and 60% clorox concentrations were not significantly different with respect to contamination frequency.

On the other hand clorox concentrations also affected callus induction frequency unlike cultivars and the interaction between cultivars and clorox concentrations. The minimum average frequency of callus induction was observed on 60% Clorox while maximum average callus induction frequency was noted on 30% clorox. The 40% clorox concentration favored 85.6% callus induction, additionally it has also been found that there were no significant differences ($P \leq 0.05$) between 30 and 40% clorox concentration in respect to callus induction frequency (Table 2).

It was concluded that 40% clorox concentration significantly reduced the contamination frequency and favored maximum callus induction frequency which is not significantly ($P > 0.05$) different from the 30% clorox (Table 2). 60% clorox reduced the contamination frequency significantly but set a negative effect on callus induction frequency. Moreover it has also been observed that 60% clorox retarded the growth and development of explants in all three cultivars. The 40% clorox was found successful in all three cultivars for surface sterilization of explants and maximum callus induction.

2, 4-D alone has no significant ($P> 0.05$) effect on the calli initiation and induction, similar results were also obtained for all the three cultivars. The interaction of 2, 4-D and cultivars was found more important in calli initiation and induction (Table 3). The cultivar IRRI-6 took minimum number of average days to calli initiation on 2 mg L^{-1} concentration of 2, 4-D. Further increase in 2, 4-D concentration for this cultivar has showed lower frequency of calli induction. At 3 mg L^{-1} the cultivar IRRI-9 showed the highest performance with minimum number of average days to calli initiation and production of the maximum number of calli. KSK-282 required 2.33 average numbers of days for calli initiation at 4 mg L^{-1} with92.22% of calli induction (Table 3). It was concluded that each cultivar responded to a particular concentration of 2, 4-D for calli initiation and induction in IRRI-6, IRRI-9 and KSK-282 as shown in Fig. 1.

Two types of calli were observed on both N6 simple and N6 supplemented media but with different frequencies. Type I callus was found yellowish, smaller in size, globular

Table 1: Composition of some important different media

Medium	Culture time	Media Composition
Callus Induction Media	7-11days	N6 salts with vitamins (Chu *et al.,* 1975), 2,4-D (2 mg/L, 3 mg/L and 4 mg/L), Casein hydrolysate 0.3 g/L, Myoinositol 0.1 g/L, Sucrose 3%, Gellan Gum Agar 0.3%, pH 5.8
(N6 Simple and N6 supplemented)		N6 Salts with vitamins (Chu *et al.,* 1975), 2,4-D (2 mg/L, 3 mg/L and 4 mg/L), Casein hydrolysate 0.3 g/L, Myoinositol 0.1 g/L, Sucrose 3%,Glycine 1g/L, Proline 2.8g/L, MES 0.5g/L, Gellan Gum Agar 0.3%, pH 5.8
Regeneration media (RM I, II and III)	3-4 weeks	MS salts with vitamins (Murashige and Skoog, 1962), 0.1 g/L Myoinositol, Sucrose 3%, hygromycin 50 mg/L, Gellan Gum Agar 0.3%, pH 5.8, Kinetin 0.5 mg/L, NAA:BAP (RM I=1:2, RM II=1:3 and RM III=1:4)
Rooting Medium	2 weeks	MS Salts, Sucrose 3%, IBA 1mg/L, hygromycin 25mg/L, Gellan gum agar 3%, pH 5.8

Table 2: Effect of different concentrations of clorox on the contamination frequency and callus induction frequency

Cultivars	Contamination frequency at different clorox concentrations			Cultivar means	Callus induction frequency at different clorox concentrations			Cultivar means
	30	40	60		30	40	60	
IRRI-6	86.60 a	20.00 b	23.33 b	43.33 A	90.00 a	86.67 a	45.56 b	74.1 A
IRRI-9	93.33 a	26.67 b	23.33 b	47.78 A	86.67 a	84.44 a	46.67 b	72.6 A
KSK-282	80.00 a	33.33 b	30.00 b	47.78 A	84.44 a	85.56 a	42.20 b	70.7 A
Treatment Means	86.67 A	26.67 B	25.56 B		87.00 A	85.56 A	44.90 B	

Table 3: Synthetic auxin effect on days to calli initiation and calli induction frequency in three different rice cultivars

Cultivars	Days to callus initiation at various 2-4,D concentrations			Cultivar means	Callus induction frequency at various 2-4,D concentrations			Cultivar means
	2	3	4		2	3	4	
IRRI-6	3.00 bc	3.67ab	4.00 a	3.55 A	93.33 a	85.56 bc	82.22 c	87.03 A
IRRI-9	3.67 ab	2.33 c	4.00 a	3.33 A	83.33 c	91.11ab	82.22 c	85.55 A
KSK-282	3.67 ab	4.00 a	2.33 c	3.33 A	83.33 c	82.22 c	92.22 a	85.92 A
Treatment Means	3.44 A	3.33 A	3.44 A		86.67 A	86.29 A	85.55 A	

Table 4: Effect of N6 simple and N6 supplemented media on type of callus production in three rice cultivars

Cultivars	Type 1 callus induction frequency		Cultivar means	Type 1I callus induction frequency		Cultivar means
	N6	N6 (S)		N6	N6 (S)	
IRRI-6	38.13 c	67.35 a	52.74 A	61.87 a	32.47 b	47.26 A
IRRI-9	42.65 c	69.51 ab	56.08 A	57.35 a	30.49 b	43.92 A
KSK-282	46.61bc	50.53 abc	48.57 A	50.50 ab	49.47 ab	49.99 A
Treatment Means	42.46 B	62.47 A		56.57 A	37.53 B	

Table 5: Effect of cultivar, infection time and co-cultivation period on *GUS* expression

Cultivar	Co-cultivation (days)	Infection Time (Minutes)			Co-cultivation means	Cultivar means
		5	10	15		
IRRI- 6	1	16.67 h	31.67 ef	46.67d	31.67 d	35.28 C
	2	23.33 g	36.67 e	56.67 c	38.89 c	
IRRI -9	1	31.67 ef	36.67 e	66.67 b	45.00 b	46.67 B
	2	26.67 fg	46.67 d	71.67 b	48.34 ab	
KSK-282	1	36.67 e	46.67 d	71.67 b	51.67 a	51.38 A
	2	31.67 ef	35.00 e	86.67 a	51.11 a	
Treatments mean		27.78 C	38.89 B	66.67 A	1 day: 42.78 B; 2 days: 46.11 A	

Means followed by same letters are not significantly different at 5% probability level

Data followed by capital alphabets represent treatments and cultivars means while data followed by small alphabets denote the individual values as an average of three replicates

and compact (Fig. 3a), while the type II callus was found larger in size, loose and friable (Fig. 3b). N6 enriched with supplements produced the highest number of type I calli for all three cultivars compared to N6 media (Table 4). The results showed that supplements like proline, glycine and MES in media greatly enhanced the callus quality and structure that favored the type I callus over type II callus.

The transformed *Agrobacterium* with pCAMBIA 1301was confirmed through PCR using specific primers of *GUS* gene that amplified the 760 bp fragment (Fig. 2). Amplified fragment showed that the pCAMBIA vector has been successfully incorporated in the *Agrobacterium tumefaciens* strain EHA-105.

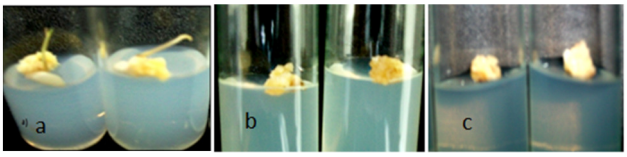

Fig. 1a: Callus induction in IRRI-6; **(b)** callus induction in IRRI-9; **(c)** callus induction in KSK-282

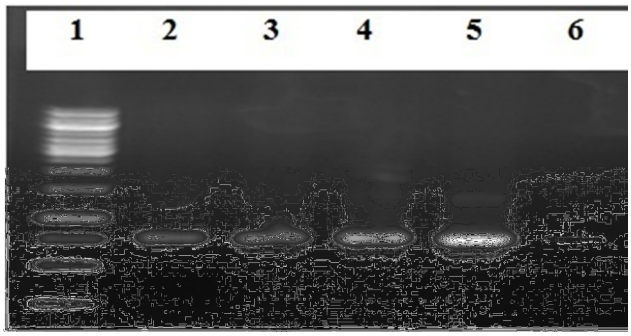

Fig. 2: Agarose gel electrophoresis of PCR amplified products from *Agrobacterium tumefaciens* strain EHA-105 transformed with pCAMBIA. Lane 1, 1 kb DNA ladder (Fermentas); lane 2 to 5, transformed *Agrobacterium tumefaciens* strain EHA-105; Lane 6, Untransformed *Agrobacterium tumefaciens* strain EHA-105

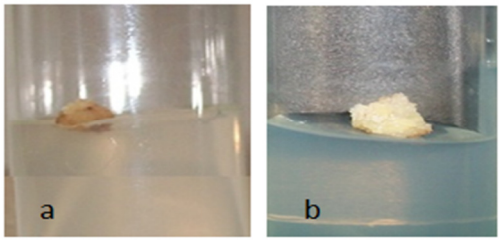

Fig. 3a: Type I callus (Yellowish, smaller in size, globular); **(b)** type II callus (whitish, loose and friable)

The proper stage of callus for each cultivar was determined and in our experiments the 13 days older calli of IRRI- 6 (Fig. 4a) showed the best response for the transformation experiments (Fig.4b). Reference to the fig. 4c, the 14 days older calli of IRRI-9 showed better transformation efficiency and *GUS* expression compared to eleven and thirteen days older calli (Fig. 4d). The eleven days older calli (Fig. 4e) of KSK-282 showed better transformation efficiency and depicted the strong blue signal of *GUS* expression (Fig. 4f).

Infection times and co-cultivation periods were significantly different from each other with respect to transformation efficiency at 5% probability level (Table 5). Similar results were also obtained for cultivar × infection time, cultivar × co-cultivation period, infection time × co-cultivation periods and cultivar × infection time × co-cultivation periods (Table 6).

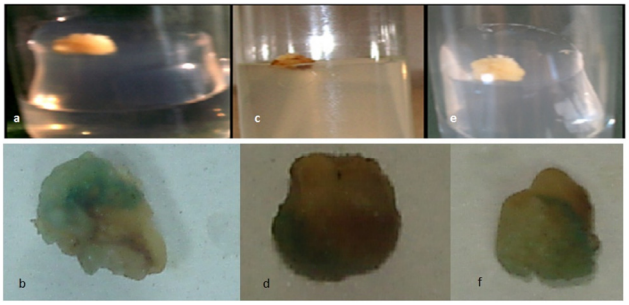

Fig. 4a:13 days older callus of IRRI-6; **(b)** *GUS* expression in IRRI-6; **(c)** 14 days older callus of IRRI-9; **(d)** *GUS* expression in IRRI 9; **(e)** 11 days older callus of KSK-282; **(f)***GUS* expression in KSK-282

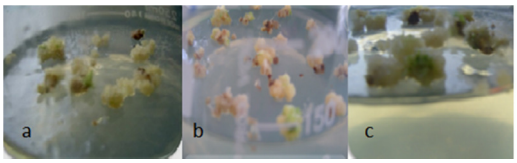

Fig. 5a: IRRI-6 on RM I **(b)** KSK-282 on RM II **(c)** IRRI-9 on RM II

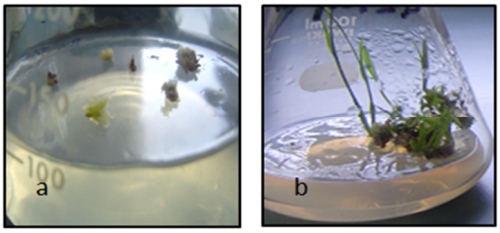

Fig. 6a: Calli on hygromycin selection media; **(b)** multiple shoot regeneration on media

The minimum *GUS* positive transient expression was observed at 5 min infection time and one day of co-cultivation period for all three cultivars. As we have increased the infection time and co-cultivation period, the number of *GUS* positive explants significantly increased. At 15 min of infection time and 2 days of co-cultivation period, the maximum numbers of calli were transformed for KSK-282 followed by the IRRI-9 and IRRI-6 (Table 5).

Statistically analysis revealed that significant differences ($P<0.001$) exist among cultivars for regeneration efficiency on different regeneration media, while effect of different regeneration media was found in-significant ($P>0.05$). The interaction between the cultivars and media had significant effect ($P<0.001$) on the regeneration of all three cultivars (Table 7). The cultivar IRRI-6 showed better regeneration on RM I followed by IRRI-9 and KSK-282. KSK-282 was found to have better regeneration on the RM II compared to the RMI and RM III (Table 7). IRRI-9 had also given maximum average regeneration on the RM II as compared to RM I and RM

Table 6: Percentage of sum of square for ANOVA

SOV	%SS
Cultivar	13.01**
Infection	76.09**
Co-cultivation	0.79**
Cultivar× Infection	3.57**
Cultivar × Cocultivation	0.72*
Infection time × Cocultivation	1.64**
Cultivar × Infection × Cocultivation	2.43**
Error	1.76**

%SS followed by ** and* within the same column are significantly different at (p<0.001) and (p< 0.01), respectively

Table 7: Establishment of regeneration on different Media

Cultivars	Different Regeneration media			Cultivar Means
	RM I	RM II	RM III	
IRRI-6	70.00 ab	43.33 de	63.33 abc	58.89 A
IRRI-9	60.00 abc	73.33 a	50.00 cd	61.11A
KSK-282	23.33 f	56.67 bcd	30.00 ef	36.67 B
Treatment Means	51.11 AB	57.78 A	47.78 B	

Means followed by same letters are not significantly different at 5% probability level

Data followed by capital alphabets represent treatments and cultivars means while data followed by small alphabets denote the individual values as an average of three replicate

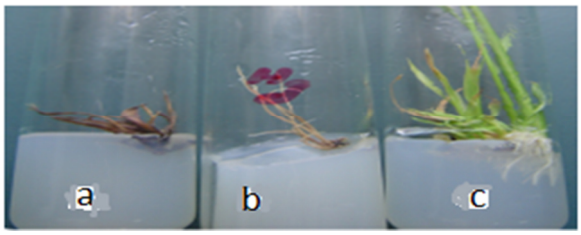

Fig. 7a-b: Untransformed putative plantlets on hygromycin selection media; **(c)** transformed putative plantlets on hygromycin selection medium

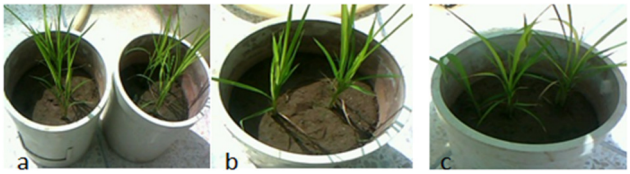

Fig. 8a-c: Established rice transgenic lines in glass house

III media.

The regeneration of calli of IRRI-6 on the RM I have shown in the Fig. 5a. The Fig. 5 band c depicted the regeneration of the KSK-282 and IRRI-9 on the RM II media respectively. The transformed calli were regenerated on selection media as shown in Fig. 6a-b.

The transformed and untransformed putative plantlets on the rooting media having hygromycin as a selectable marker are shown in Fig. 7a, b and c.

The plantlets were then shifted to the soil and vermiculate mixture for further growth and development (Fig. 8a-c).

The confirmations of transgenic plants were carried out by PCR using *GUS* specific primers which amplified 760 bp fragments (Fig. 9).

Agarose gel electrophoresis of total RNA extracted from transgenic cultivars (Fig. 10). The *GUS* gene was amplified from reverse transcription reaction product of each sample with similar condition as described earlier (Fig. 11).

Discussion

Stable genetic transformation for indica rice cultivars is important and has been shown to be affected by various factors (Cheng *et al*., 2004). A good number of rice cultivars tested for regeneration were proved recalcitrant to *Agrobacterium* mediated transformation due to the complex mechanism of T-DNA delivery, genome complexity and biochemical process involved (Gelvin, 2010). Moreover, regeneration through callus also posed difficulties due to genotypic susceptibility of rice explants to *Agrobacterium* strain which resulted in the browning or cell death (Dan, 2008). We have tested three cultivars of rice i.e., IRRI-6, IRRI-9 and KSK-282 for better quality of callus induction and regeneration while considering different parameters like type of callus, culture media, their chemical composition and plant hormones.

Sodium hypochlorite has been routinely used in tissue culture to kill microorganisms from the surface of explants (Nakagarwara *et al*., 1998). In the present study three different levels of clorox i.e., 40, 50 and 60% were evaluated for the surface sterilization of rice seeds. It has been found that 40% clorox concentration significantly reduced contamination rate and produced the maximum number of calli in all the three cultivars. Our results slightly deviated from the findings of Oyebanji *et al.* (2009) and Li *et al.* (1993),who obtained better surface sterilization in different rice cultivars at 45 and 50% clorox. This variation may be due to genotypic dependent responses of explants.

Synthetic auxin 2, 4-D acts as an initiator for the formation of callus from mature seeds of rice (Karthikeyan *et al*., 2009). Higher tissue culture responses were also recorded from immature rice seeds (Li *et al*., 1993), but seasonal limitations reduce its utility for *in vitro* purposes (Ge *et al*., 2006). In the current study mature seeds of three different rice cultivars i.e. IRRI-6, IRRI-9 and KSK-282 were used for the production of calli on various concentrations of 2,4-D. The highest number of average calli for IRRI-6 were observed on 2 mg L^{-1} of 2, 4-D compared to 3 and 4mg L^{-1} of 2, 4-D. Cultivar IRRI-9 produced the highest number of calli on 3 mg $L^{-1}$of 2, 4-D. Rashid *et al.* (2003) reported that use of 2.5 mg $L^{-1}$of 2, 4-D in Basmati rice is sufficient for callus induction. KSK-282 cultivar showed the highest callus induction on 4 mg $L^{-1}$of 2, 4-D. All three cultivars in present study responded to a particular concentration of 2, 4-D for callus initiation and induction. The similar high genotype specific responses

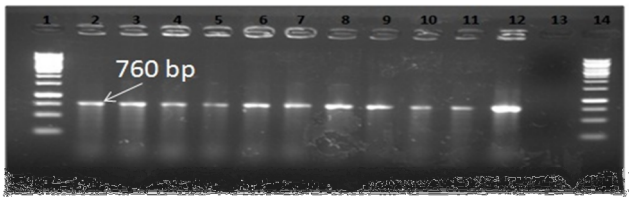

Fig. 9: Confirmation of transgenic plants through PCR. Lane 1 and14, 1 kb DNA Ladder (Fermentas); lane 2 to 5, *GUS* transgenic lines of IRRI-6; lane 6 to 8, *GUS* transgenic lines of KSK-282; lane 9 to 11, *GUS* transgenic lines of IRRI-9; lane 12, plasmid positive sample; lane 13, negative control

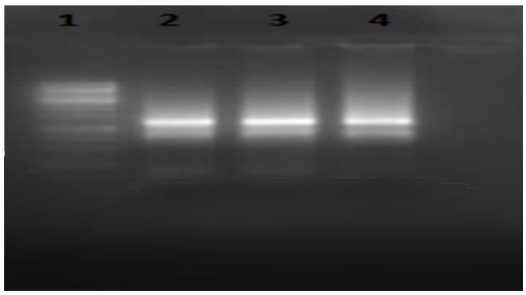

Fig. 10: Agarose gel electrophoresis of total RNA from three different rice cultivars. Lane 1, 1Kb DNA ladder; lane 2, total RNA from the IRRI-6; lane 3, total RNA from the IRRI-9 and lane 4, total RNA from the KSK-282

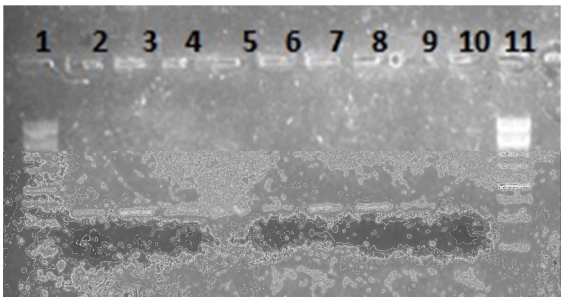

Fig. 11: Agarose gel electrophoresis of reverse transcriptase PCR amplified product**.** Lane 1 and 11, 1Kb DNA ladder; lane 2, 3 and 4, RT-PCR amplified product from three different transgenic lines of IRRI-6; lane 5, 6 and 7, RT-PCR amplified product from three different transgenic lines of IRRI-9; lane 8 and 9, RT-PCR amplified product from two different transgenic lines of KSK-282; lane 10, Negative control, untransformed KSK-282

were also observed previously in six different elite indica rice cultivars grown in Pakistan (Joyia and Khan, 2013).

The use of culturing media has direct impact on callus induction and their quality (Lee *et al*., 2002). Callus induction and shoot regeneration are the limiting factors for the development of transgenic rice. In the present study we have tested the effect of two different media (N6 simple and N6 enriched with supplements) for callus induction of three rice cultivars. N6 enriched medium produced compact mass, nodular shape of calli suitable for genetic transformation. The organic supplements like proline, glycine and MES (2-*N*-morpholino ethane sulfonic acid) in the N6 medium have the capability to increase the quality of callus formation and their regeneration in rice cultivars (Parfitt *et al.*, 1988; Larher *et al*., 1993; Perez-Alfocea *et al*., 1993; Moghaddam *et al*., 2000; Wagiran *et al*., 2008). Manipulating the culture media Quiroz-Fogieroa *et al.* (2006) also obtained dry, compact and smaller size of calli in rice cultivars.

Transformation efficiency in rice depends on the age of calli (Joyia and Khan, 2013). Three weeks older calli has been shown to be optimum stage for efficient transformation in rice (Rafique *et al.,* 2010). Raja *et al*. (2009) reported that transformation efficiency decreases with the increase of calli age and six weeks older calli were completely recalcitrant to genetic transformation. In our experiments the 13 days older callus of IRRI- 6 found suitable for the transformation experiments while 14 and 11 days older calli of IRRI-9 and KSK -282 respectively showed better *GUS* expression.

Regeneration media for indica rice contained different combination of organic compounds, growth regulators, inclusion of nitrogen and carbohydrate source (Ge *et al*., 2006). In our study we have investigated regeneration of explants of three different rice cultivars on various regeneration media. It was observed that different rice cultivars have responded on different regeneration media. The cultivar IRRI-6 showed optimum regeneration on RM I (1 mg/L NAA, 2 mg L^{-1} BAP) followed by IRRI-9 and KSK-282. KSK 282 and IRRI-9 were found to have better regeneration on RM II (1 mg/L NAA, 3 mg/L BAP) compared to the RMI and RM III.

Hussain *et al*. (2010) obtained higher regeneration in two rice cultivars (JP-5 and GNY-53) by using 1 mg L^{-1} NAA and 2 mg/L BAP hormonal combination, while same study also depicted that Basmati-370 showed maximum regeneration on 1 mg/L NAA and 4 mg/l BAP. Rashid *et al*. (2004) secured higher regeneration on 1.0 mg/L NAA and 5.0 mg/L BAP from calli of different varieties of indica rice. BAP and NAA were found essential for initiation of shoots (Azria and Bhalla, 2000). Higher shoots were resulted with BAP as compared to Kinetin in Spanish rice varieties (Pons *et al*., 2000).

Infection time is a crucial step of genetic transformation. In the present study three rice cultivars were subjected to three different infection times (5, 10 and 15 min) and two co-cultivation periods, 1 and 2 days. It has been found that 15 min infection time and 2 days co-cultivation period have given maximum transformation efficiency in all three rice cultivars. The same results were also observed by Samiphak and Siwarungson (2006) in different rice transformation experiments. Bernal *et al*. (2012) used 3 days of co-cultivation period and obtained improved transient *GUS* gene expression compared to 5 days of co cultivation period. Ozawa (2009) also noticed

that prolonged co-cultivation period of more than 3 days can cause browning in calli and subsequently decrease the frequency of transformation.

In summary, efficient and reproducible regeneration system has been established for all three recalcitrant rice cultivars by investigating different parameters that affect the transformation and regeneration efficiency. Modifications in cultural conditions for better quality of callus production and regeneration were successful in the transformation of IRRI-6, IRRI-9 and KSK-282. The resultant putative transgenic plants were confirmed by PCR using *GUS* specific primers. Putative transgenic plants were analyzed for expression by reverse transcriptase polymerase chain reaction (RT-PCR). Our study can be efficiently utilized for the genetic transformation of any economically important trait in above mentioned cultivars. The optimized protocols can also be extended for other rice cultivars as well.

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