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RESEARCH PAPER

Optimization of *Agrobacterium* mediated callus-based transformation protocol for rice (Super Basmati) and GUS expression

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Key Message: This study optimized an Agrobacterium-mediated callus-based transformation protocol for rice (Super Basmati), enhancing GUS gene expression efficiency. The findings indicate the importance of optimizing transformation conditions to improve the genetic modification of rice cultivars.

Abstract

Rice (*Oryza sativa* L.) is a staple food in many countries of the world. The world population is increasing at a tremendous rate. The demand of food is increasing but supply is limited due to decrease of agriculture land, biotic and abiotic stresses. There is need of time to increase the yield of food crops including rice by incorporating resistance genes that will improve the ability of cultivar to resist biotic and abiotic stresses. *Agrobacterium* mediated callus-based transformation is one of the techniques for insertion of gene of interest. This study was an attempt to evaluate the response of four cultivars (Super Basmati, Basmati 370, Basmati 385 and Shaheen Basmati) of rice for regeneration and transformation on the basis of hygromycine. The cultivar super basmati responded best response so further GUS expression of this cultivar was done to check the transformation efficiency. The EHA101 *Agrobacterium* strain, plasmid containing GUS gene was used to evaluate the transformation rate. The factors affecting transformation process were optimized. The results revealed that 500 mg/L of cefotaxime was best concentration to decontaminate the left-over bacteria. The 50 mg/L of hygromycine was optimized as lethal dose for selection of transformed calli. The (100 µM/L) of acetosyringone in cocultivation media enhanced the ability of *Agrobacterium* to deliver the required gene in the calli. Out of four cultivars of rice, super basmati showed the best response for regeneration and transformation. The 24% GUS expression was recorded in the leaves of super basmati.

Keywords: Agrobacterium, GUS expression, Hygromycine, Rice, Super basmati, Transformation

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Introduction

Rice (*Oryza sativa* L.) is a cash crop and staple food of many countries in world (Ibrahim et al., 2016; Ali et al., 2019). It belongs to the family poaceae. In nutrition point of view, it contains protein, riboflavin, thiamine, vitamins and niacin. It is cultivated in a wide range of habitats such as from topical, subtropical regions and dry land but frequently grown in water (Ayres & Park, 1994). The two sub-species i.e. Indica and Japonica are being cultivated in various regions of the world (Sharan et al., 2004). The starch contents vary in both grains of indica and japonica. Starch consists of amylopectin and amylose (Shimada et al., 1993). Pakistan is one of the leading rice producing countries (Rashid et al., 1996). Rice grown in Pakistan comprises of Basmati (high quality aromatic rice), IRRI (coarse rice) and short grain cold tolerant (Xiao et al., 1996). There are two cultivars of rice *Oryza sativa* and *Oryza glaberrima*. *Oryza sativa* has three races: *Oryza sativa* sinica (japonica) *Oryza sativa* indica and *Oryza sativa* javanica. Indica rice including basmati varieties are predominantly cultivated in Pakistan. There are a number of basmati varieties exist. Such as Basmati-370, Basmati-385 and Super basmati. Basmati rice production is limited by various biotic (diseases and insects) and abiotic (unfavorable soil temperature and water conditions) factors (Herdt, 1991).

There are different diseases of rice either they are transmitted by Insects or bacteria, but bacterial blight (BB) disease is the most dangerous disease. It is caused by *Xanthomonas oryza* pv. Oryza (xoo), which is one of the oldest known diseases and was first noticed by the farmers of Japan in 1884 (Tagami & Mizukami, 1962). This disease can be controlled by reducing the susceptibility of the plant to infection, seedling damage, balanced fertilizers and use resistant varieties. The most economical and effective method to control this disease is to incorporate the resistant gene into cultivars. The two bacterial blight resistance genes *Xa21* and *Xa1* were isolated using a map based cloning strategy (Song et al., 1995; Yoshimura et al., 1998).

Tissue culture is one of the most important techniques being used for rapid and pathogen free plants (Jan et al., 2015;

Shah et al., 2015; Ahmad et al., 2020). Genetic transformation is a biotechnological approach in which foreign genetic material is incorporated and expressed into the cells through different methods. These different methods of genetic transformations are polyethylene glycol (PEG) mediated direct gene transfer, electroporation-mediated direct gene transfer, biolistic method, microinjection method and *Agrobacterium*-mediated callus-based transformation (Sheng & Citovsky, 1996). Tissue type, age, genotype, and susceptibility to *Agrobacterium* all play a role in the effectiveness of bacterial infection. In addition to the specific plant-bacteria interactions, *vir* genes are dependent upon various factors, which include the presence of phenolic compounds, media pH, and temperature. Without phenolic signal molecules to initiate the expression of *vir* genes, the T-DNA transfer process does not occur. Wounded plant tissues produce phenolic compounds such as acetosyringone. Which is a signaling molecule for the induction of *vir* genes (Sheng & Citovsky, 1996). Monocots lack acetosyringone due to which transformation rate is very low as compared to dicots. Keeping in view of above factors, this study was carried out, to optimize the factors affecting transformation of gene in rice. To achieve this objective, Agrobacterium strain EHA101 containing pTCL5 binary vector consisting of hygromycin phosphotransferase (*hpt*) gene, *Xa21* gene and B-glucuronidase (GUS) gene as reporter was used.

Materials and Methods

Plant material and tissue culture conditions

Initially four cultivars of rice i.e. Super Basmati, Shaheen Basmati, Basmati 370 and Basmati 385 were used to assess the tissue culture response of these cultivars. Mature seeds were used as explant for callus induction by using MS (Murashige and Skoog, 1962) and N6 (Chu et al., 1975) media supplemented with different concentrations of hormones. The tissue culture media was autoclaved before use to kill any form of life present in there.

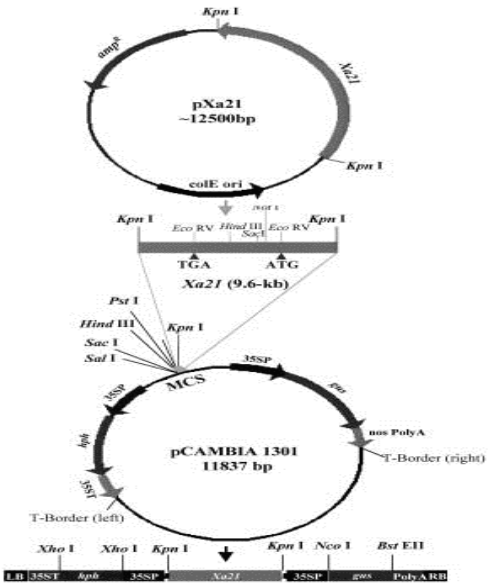

Fig.1 Plasmid diagram

Washing and sterilization of explant

The de-husked seeds were washed with tap water then sterilized with 70% ethanol for one minute. After this these were rinsed with 50% Clorox (Sodium hypochlorite) for 15 minutes. Finally, three to four times washed with sterilized water to get rid of Clorox completely. Then these seeds were placed on filter paper for drying.

Callus induction and regeneration protocol

The optimized callus induction and regeneration protocol was used for this experiment (Mehmood et al., 2016). Four cultivars were initially used for callus induction and regeneration then on the basis of best regeneration (Table 1). The super basmati transformed calli were further placed on optimized regeneration media.

Bacterial strain and transformation vectors

The *Agrobacterium tumefaciens* strain EHA-101 harbouring binary vector PTCL-5 (Fig. 1) was used in this experiment. The vector contains hygromycin phosphotransferase (*hpt*) gene, kanamycin gene, *Xa21* gene and β-glucuronidase (GUS).

Bacterial culture

Agrobacterium strain EHA101 was streaked on petri plates having agar solidified YEP (Yeast extract peptone) media supplemented with 50 mg/L of kanamycin and 50 mg/L of hygromycin. These plates were incubated at 28 °C for colonies to appear. The one clone of *Agrobacterium* was picked and mixed in a test tube containing 4 ml of liquid YEP media. This was placed for incubation on shaker incubator for 24 hours at 28 °C at 150 rpm. Next day, bacteria culture was taken in Eppendorf tube and centrifuge at 12000 rpm for three minutes. The supernatant was discarded, and pellet was resuspended in MS liquid media containing acetosyringone (100 µM/L). This MS liquid media containing *Agrobacterium* was further placed on shaker incubator for a period of 24 hours to get optimum density (0.5 - 0.6 at 600 nm).

Optimization of cefotaxime to eliminate Agrobacterium

After co-cultivation, the calli were washed three to four times with sterilized autoclaved distilled water followed by three times washing with liquid MS media containing 0 mg/L, 250 mg/L and 500 mg/L cefotaxime to decontaminate the left-over bacteria and placed on to filter paper to blot excess of liquid.

Optimization of hygromycin

The lethal dose of hygromycin was optimized by using 25 mg/L, 50 mg/L and 75 mg/L. in this experiment non-transformed calli were used. The optimized concentration (50 mg/L) from this experiment was further used to select transformed calli.

Cocultivation effect of acetosyringone on transformation rate

In co-cultivation step the optimized callus induction media (Mehmood et al., 2016) i.e. N6 media supplemented with acetosyringone (100 µM/L) and without acetosyringone was used as cocultivation media. Calli of all four cultivars were pooled and infected with *Agrobacterium* suspension for 15 minutes. These infected calli were dried on blotting paper and then transferred on petri plates having cocultivation media for co-cultivation. These petri plates were sealed with parafilm and placed in incubator for 60-72 hours in dark at 28 °C. The effect of acetosyringone was analysed on the basis of GUS expression.

Selection

In the next phase, the calli of four cultivars were transferred to selection medium consisted of N6 media supplemented with 50 mg/L hygromycine and 500 mg/L cefotaxime. These calli were incubated at 28 °C in the dark for a period of two to three weeks. The calli which were resistant to hygromycine proliferate and considered as transformed. The cultivar was selected on the basis of maximum hygromycine resistant calli

Regeneration, acclimatization and shifting of plants into pots

After two or three weeks, the transformed calli (hygromycin resistant calli) were transferred to regeneration media for plantlets or shoots formation. Regeneration media consisted of MS media supplemented with 30 g/L D-sucrose, 30 g/L sorbitol, 1 mg/L NAA and 5 mg/L BAP with 50 mg/L hygromycine and 8 g/L agar. pH of media was maintained at 5.8. After shoot regeneration, plantlets were transferred to rooting media supplemented with 0.5 mg/L of NAA for rooting for a period of two weeks. These plants having roots then shifted to tap water for a period of one week for acclimatization before shifting to soil. After that these plants were shifted in pots and placed in nurseries.

GUS assay

Histochemical GUS assay was carried out as described by Jefferson (1987). For this purpose, small pieces of leaves of regenerated plants were taken and put them in Eppendorf tube and mixed them with X-Gluc solution. This mixture was incubated at 37 °C for overnight.

Results and Discussion

In plant tissue culture and gene transformation technique, the culture media is composed of complex mixture of salts, organic supplement, carbon source, gelling agent, plant growth regulators and antibiotics. The essential elements or mineral ions are further divided into macro elements, micro elements and iron source. Iron is added in the medium as iron sulphate, Organic supplements include vitamins and amino acids. Amino acid provides a source of reduced nitrogen and ammonium ions, uptake causes acidification of medium. Casein hydrolysate can be used as a source of a mixture of amino acids. D-sucrose was used as a carbon source and agar was used as a gelling agent in this study. Auxins promote cell division and cell growth. In plants there is IAA (indole-3-acetic acid) naturally occurring auxin. But in this study 2, 4-Dichlorophenoxy acetic acid (2,4-D) was used for callus induction. Synthetic auxin like naphthalene acetic acid, of NAA, is used extensively to promote root formation on stem and leaf cuttings. In this study it was used in rooting medium as well as in regeneration medium combination with BAP. Cytokinins promote cell division; naturally occurring cytokinins are zeatin and 2ip (2-isopetyle). There are synthetic cytokinin e.g. kinetin and BAP (benzyle amino purine) First successful transformation was done in japonica rice by using mature seed-derived calli as explants (Rashid et al., 1996).

Cocultivation and selection

Cocultivation is the first step of transformation after coinfection of calli with Agrobacterium. In this study, the time for infection was fifteen minutes. This time for cocultivation was reported by other scientists and reported that maximum calli were transformed. Our experiment also confirmed the earlier studies that cocultivation media supplemented with acetosyringone enhanced the transformation rate as compared to without acetosyringone. The transformed calli were selected on selection media, supplemented with 2,4-D, hygromycine and cefotaxime.

Optimization of Cefotaxime to control bacterial growth

Cefotaxime is an antibiotic which was used to control Agrobacterium growth. Out of three concentrations 500 mg/L was the best and 100% decontaminated the left-over bacteria. While 50% growth was controlled at 250 mg/L (Table 1). Antibiotics such as cefotaxime, carbenicillin and timentin have

been used regularly in *Agrobacterium*-mediated transformation of crops following co-culture to suppress or eliminate *Agrobacterium* (Cheng et al., 1996) Although cefotaxime worked with *Agrobacterium*-mediated transformation of rice and maize initially. It was later found that suitable concentration of cefotaxime was 250 mg/L (Ishida et al., 1996).

Table 1 Optimization of cefotaxime to control bacterial growth

Cefotaxime concentration	No. of calli used	No. of calli having bacterial growth	No. of Calli with no bacterial growth	Percentage control
0 mg/L	60	60	0	0%
250 mg/L	60	30	30	50%
500 mg/L	60	0	60	100%

Each treatment had three replications, and 20 calli were used in each replication

Optimization of hygromycin as a lethal dose

Hygromycin was used to select the transformed calli. The lethal dose (50 mg/L) was optimized. Results showed that 53.33% calli become dead at this concentration (Table 2). So, it was the optimum dose which was used to select the transformed calli. Hygromycine is extensively used for rice transformation. It is considered as a selectable marker for transformation. At the selection stage, hygromycine was used in selection media for selection of transformed calli. The observation revealed that the calli which were transformed showed resistance to hygromycine and growth. Similar quantity i.e., 50 mg/L of hygromycine was reported for Basmati 370, 385 and Basmati 6129 by Rashid et al. (1996).

Table 2 Optimization of hygromycin as a lethal dose

Hygromycin concentration	No. of calli used	No. of dead calli	Percentage
25 mg/L	60	20	33.33
50 mg/L	60	32	53.33
75 mg/L	60	60	100

Each treatment had three replications, and 20 calli were used in each replication

Transformation response by rice cultivars

The callus of four rice cultivars were initially transformed and selected based on hygromycin. The data (Table 3) showed that super basmati exhibits maximum response for transformation i.e. 68.66% while minimum 57.72% showed by Shaheen basmati. The transformation difference varies due to the different genetic makeup of each cultivar. Similarly in regeneration experiment it was observed that super basmati showed the best response for regeneration. So, it was selected for further analysis like shifting of plants to glass house and then for GUS expression. The other three cultivars were dropped due to low regeneration and transformation rate.

Table 3 Transformation efficiency on the basis of hygromycine (50 mg/L)

Varieties	No. of calli on cocultivation	No. of calli on selection media (a)	No. of Hg resistant calli (b)	Transformation efficiency Hg + % (b/a)
Basmati 385	450	450	290	64.44
Super Basmati	450	450	309	68.66
Basmati 370	450	450	306	68
Shaheen Basmati	450	450	260	57.72

Hg = Hygromycine; Three replications were used for each variety and 150 calli were used in each replication

Effect of Acetocyringone

Acetosyringone is a phenolic compound which is naturally released by wounded dicot plants, but it is absent in monocots (Shah et al., 2016). This chemical stimulates the vir gene to transfer TDNA into the host cell. In this study 100 µM/L of acetosyringone was used to check its response for transformation. The results revealed that it has positive effect and enhance the transformation of gene into rice cultivar (super basmati). There was 73.33% hygromycine resistant calli were recorded (Table 4). Sharan et al. (2004) found that acetosyringone plays a crucial role for improvement and efficient transformation. They used *Agrobacterium tumefaciens* strain EHA 105 carrying binary vector pCAMBIA1301 for transformation in two cultivars of indica rice, KHR-46 and HKR-126 and reported that high concentration of acetosyringone in the *Agrobacterium* culture and co-cultivation medium proved for successful transformation. Rashid et al. (1996) also reported high transformation efficiency with acetosyringone.

Azhakhanandum et al. (2000) developed an *Agrobacterium*-mediated transformation procedure for the three major indica, japonica and javanica rice sub-species. In this study, acetosyringone concentrations (50-300 µmol/L) were evaluated, 100 µmol / L was the optimum concentration. Rashid et al. (1996); Hiei et al. (1997) reported that when acetosyringone was omitted, the level of transient GUS expression was low and stable transformed plants could not be regenerated.

Table 4 Effect of acetosyringone on transformation efficiency in Super Basmati on the basis of hygromycin resistant calli

Media used (N6)	Number of calli used (A)	No. of calli with blue spots and hygromycin resistant calli (B)	Hygromycin resistant calli % (B/A) × 100	Total number of blue spots (C)	Average number of blue spots per calli (C/B)
Without acetosyringone	45	9	20	12	1.33
With acetosyringone	45	33	73.33	51	1.54

Regeneration response of rice cultivars

The data in Table 5 showed that super basmati exhibits maximum regeneration response (58.33%) when its calli were not transformed. While the other cultivars showed less regeneration response on regeneration media. So, the calli of super basmati were used to transformed and proceed the further steps of experiment and to check the GUS expression. The data in Table 6 presents the regeneration response of transformed calli of super basmati. In this case the regeneration percentage was 44.44%. These results also indicate that the regeneration ability of transformed calli decreased as compared to non-transformed calli. Because the transformed calli were under the stress of cefotaxime and hygromycine at the selection step. These antibiotics have a negative effect on the health of cells and mostly non transformed cells become dead and necrosis starts. Some calli showed browning coloration. The transformed cells have bright colour and showed green spots before shooting to appear (Fig. 2). The shooting started from these green spots before rooting. (Fig. 3). The data in Table 7 showed that 32 plantlets were shifted on rooting media having 70 shoots. Hygromycin was used in rooting media so only 25 plants survived while 7 plants become dead. After root formation they were shifted in tap water in test tubes for acclimatization then they were shifted in pots and placed in glass house. (Fig. 4, 5, 6, 7).

Table 5 Comparison of regeneration response of cultivars of rice

Varieties	No. of calli used	No. of calli having green spots	Plantlet formation	Plantlet formation %
Basmati 370	60	16	13	21.66
Basmati 385	60	14	19	31.66
Super Basmati	60	23	35	58.33
Shaheen Basmati	60	16	21	35

Three replications were used for each variety and 20 calli were used in each replication.
(Regeneration media was used as MS media with 1 mg/L NAA and 5 mg/L BAP)

Table 6 Regeneration of Transformed calli of super basmati

Replication	No. of calli used	Browning of calli	Proliferating calli	Calli having green spot	Plantlet formation	Regeneration percentage
1	25	3	22	12	10	
2	25	5	20	11	08	
3	25	3	22	16	14	
	75	11	64	39	32	44.44%

(Regeneration media was used as MS media with 1 mg/L NAA and 5 mg/L BAP)

Table 7 Plants on rooting media

No. of plants shifted to rooting medium	No. of shoots	Hygromycin resistant plant	No. of plants became dead	% of hygromycin resistant plants on rooting media
32	70	25	7	78%

Azhakhanandum et al. (2000) established regeneration system via somatic embryogenesis for three cultivars i.e. indica, japonica-javanica and indica-javanica. They used MS based medium containing 0.5% sucrose with either 2.0 mg/L BAP or with 2.0 mg/L kinetin and 0.5 mg/L NAA for initial ten days in dark period for germination of somatic embryo. It was observed that increased in agarose concentration in the regeneration medium from 0.4% to 1.0% (w/v) for 10 days, stimulated shoot regeneration. Rashid et al. (2000) reported that media containing NAA 1 mg/L and BAP 5 mg/L was found to be the optimal for regeneration. It appeared that presence of a high concentration of BAP enhanced the growth of the plant and low concentration of NAA proved to be a weak auxin. Khatun et al. (2003) studied *in vitro* regeneration of callus, induced from mature scutella of four genotype of rice i.e. Lx297, IR64, V19 and IR64-1-1-4. Considering green spots, roots and necrosis in regeneration medium, it was found that R-MSK2 media combination performed better in embryogenic calli (green spot, 36.11%) as well as production of non-embryogenic calli (calli with root necrosis = 69.44%) than N6-MSK2 and MS-MSK2 media. Pipatpanukul et al. (2004) studied that RD6 show suitable regeneration on N6 medium supplemented with 3% (w/v) sucrose, 2.5 µM IAA, 18 µM BA and 0.8% agar. Islam et al. (2005) studied the regeneration of commercial hybrid lines of IR-69690. They used N6 basal medium supplemented with 2, 4-D (1 mg/L), NAA (2 mg/L) and Kinetin (1 mg/L). This media has better response as compared to other. Shahnewaz et al. (2004) reported the effect of various concentrations of sucrose 0, 1, 2, 3, 4, 5, 6%, in N6 media supplemented with 2.0 mg/L 2, 4-D, 0.5 mg/L Kn and 2.5 mg/L NAA. They obtained 65% plant regeneration at 4% sucrose. The highest concentration of sucrose (6% and above) in the culture medium not only resulted in the percentage of callus induction but also prompted the regeneration of albino plants. Cho et al. (2004) optimized the *in vitro* culture system for regeneration efficiencies of Korean rice cultivars by using different medium and shoots regeneration (0-88.7%) were observed among the rice cultivars. It was observed that MS medium was superior to N6 medium for shoot regeneration. The calli of highly regenerated cultivars grew faster and showed higher rates of green tissue formation and shoot regeneration. Khaleda & Al-Forkan (2006) reported the best plant regeneration on LS based medium supplemented with 2 mg/L BAP and 1.5 mg/L 2,4-D produced the highest 72% regeneration frequency. Noor et al. (2005) studied that regeneration of super basmati was 90% with MS medium supplemented with NAA 1 mg/L and BAP 2.5 mg/L and basmati 385 shows 83% regeneration on MS medium supplemented with NAA 1 mg/L and BAP 5 mg/L.

GUS expression

Small pieces of leaves from six plants were carried out with X-Gluc solution for Gus expression. Out of 25, six leaves showed a positive Gus expression and thus 24% GUS expression was recorded (Table 8; Fig. 8). GUS expression studies were also conducted previously like Sharan et al. (2004) optimized the condition for transformation in two indica rice HKR-6 and HKR-126. They used *Agrobacterium* strain EHA 105 containing binary vector pCAMBIA 1301 which have been proved to be efficient for transformation. The percent transient GUS expression found to be higher in cultivar HKR-126 (44.4%) as compared to HKR-46 (28.9%). Qian et al. (2004) reported that transient GUS expression was detected three days after co-cultured calli, with variations ranging from 9.6% to 71.8%. Callus induced was highly susceptible to *Agrobacterium* and callus on MBA 0.2 gave the highest rate of GUS expression. However calli induced on N6, MS, MB medium were poorly susceptible to the Agrobacterium with only 10% GUS transient expression. A simple and highly efficient rice transformation system was established by Li and Li (2003) based on the studying of factors influencing the *Agrobacterium*-mediated rice transformation. The putative transgenic plants were confirmed by GUS assay and southern-blot analysis.

Table 8 Gus expression analysis

No. of leaves used in GUS expression	No. of leaves showing GUS expression	GUS expression percentage
25	6	24%

Fig. 2 Green spots on calli

Fig. 3 Shoot formation from green spots calli

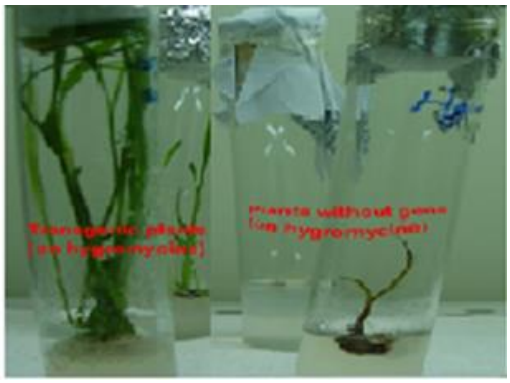

Fig. 4 Hygromycin resistant transgenic plants

Fig. 5 Plants of Super Basmati on rooting media

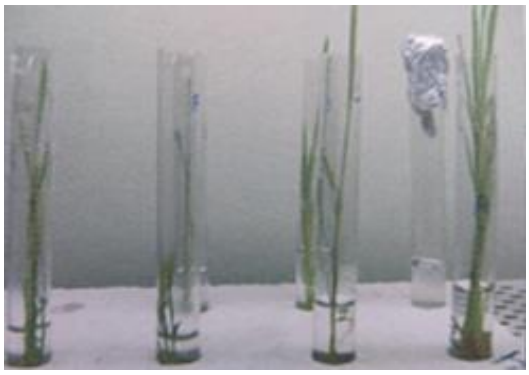

Fig. 6 Acclimatization of transgenic plants

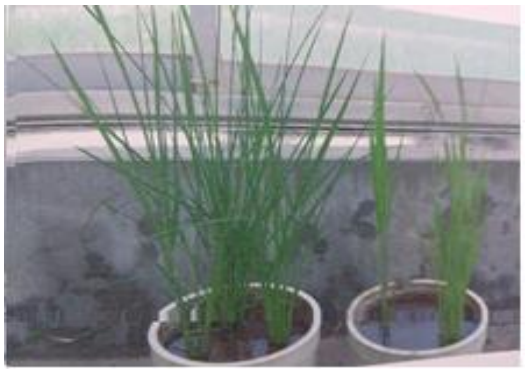

Fig. 7 Transgenic plants in pots

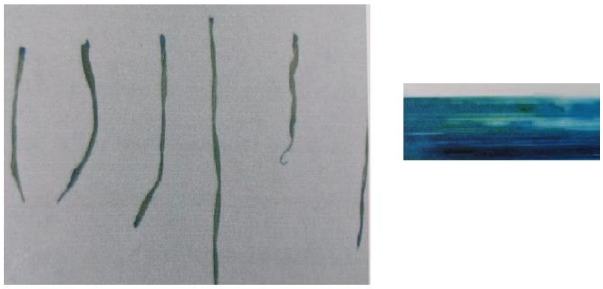

Fig. 8 Gus expression

Conclusion

Genetic transformation of rice has been an important area of research in past few years. It is an important genomic tool in which *Agrobacterium tumefaciens*-mediated transformation is a suitable delivery method for target gene. It is a simple, low-cost method of genetic transformation. Rice (*Oryza sativa* L.) is the primary cereal crop in the world. Pakistan is one of the largest rice producing countries having annual production of more than five million tons but the main problems in the production are biotic and abiotic stresses which limit the productivity of the rice. The four cultivars of rice (Super Basmati, Shaheen Basmati, Basmati 370 and Basmati 385) were used. The Agrobacterium strain EHA-101 harbouring binary vector pTCL5, containing hygromycin phosphotransferasse (*hpt*) gene, conferring resistance to hygromycin as selectable marker, Xa21 gene resistance to bacterial blight, and ß-glucuronidase (GUS) gene as a reporter. Super Basmati showed maximum hygromicine (50 mg/L) resistant calli (68.66%) on selection media than other three cultivars. In regeneration medium for shooting two-growth regulator BAP 5 mg/L NAA 1 mg/L were used. In rooting medium 0.5 mg/L of NAA was used supplemented with hygromycine (50 mg/L). Thirty-two regenerated plants were transferred in rooting medium for root induction, but twenty-five plants showed resistance to hygromycine. Later, GUS expression of leaves of six plants were done. Only four plants show GUS expression. This study will be helpful in future for other researcher to genetically manipulate rice for economically important traits.

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