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Research Article

High-frequency plant regeneration from transgenic rice expressing *Arabidopsis thaliana* Bax Inhibitor (*At*BI-1) tissue cultures

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Abstract Genetic transformation was affected by material of explant, age of callus, and medium of regeneration. Two rice seed cultivars (Ilpum and Baekjinju) and mediums were investigated in this study for enhancing regeneration of transgenic rice expressed *At*BI-1 gene encoding the *Arabidopsis thaliana* Bax inhibitor. Regeneration rate of Ilpum rice transformant in gelrite of 5 and 8 g were 27.4% and 18.0%, respectively. In Baekjinju, regeneration rate of transformant was 5.4% and 4.3% in 5 and 8 g gelrite, respectively. The highest number of transformant plant in this study was regenerated from Ilpum cultivar on MS medium (30.4%) and was applied for the subsequent experiment. The callus regeneration rate of transformant were 40.7% in callus infection of up-side, it was higher regeneration then in the down-side (3.9%). The regeneration rate of callus of 25 days and 35 days were 14.7% and 38.6%, respectively. The most important application of this work is in genetic transformation of rice, particularly for improvement transgenic plant tissue culture protocol with high frequency of plant regeneration.

Keywords *Agrobacterium*-mediated, Transformation, Rice, Regeneration, *At*BI-1

Introduction

Rice is one of the basic staple foods for more than half human population of the world's (Juliano 1985), which is most interesting for the study of molecular breeding such as behavior of transgenic plants. Genetic engineering of plant have been popular used for various purposes such as to increase yield and tolerant to environmental stresses, and was useful to maintain excellent genes. The target genes can be introduced directly from various species of plants to engineer a GM crops (Kim et al. 2009). Previous study has reported that some method had been developed for the producing of new transgenic rice plants such as protoplast (Datta et al. 1990), gene gun (Christou et al. 1991), and *agrobacterium* (Hiei et al. 1994) to insert the gene(s) into the plant cells. *Agrobacterium*-mediated plant transformation method was most popular, relatively easy, and widely used method to introduce a small number of DNA cloning of the gene into the chromosome of a monocotyledonous plant. Tissue culture has been commonly used for transformation procedures to generate transgenic crops such as corn, soybeans, cotton, canola crops, rice and maize (James et al. 2011). Transgenic rice had been successfully developed by using mature embryos of rice as explants (Hiei 1994), young callus tissue of *indica* rice (Manimaran et al. 2013), inflorescence tissue (Dong et al. 2001), green tissues induced from mature embryos (Cho et al. 2004). Besides, other explant such as scutellum-derived calli was mostly used as source for transformation to increasing of transgenic rice regeneration efficiency (Hiei et al. 1994; Kant et al. 2001). Heyser et al. (1983) reported that the parents of a plant genotype, medium composition, material explant, and culture method affected the frequency of plant tissue culture regeneration. To investigated the effect of the callus aged, callus type, and MS medium composition on regeneration of rice transformants, we cultivated two transformants of *japonica* rice (Ilpum and Baekjinju) inserted the *At*BI-1 *Arabidopsis thaliana* Bax Inhibitor-1 to understand its efficiency and gene stability of rice mutants. The present study describes the simple procedures for the establishment of different types of japonica rice varieties, MS medium composition, types of callus, and callus aged to enhanced regeneration and transformation efficiency.

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Materials and methods

Plant material and plasmid construct

Mature seed of japonica varieties (*Oryza sativa* L.) cv. Ilpum and Baekjinju obtained from Plant Molecular Breeding Lab. were used in this study. The binary plasmid pBin19 T-DNA region was transform into *Agrobacterium* strain LBA4404 (pSM-GFP) by triparental mating method (Lichtenstein and Draper 1985). The binary plasmid pBIN19 has *gfp* and *npt* as reporter and plant selection marker genes, respectively, which are driven by CaMV 35S promoter and *npt* II gene outside the T-DNA region as bacterial selection marker (Fig. 1)

Agrobacterium-mediated rice transformation

Dehulled mature seeds were sterilized with 70% EtOH for approximately 90 seconds with vigorous shaking then washed in autoclaved ddH_2O for three times, and followed by incubation in 1% NaOCl for 30 minute with shaking 150 rpm. The samples were washed with ddH_2O for 3 times, then dried on sterile filter paper for an hour in room temperature and pre-culture on callus induction medium (4.4g/L MS, 2 mg/L 2,4-D, 30.0 g/L sucrose, 2.0 g/L casein, 5.0 g/L gelrite pH 5.8) at 26±1℃ under continuous dark condition for 30~50 days.

Agrobacterium tumefaciens strain LBA4404/pBin19-*At*BI-1-GFP (Fig. 1) was cultured in LB (10.0 g/L Tryptone, 5.0 g/L yeast extract, 10.0 g/L NaCl, 15.0 g/L Bacto^{TM}agar, 50 mg/L kanamycin, 50 mg/L streptomycin) at 30°C in a shaker incubator for 72 hour then adjusted to 0.8 $O.D._{600}$. Callus was divided into 2 pieces from scutellum as standard for cutting the calli, was separated to Up (white, hard) and Down (yellowish, soft) sides (Fig. 2). Each calli were divided to 1 ~ 5 mm diameter, put on moderate concentration of *Agrobacterium* (0.8 OD_{600}) for 3 min and dried on sterile filter paper for few minutes. The calli were inoculated on the co-cultivation media at 26±1°C under continuous dark condition for 3 days. Callus were selected in the selection medium (4.4 g/L MS, 1.0 mg/L NAA, 5.0 mg/L kinetin, 2.0 g/L casein, 30.0 g/L sucrose, 50.0 mg/L kanamycin, 250.0 mg/L carbenicillin, 6.0 g/L gelrite, pH5.8), indicated that it was infected by *agrobacterium*. Putative transformants would appear from calli and it was transferred to test tubes containing antibiotic to obtain the resistance callus for growth leaves and root. After 3 weeks in a test tube, the Putative transformants grew to complete plants.

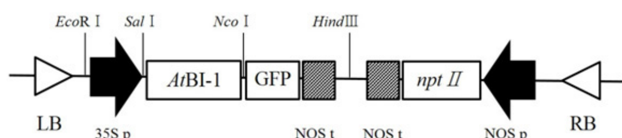

Fig. 1 Linear map of binary vector (pBin19-*At*BI-1-GFP). 35S: *Ca*MV 35S, *At*BI-1: *Arabidopsis thaliana* Bax Inhibitor-1, GFP: Green Fluorescent Protein gene, NOS : Nopaline Synthase terminator, *Npt*2: Neomysin phosphotrasferase gene, LB: T-DNA left border, RB: T-DNA right border

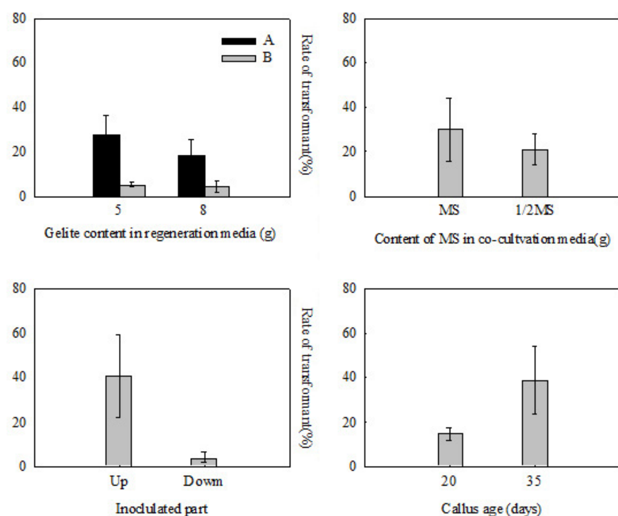

Fig. 2 Part of callus used for infection. A : Callus formation from mature seed, B : Primary Callus of up-side, C : Callus of down-side

Polymerase chain reaction analysis for check transformants

Leaves of putative transformants were harvested. Genomic DNA extracted on 3~4 leaf stage using DNeasy® Plant Mini Kit (QIAgen, Germany). Polymerase Chain Reaction (PCR) analysis was then performed using specific primers for the *At*BI-1 gene (*At*BI F: 5'-ATG GAT GCG TTC TCT TCC TT-3', *At*BI R: 5'-CAG CCC CTC AGT TTC TCC TT-3') PCR was conducted to the following conditions: pre-denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute and then post-extension at 72°C for 5 minutes. Amplified band size to PCR was checked with 15 bp ~ 13 kb QX DNA size marker by QIAxcel (QIAGEN Co., Germany).

Results and discussion

The first tested the callus induction and regeneration frequency of different varieties, medium and age of callus to increase the efficiency of transformation of rice. Two mature seeds of *japonica* rice varieties (Ilpum and Baekjinju) were cultured on callus induction medium for 20 days. The young callus

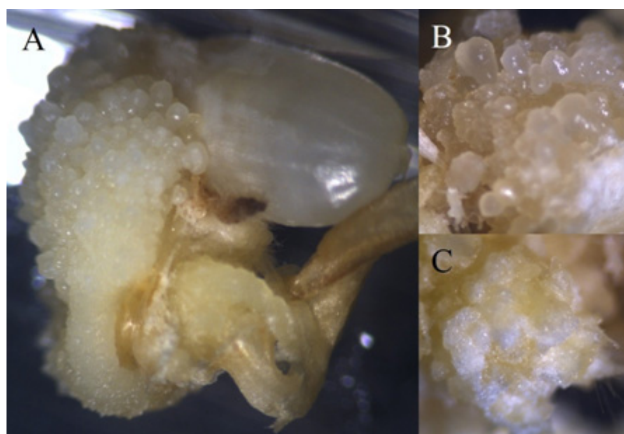

Fig. 3 Reganeration rate of transformants. A: Ilpum, B: Baekjinju

appeared from scutellum on 7 day and proliferated until 30 days incubation on the same medium. The compact primary callus from scutellum was initiated on the 6th day after inoculation in the callus induction medium (Fig. 3A). Embryogenic calli formed yellowish to white and hard to soft on the 6th day. The callus size was gradually increased, which the changes in morphological callus were occurred. From 10 days of incubation onward, the secondary callus was formed. The primary (Fig. 3B) and secondary (Fig. 3C) callus were selected for transformation.

The callus induction frequencies of different varieties showed that the Ilpun variety had high frequency regeneration than that of Baekjinju variety was cultivated in induction medium contained 5 g/L gelrite (Table 1). However, the callusing frequency of two rice varieties (Ilpun and Baekjinju) in induction medium contained 5 g/L gelrite were 67.3% and 50.6%, respectively. Gelrite treatment at different concentrations caused the differences in the rate of callus and plants regeneration, but it has the same effect on the both of varieties. High concentration of gelrite decreased the level of callus induction and plant regeneration. The ability of explants to form the callus depended on the influence of rice varieties. Ilpun had high ability to produced callus in the induction medium contains 5 g/L of gelrite, than was used for continuous observation. The high concentration of gelrite contributed the plants regeneration, but it had decreased the plant regeneration at concentrations greater than 5 g/L.

This study demonstrated that the concentration of nutrients had important effects on the callus and plant regeneration of transgenic rice (Table 2). Two composition of medium (MS and 1/2MS) were tried to obtained the high frequency plant regeneration from Ilpum variety mature seed explants. Five hundred seed were cultured on normal MS medium and then after 30 day on the dark condition, the callus from scutellum

Table 1 Regeneration rate of transformants in different gelrite concentration

Cultivar	Content of gelite (g)	No. of seed cultured	No. of seed for infection	No. of calli co-cultivated (%)	No. of calli survived (%)	No. of green spot (%)	No. of albino plant (%)	No. of plant (%)	No. of plant acclimatized (%)
Ilpum	5	500	439	538.7±2.5[a] (100.0)	362.7±28.5 (67.3±5.3)	312.7±24.5 (58.1±4.6)	44.0±12.3 (8.2±2.3)	175.0±44.2 (32.4±8.1)	148.0±46.8 (27.4±8.6)
	8	140	100	123.0±2.6 (100.0)	72.3±9.2 (58.6±6.7)	58.7±8.8 (47.5±6.5)	12.7±5.8 (10.2±4.7)	40.7±7.6 (33.1±6.4)	22.3±9.2 (18.0±7.4)
Baekjinju	5	538	351	637.7±99.3 (100.0)	326.0±45.6 (50.6±2.9)	114.3±31.6 (17.3±3.8)	20.0±4.6 (3.1±0.5)	51.7±23.1 (7.6±3.2)	35.0±8.7 (5.4±1.0)
	8	240	125	156.7±2.5 (100.0)	52.0±18.6 (33.4±12.3)	23.0±15.5 (14.9±10.1)	16.3±14.9 (10.6±9.7)	7.7±5.2 (5.0±3.4)	6.7±4.2 (4.3±2.7)

[a]mean±standard deviation.

Table 2 Regeneration rate of transformants in different MS concentration

Media	No. of seed cultured	No. of seed for infection	No. of calli co-cultivated (%)	No. of calli survived (%)	No. of green spot (%)	No. of albino plant (%)	No. of plant (%)	No. of plant acclimatized (%)
MS	500	439	383.0±4.0[a] (100.0)	268.0±9.0 (70.0±2.7)	231.0±13.1 (60.3±3.3)	37.7±15.2 (9.8±3.9)	116.3±61.0 (30.4±15.9)	115.0±56.8 (30.0±14.8)
1/2MS	500	439	155.7±4.5 (100.0)	94.7±24.2 (60.8±15.4)	81.7±21.5 (52.5±13.7)	6.3±2.5 (4.1±1.0)	58.7±26.7 (37.3±16.5)	33.0±11.3 (21.1±7.0)

[a]mean±standard deviation.

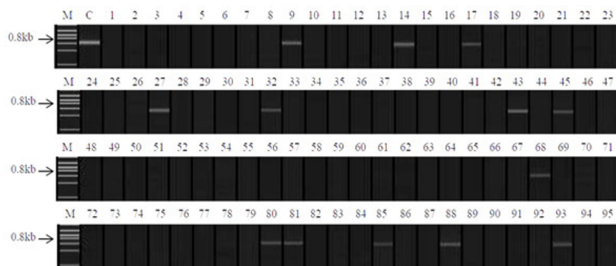

Fig. 4 PCR analysis results. M: 15bp~3kb QX DNA size marker, C: DNA of *At*BI-1 plasmid, 1: non-transformant, 2~95: sample plants estimated transformants

was separated from whole seed for *Agrobacterium* infection about 439 seeds. After *Agrobacterium* infection, the callus were co-cultivated normal MS medium resulted the highest percentage of callus regeneration. Percentage of callus, green spot, albino, and plant after acclimatization in normal MS medium were 70%, 60.3%, 9.8%, and 30.4%, respectively. The decreasing of nutrients in induction medium produced a lower of callus (60.8%) and plant after acclimatization (21.1%), which significantly was influenced callus induction and plant regeneration. The result of best composition of MS medium contained gelrite was used for next experiments.

Ilpum variety was preferred to the callus cultivation, and then it was cultured in the induction medium containing 5 g/L gelrite to obtain the primary and secondary callus for its regeneration ability observation. For each experiment, 439 calli were infected with *Agrobacterium* suspended with MS solution for 1 min followed by co-cultivation in dark for 72 h. The transformed calli were washed in sterile water followed by MS callus induction liquid medium supplemented with 1 mg/L of neomysin and detected for transient GFP expression. Putative transgenic calli of Ilpum-*At*BI-1 gene expression were selected from co-cultivation medium contained antibiotic. Primary callus were cultivated in selection medium produced a high percentage of calli survived (87.4%) compared than that of secondary callus (27.1%) (Table 3). After 7 days in regeneration medium, the percentage of plant regeneration derived from primary and secondary callus decreased approximately 40.4% and 6.0%, respectively. Plant derived from primary callus had a higher life ability and more stable than its derived from secondary callus during acclimatization course.

Medium, variety, and source of explant affected the high efficiency of transformation, we also tested the callus induction and the regeneration frequency of different calli aged of japonica rice variety Ilpum-*At*BI-1 gene. Dehulled rice seeds were cultured on callus medium for 30 days. *Agrobacterium* were infected after callus proliferation, when the callus aged on 20 and 35 days. The percentage of calli survived of 20- and 35-day-after co-cultivated were 66.8% and 66.6%, respectively, showed was not significantly different. The 35-day-old callus produced high frequency of plant regeneration and after acclimatization, 50.1% and 38.6%, respectively, was significantly different compared those of the 20-day-old callus.

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Table 3 Regeneration rate of transformants in different inoculated part

Inoculated part	No. of seed cultured	No. of seed for infection	No. of calli co-cultivated (%)	No. of calli survived (%)	No. of green spot (%)	No. of albino plant (%)	No. of plant (%)	No. of plant acclimatized (%)
Up	500	439	272.3±3.2[a]	238.0±4.0	211.7±10.9	18.0±11.9	109.7±48.7	110.7±50.9
			(100.0)	(87.4±1.2)	(77.7±3.7)	(6.6±4.3)	(40.4±18.0)	(40.7±18.8)
Down	500	439	110.7±1.2	30.0±4.6	19.3±2.4	19.7±4.1	6.7±2.6	4.3±2.4
			(100.0)	(27.1±4.2)	(17.4±2.1)	(17.8±3.7)	(6.0±2.4)	(3.9±2.1)

[a]mean±standard deviation.

Table 4 Regeneration rate of transformants in different callus age

Callus age (days)	No. of seed cultured	No. of seed for infection	No. of calli co-cultivated (%)	No. of calli survived (%)	No. of green spot (%)	No. of albino plant (%)	No. of plant (%)	No. of plant acclimatized (%)
20	260	215	265.3±5.0[a]	177.0±19.9	147.7±36.6	32.0±3.6	37.3±14.2	39.0±15.1
			(100.0)	(66.8±8.5)	(55.8±14.3)	(12.1±1.4)	(14.1±5.6)	(14.7±5.7)
35	240	224	273.3±57.5	185.7±76.0	165.0±71.6	12.0±7.2	137.7±66.1	109.0±74.2
			(100.0)	(66.6±21.7)	(59.3±21.7)	(4.2±1.7)	(50.1±22.7)	(38.6±26.1)

[a]mean±standard deviation.

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