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ESTABLISHMENT OF *AGROBACTERIUM*-MEDIATED BT GENE TRANSFORMATION SYSTEM IN MAT RUSH (*JUNCUS EFFUSUS* L.)

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Abstract

Agrobacterium-mediated genetic transformation was explored for inducing insect resistance in mat rush (*Juncus effusus* L.). Firstly, the callus regeneration system was established, and secondly the *Agrobacterium tumefaciens* strain EHA105 with plasmid pKUB carrying *cryIA(b)*, *hph* and *gus* genes, coding for hygromycin phosphotransferase and β-glucuronidase, respectively, was used for transformation. Results indicated that 150 μM acetosyringone in both of the induction and co-cultivation media increased the frequency of GUS^+ calli, and OD_{600} value 0.4 of *Agrobacterium* enhanced the rate of GUS^+ calli, significantly. The infection duration of 45-60 min, co-cultivation duration of 4 days, and infection in the 12^{th} or 16^{th} day were the best combination for transformation. *Agrobacterium*-treated plants achieved a transformation frequency of 9.56 % to the highest. Transient GUS expression and hygromycin-resistance tests were practicable and reliable for selecting the positive transformants. This transformation system was found suitable for manipulating the desired traits through transgenic techniques, as the transgenic *Juncus effusus* L. plants were produced from seeds to embryogenic calli, then to plantlets. The *Agrobacterium* - mediated transformation of *Bt* gene into mat rush with some optimizations is highly successful and reproducible as experienced from the established protocol.

Introduction

Wetland plant mat rush (*Juncus effusus* L.) is an ecologically and economically important plant, and is widely used in water gardens, mitigation and construction of wetlands. It is planted for its stem, which can be used as raw material of woven products, such as straw mats, seats hats basket making, thatching, weaving mats and many others (Wright, 1992). It is widely damaged by insects, and no insect-tolerant genetic resource is available in this species. Recently, transgenic techniques are preferred for the genetic transformation of desired traits in plants. *Agrobacterium*- mediated transformation is the most commonly used method for transferring foreign genes into plants cells (Shrawat & Lorz, 2006).

Insect resistance is developed in crops through the introduction of *Bacillus thuringiensis* (*Bt*) crystal protein (*cry*) gene, which confers resistance to major pests (Estruch *et al.*, 1997). The *cryIA(b)* gene has been introduced into many plant species; including rice (Ye *et al.*, 2003), cotton (Stewart *et al.*, 2001) and sugarcane (Arencibia *et al.*, 1997). Although *Juncus effusus* is an important economic crop, especially in China and Japan where it is grown on large scale; however, so far little attention has been paid for its callus induction and genetic transformation. We already have established an efficient protocol for callus induction and regeneration of this plant. Since there had been no reports about the transfer of *Bt* gene to mat rush mediated by *Agrobacterium*, it was considered important to investigate and optimize the different factors, which influences the transformation frequency for the establishment of successful transformation system. Here for the first time, we report the successful production of transgenic mat rush by optimizing the procedures of transformation and plant regeneration.

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Materials and Methods

Callus induction: Seeds of two cultivars of *Juncus effusus* L., viz. Nonglin-4 and Yinlin-1 were surface-sterilized sequentially with 316 µM $KMnO_4$, 70% ethanol, 1.0% NaOCl + 2 drops Tween-20, and 3 rinsings of sterile water (Zhang *et al.*, 2003). Seeds were germinated on hormone-free MS medium (Murashige & Skoog, 1962) with 0.8% agar under 16/8 h (light/dark) light at 19±2ºC. The basal 1-2 mm caudex of the seedlings was cut and cultured on MS callus induction medium supplemented with 20 g L^{-1} sucrose, 8 g L^{-1} agar, 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} 6-benzylaminopurine (BA) at 19 ºC in the dark. After 30 d, calli were sub-cultured twice in the same medium (Xu *et al.*, 2009b). Then embryogenic calli were cultured in the callus multiplication media with different hygromycin concentrations (25, 50, 75 and 100 mg L^{-1}) to find out the feat concentration for the selection of resistant calli. After 8 weeks of multiplication, the whitish-yellow-colored compact nodular calli were selected to culture on pre-medium (MS + 2,4-D 2 mg L^{-1} + BA 0.5 mg L^{-1} + AS 100 µmol L^{-1} + 2% sucrose + 0.8% Agar, pH 6.0) for three days. Then the calli were infected by *Agrobacterium* suspension with different OD values for 5~60 min.

***Agrobacterium* multiplication:** *Agrobacterium tumefaciens* EHA105 contained plasmid pKUB with T-DNA region of binary vector (Fig. 1) was obtained from Prof. Illimar Altosaar, Department of Biochemistry, University of Ottawa, Canada. The T-DNA region of binary vector included a neomycin phosphotransferase gene (*npt II*) under the control of nopaline promoter (*Pnos*), a hygromycin B phosphotransferase gene (*hpt*) and a β-glucuronidase (GUS) gene both under the control of CaMV35S promoter (*P35S*) (Cheng *et al.*, 1998). *Agrobacterium tumefaciens* was cultured in 5 mL YEP liquid medium with 50 mg L^{-1} hygromycin at 28ºC with overnight shaking at 180 rpm. This culture (5 mL) was transferred to 50 mL pre-culture medium with acetosyringone 100 µM and 50 mg L^{-1} hygromycin, and shaked at 180 rpm for 36 h at 28ºC. *A. tumefaciens* cells were harvested by centrifugation at 5000 rpm for 10 min., at 4ºC, and resuspended in a liquid suspending medium (OD_{600} 0.2-0.3) with 100 µM acetosyringone. The multiplied bacterial culture was used for infecting *Juncus effusus* L., calli.

Gene transformation and plant regeneration: Embryogenic calli were immersed in the *A. tumefaciens* suspension for 5-60 min, and then transferred to callus induction medium supplemented with 0-250 µM acetosyringone. After co-cultivation for 2-8 days, calli were transferred into callus induction medium with 250 mg L^{-1} carbenicillin. They were grown for 2 days at 19ºC in the dark to restrict the bacterial growth and to allow the calli recovery from co-cultivation shock (Zhao *et al.*, 2002). The calli were then transferred to MS selection medium supplemented with 2 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BA, 20 g L^{-1} sucrose, 8 g L^{-1} agar, 250 mg L^{-1} carbenicillin and 50 mg L^{-1} hygromycin. The calli were cultured in the dark at 19ºC and subcultured into the same medium once every week.

After seven subculturings, the putative transgenic calli were used for plant regeneration which proceded in two steps (Xu et al., 2009b). First, the embryogenic calli were cultured in the regeneration MS medium-1 (0.5 mg L^{-1} BA , 1 mg L^{-1} KT, 250 mg L^{-1} carbenicillin and 50 mg L^{-1} hygromycin) for 10 days. In the second step, the calli was transferred to regeneration MS medium-2 (3 mg L^{-1} IAA, 0.5 mg L^{-1} BA, 1 mg L^{-1} KT, 250 mg L^{-1} carbenicillin and 50 mg L^{-1} hygromycin) until the shoots were visible. The putative transgenic shoots were transferred to the 1/2 MS rooting medium (20 g L^{-1} sucrose, 8 g L^{-1} agar and 50 mg L^{-1} hygromycin). The resistant caudexs were cut to test the transient GUS expression. After 30 d growth in the culture medium, the resistant plants were transferred into pots with soil-perlite mixture after seedling hardening for about 3 days.

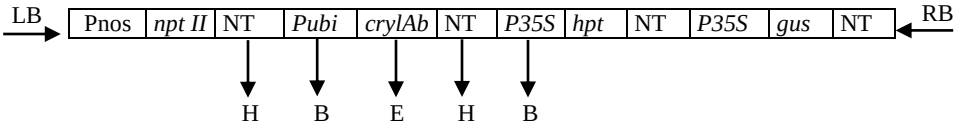

Fig. 1. The T-DNA region of pKUB binary vector in *A. tumefaciens* used for *J. effusus* L. transformation. *Pnos*: nopaline promoter, *Pubi*: ubiquitin promoter, *P35S*: CaMV35S promoter, *npt II*: neomycin phosphotransferase gene, *hpt*: hygromycin phosphotransferase gene, LB: left boundary, RB: right boundary, H: *Hind III*, B: *Bam*H I, E: *Eco*R I

GUS histochemical assay: The GUS histochemical assay was carried out for putative transgenic calli and plantlets (Rueb *et al.,* 1989) with some modifications. Tissues were soaked for 6-10 h at 37ºC in the 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) coloration medium (100 mmol L^{-1} Sodium phosphate buffer, 10 mmol L^{-1} EDTA, 1 mmol l^{-1} X-gluc and 0.1% Triton) having pH 7.0. Then the tissues were dried with sterile blotting-paper to remove excess coloration medium, and immersed in anhydrous ethanol for assaying the transient GUS expression.

Polymerase chain reaction: The mat rush genomic DNA was extracted from putative transgenic leaf tissues according to the CTAB method (Doyle *et al*., 1988). The *cryIAb* gene primer set, 5'-GCAACCATCAATAGCCGTTACA-3' and 5'- GTCAATGGGATT TGGGTGATTT-3', which produces a 0.872-kb fragment within the *cryIAb* gene, and the *gus* gene primer set, 5' GGGATCCATCGGAGCGTAATG 3' and 5' GCCCACAGCAGCAGTTTCATA 3', which amplifies a 0.563-kb fragment within the *gus* gene, were used for polymerase chain reaction (PCR) analysis. Each 25 µL reaction system contained 4 ng genomic DNA, 0.5 uM of each primer, 0.2 mM dNTP, 10 × Taq DNA polymerase reaction buffer, and 1U Taq DNA polymerase (Sheng Gong, China). The PCR protocol was set as: 4 min at 94ºC, 30 cycles of 30 s at 94ºC, 1 min at 54ºC (for *crylAb*) or 50ºC (for *gus*), and 80 s at 72ºC with a final extension of 8 min at 72ºC. The amplified PCR products were used for electrophoresis analysis in a 1% (w/v) agarose gel, and photo-documented under UV light (Lee *et al.,* 2006).

Statistical analysis: Data were analyzed using the SAS statistical software. Where the *F*-test showed significant differences among means, multiple range tests were performed at the 0.05 level of probability (Steel *et al.,* 1997).

Results and Discussion

Effect of co-cultivation duration on transient GUS expression: The co-cultivation duration is one of crucial factors influencing *Agrobacterium*-mediated gene transfer in plants and depends largely upon the *Agrobacterium* strain and the explant used for transformation. Generally, 2-3 days co-cultivation is considered suitable for *Agrobacterium*-mediated transformation, but in certain monocotyledonous plants (Toyama *et al.,* 2003; Akutsu *et al.,* 2004) 4–9 days have been described. In the present study, after infection by *A. tumefaciens*, calli of both genotypes of *J. effusus* were cultured in the subculture medium for 2-8 days. Co-cultivation period affected the transient GUS expression significantly, that was lowest for 2-day of co-cultivation (Fig. 2). Extension of the co-cultivation period from 2 to 4 day significantly enhanced the frequency of explants showing GUS^+ activity. There was gradual decrease in GUS expression frequency of explants when co cultivation was further increased from 4 to 8 d. It has been reported that too short or too long co-cultivation duration reduced the GUS expression in orchard grass (Lee *et al.,* 2006; Hasan *et al*., 2008).

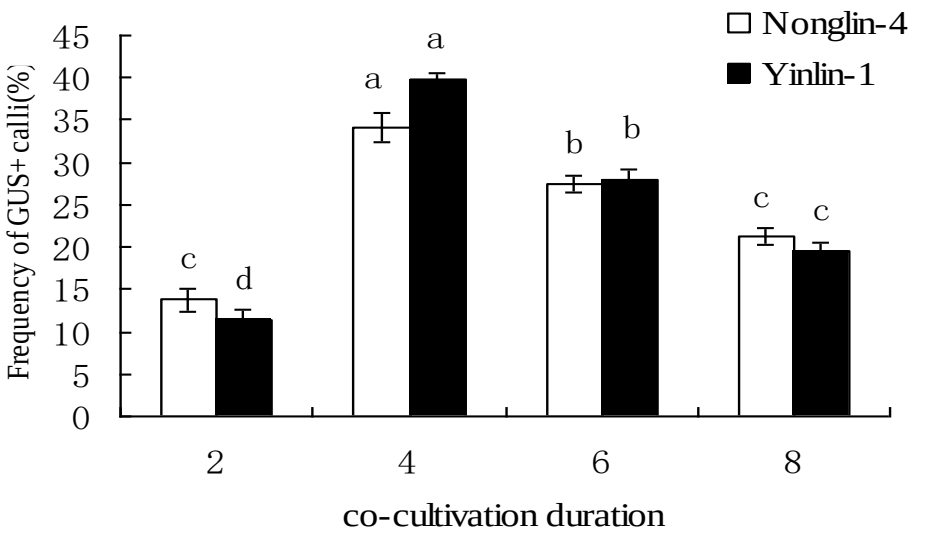

Fig. 2. Effect of co-cultivation duration (days) on transient GUS expression in seed-derived calli of *J. effusus* L. Acetosyringone 150 μM was supplemented in both the inoculation and co-cultivation media. Different letters indicate significant differences at $p<0.05$.

Effect of acetosyringone on the transformation efficiency: Acetosyringone is thought to be an external signal molecule that induces *vir* genes and also the key factor in *Agrobacterium*-mediated transformation (Guo *et al.,* 1998). It is known to enhance T-DNA insertion by *A. tumefaciens* into recalcitrant plant hosts (Godwin *et al.,* 1991). Here, the transformation efficiency was estimated by the transient GUS expression of transgenic calli 3 days after co-cultivation with *A. tumefaciens.* Increasing the concentration of acetosyringone from 50 to 150 mg L^{-1} enhanced the frequency of GUS^+ expression significantly (Fig. 3). However, further increase beyond 150 mg L^{-1} caused a sharp reduction in GUS^+ expression frequency in both genotypes. The lowest frequency GUS^+ expressing calli were recorded without using acetosyringone. With 100 μM concentration of acetosyringone in orchard grass calli, Lee *et al.,* (2006) obtained the highest frequency of GUS-expressing calli to both inoculation and co-cultivation media.

Effect of *A. tumefaciens* concentration on transient GUS expression: Optimum concentration of *A. tumefaciens* in the media is important as low amount may result in reduction of GUS^+ expression while higher concentration may cause contamination of explant tissues. It depends upon the *Agrobacterium* strain, plant tissue used (Gurlitz *et al.,* 1987). Results revealed that concentration of *A. tumefaciens* in the medium affected the rates of calli survival and transient GUS^+ expression significantly (Fig. 4). Survival rate of Nonglin-4 genotype calli decreased, but frequency of transient GUS expression enhanced by increasing the concentration of *A. tumefaciens* from 0.2 to 0.4 of OD_{600}. With OD_{600} values from 0.4 to 0.8, the frequency of transient GUS expression reduced significantly, because it might have resulted in the assembling of bacteria themselves and difficulty of binding to calli. The highest rate of transient GUS expression was about 40% at *A. tumefaciens* concentration of 0.4. Results reported in wheat (Amoah *et al.,* 2001) and *Prunus cerasus* L. (Song & Sink, 2005) conform to our findings.

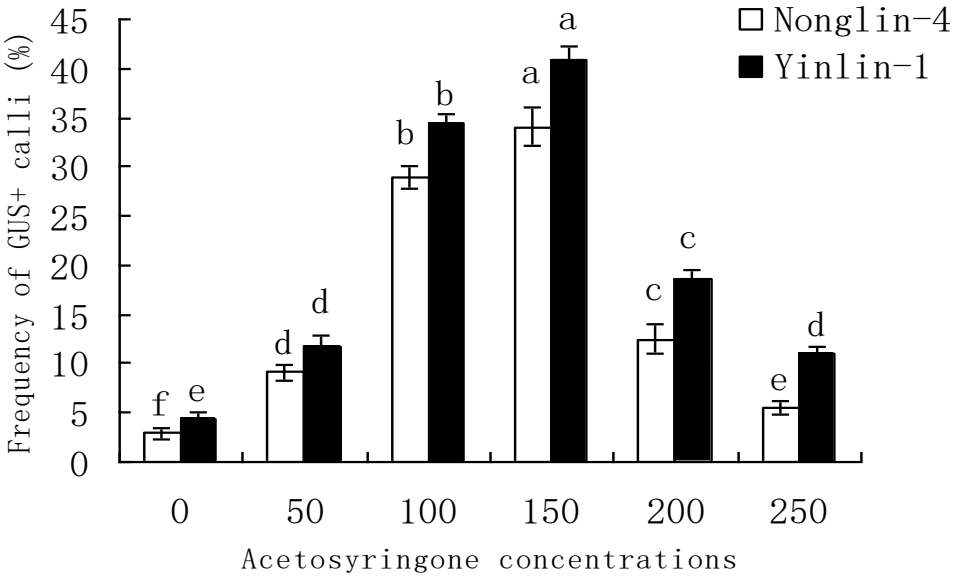

Fig. 3. Effect of acetosyringone concentration on transient GUS expression in seed-derived calli of *J. effusus* L.
Data were recorded after 3 days of co-cultivation with *A. tumefaciens* strain EHA105. Values represent the mean (±S.D.) of six independent experiments. Acetosyringone was supplemented in both the inoculation and co-cultivation media. Different letters indicate significant differences at $p<0.05$.

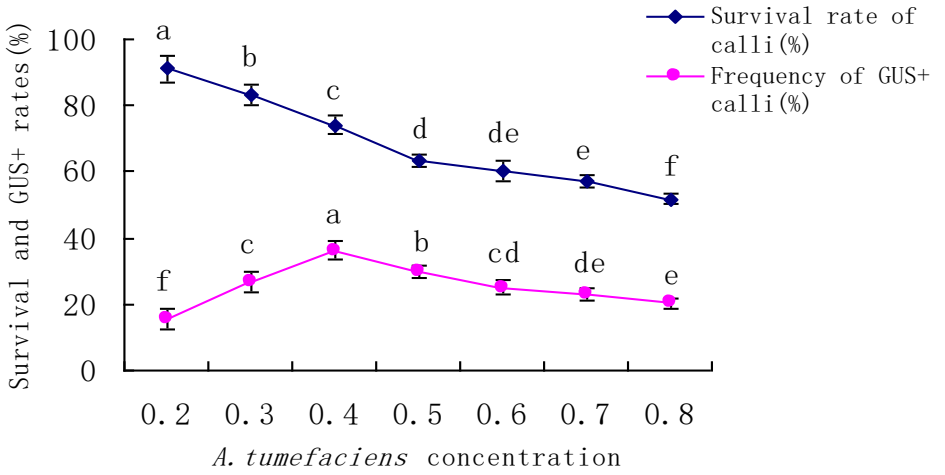

Fig. 4. Effect of *A. tumefaciens* concentration (OD_{600}) on the rate of calli survival and transient GUS expression in Nonglin-4 genotype of *J. effusus* L.
Different letters indicate significant differences within the same analysis at $p<0.05$.

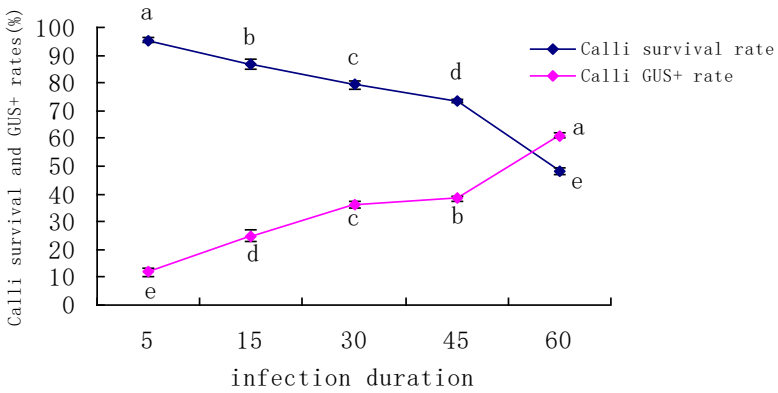

Fig. 5. Effect of different infection durations on calli survival and GUS$^+$ rates (%) in *J. effusus.* Data were recorded after being immersed in *A. tumefaciens* solution for the different time intervals. Values represent the mean (±S.D.) of five independent experiments. Different letters indicate significant differences within the same treatment at $p<0.05$.

Table 1. Effect of different infection stages of calli on the regeneration of *J. effusus* L., genotype Nonglin-4.

Infection stage (days)	Regeneration rate (A, %)	Hygromycin-resistant rate (B, %)	Transformation rate (A×B/100, %)
0	0.56 ± 0.93 e	95.00 ± 7.07 a	0.53 ± 0.63 d
4	2.04 ± 0.84 d	78.89 ± 2.72 b	1.61 ± 0.60 c
8	6.11 ± 1.17 c	73.33 ± 6.20 c	4.48 ± 0.87 b
12	13.02 ± 1.66 b	65.31 ± 2.37 d	8.50 ± 1.07 a
16	15.56 ± 0.99 a	61.47 ± 1.33 e	9.56 ± 0.53 a

Data were recorded after being influenced by *A. tumefaciens* at different times. Values represent the mean (±S.D.) of five independent experiments. Different letters indicate significant differences within the same column at $p<0.05$

Effect of *A. tumefaciens* infection duration on transient GUS expression: Different infection durations affected the calli survival and GUS$^+$ rates significantly (Fig. 5). Longer duration of *A. tumefaciens* infection damaged the callus tissues, while at shorter duration the transient GUS expression was not obvious. Frequency of calli survival reduced with the prolonging of infection duration. The GUS$^+$ rate increased along with the prolonging of the infection duration. In order to get the balance of calli survival rate and GUS$^+$ rate, 45-60 min infection duration was found appropriate.

Effect of different infection time on the plant regeneration: *Juncus effusus* L. calli were co-cultivated with both *Agrobacterium tumefaciens* containing or lacking the binary vector pKUB (Fig. 1), and kept for 20 days. Although some growth of *Juncus effusus* L., occurred in control, however, the calli capable of sub-culture to fresh medium supplemented with 50 µg mL^{-1} hygromycin B were only obtained from co-cultivation with *A. tumefaciens* containing pKUB. In the beginning, the hygromycin-resistant rate was higher and the regeneration rate was very low (Table 1). The regeneration rate enhanced with the progressing of infection time. The hygromycin-resistant rate was opposite to the regeneration rate. So the infection process suited at the 12th or 16th day after subculture when the transformation rate was significantly higher than that of others viz. 8.50 and 9.56%, respectively.

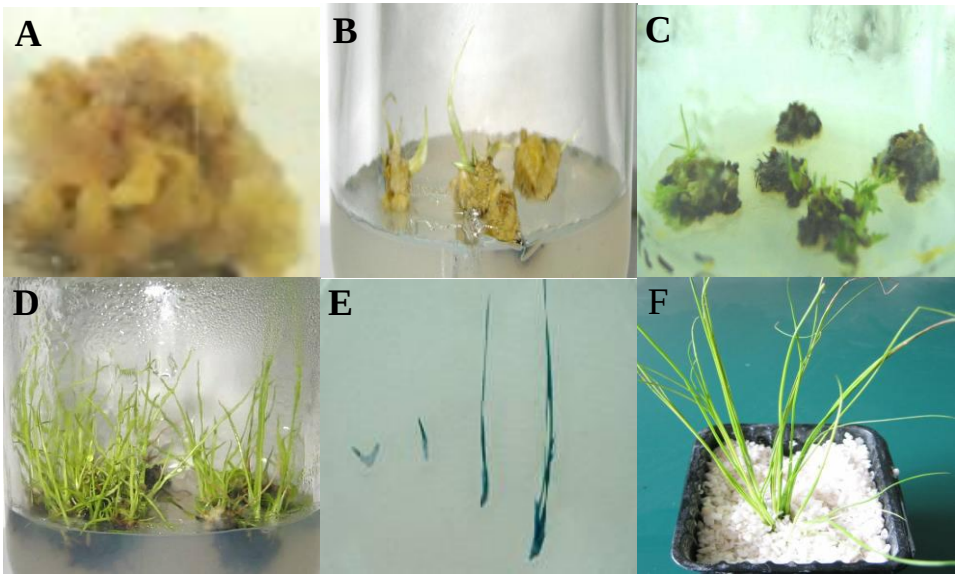

Fig. 6. Hygromycin-resistant calli and transgenic plants obtained by *Agrobacterium* mediated transformation of *Juncus effusus* L.
A. hygromycin-resistant embryogenic callus; B. regenerated plants from embryogenic calli in the first three days; C. plants regenerated from embryogenic calli after one week; D. plants regenerated from embryogenic calli after two weeks; E. transient GUS expression in regenerated plants; F. transgenic plant in the pot.

Selection and molecular confirmation of transgene expression: The putative transgenic calli were subjected to GUS histochemical assay for confirming the presence of transgene. The calli showing blue staining revealed the T-DNA, had successfully integrated into the genome of *Juncus effusus* L. After 7 sub-culturings, hygromycin-resistant embryogenic calli of Nonglin-4 were selected for regeneration experiment (Fig. 6A). The regenerated plants developed from resistant calli after 3 days (Fig. 6B), 1 week (Fig. 6C) and 2 weeks (Fig. 6D) in the regeneration media. Then these plants were transferred to 1/2 MS medium with 50 mg L^{-1} hygromycin for selection. Survived plantlets obtained from the resistant calli of genotype Nonglin-4 were used for GUS histochemical assay. Nearly all of the regenerated plants with hygromycin-resistance showed positive GUS expression (Fig. 6E). These resistant plants were used for further PCR confirmation for both cultivars viz. Nonglin-4 and Yinlin-1. For the PCR confirmation, two independent PCR analysis were carried out using the primer sets of *Bt* and *gus* genes. Non-transformed plants of Nonglin-4 and Yinlin-1 were used for the negative control. Electrophoresis revealed the expected 0.872 and 0.563 kb bands for *Bt* and *gus* genes respectively in both cultivars (Figs. 7, 8), which were not detected in control plants. Plantlet regeneration from PCR-positive embryogenic tissues of hybrid firs was also reported by Xu *et al.*, (2009a). The resistant plants were transferred into soil after seedling hardening for 3 days.

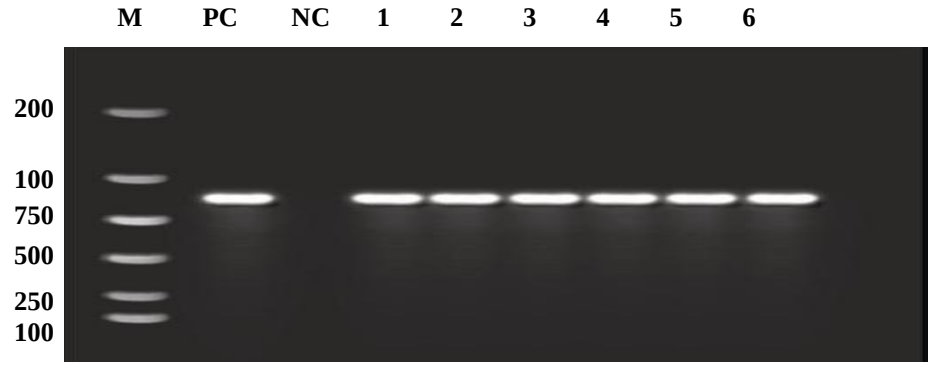

Fig. 7. PCR detection results of *Bt*-transgene.
PCR amplification was conducted with *Bt* specific primer for production of a 872-bp (*Bt*) fragment using genomic DNA of samples as the templates. Lane M: DL 2000 marker; NC: wild type used as negative control, PC: pKUB used as positive control. Lanes 1-3: transgenic plants of Nonglin-4; Lanes 4-6: transgenic plants of Yinlin-1.

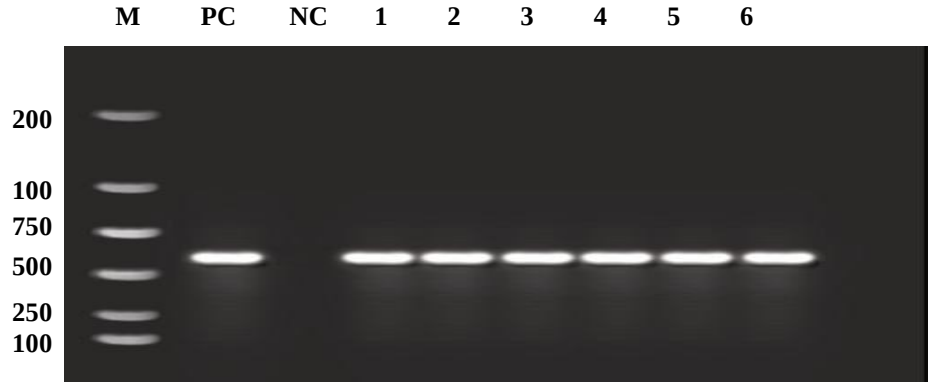

Fig. 8. PCR detection results of *gus*-transgene.
PCR amplification was conducted with *gus* specific primer for production of a 563-bp (*gus*) gene fragment using genomic DNA of samples as the templates. Lane M: DL 2000 marker; NC: Wild type used as negative control; PC: pKUB used as positive control; Lanes 1-3: transgenic plants of Nonglin-4; Lanes 4-6: transgenic plants of Yinlin-1.

Conclusion

Earlier, there was no protocol available for the transformation of *Bt* gene into mat rush *(Juncus effusus* L.) mediated by *Agrobacterium*. Therefore, an efficient system of gene transformation mediated by *Agrobacterium* for *Juncus effusus* L., was developed by optimizing different factors. The transient GUS expression and hygromycin-resistance tests were facile and reliable for selecting the positive transformants. Through this protocol, transgenic *Juncus effusus* L., plants were produced from seeds to embryogenic calli, then to plantlets. This transformation system may help to manipulate the desired traits though transgenic techniques. So, the currently developed protocol of *Agrobacterium*-mediated transformation of *Bt* gene into mat rush with some optimizations is highly successful and reproducible.

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