Molecular and Cytogenetic Analysis of Transgenic Plants of Rice (*Oryza sativa* L.) Produced by *Agrobacterium*-mediated Transformation

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ABSTRACT

To demonstrate the importance of transformation efficiency in independent event, molecular and cytogenetic analysis were conducted with genomic DNA and chromosome of transgenic plants produced by *Agrobacterium tumefeciens* LBA4404 (pSBM-PPGN: *gusA* and *bar*). Selection ratios of putative transgenic calli were similar in independent experiments, however, transformation efficiencies were critically influenced by the type of regeneration media. MSRK5SS-Pr regeneration mediun, which contains 5 mgL^{-1} kinetin, 2% (w/v) sucrose in combination with 3% (w/v) sorbitol, and 500 mgL^{-1} proline, was efficient to produce transgenic plant of rice from putative transgenic callus in the presence of L-phosphinotricin (PPT). With MSRK5SS-Pr medium, transformation efficincies of Nagdongbyeo were significantly enhanced from 3.7% to 6.3% in independent callus lines and from 7.3% to 19.7% in plants produced, respectively. Stable integration and expression of *bar* gene were confirmed by basta herbicide assay, PCR amplification and Southern blotting of *bar* gene, and fluorescence *in situ* hybridization (FISH) analysis using pSBM-PPGN as a probe. In Southern blot analysis, diverse band patterns were observed in total 44 transgenic plants regenerated from 20 independent PPT resistant calli showing from one to five copies of T-DNA segments, however, the transformants obtained from one callus line showed the same copy numbers with the same fractionized band patterns.

Key words **:** *Agrobacterium*-mediated transformation, Embryogenic callus, Rice, Transformation efficiency, FISH, Southern blotting analysis

INTRODUCTION

Agrobacterium-mediated transformation is very useful tool for introducing the novel genes into various plant species including monocotyledonous and dicotyledonous plants. Especially, rice (*Oryza sativa* L.) has been one of the most attractive target to produce transgenic plant for enhancing agronomically valuable traits by genetic transformation because of its importances as a staple food and a model plant for genomic studies. However, rice had been precluded in application of the system until early 90's, since this bacterium was only known as parasite to dicotyledonous plants. Since the efficient

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transformation system was established by employment of acetosyringone (AS), which activates *vir* genes of *Agrobacterium* to target explants (Hiei *et al.*, 1994), *Agrobacterium*-mediated transformation has been considered one of the most reliable tools for transformation in rice. This system is efficient and economical in manipulation. And stable integration and expression of transgenes in host plant with low copy were expected compared to abiological method (Ke *et al.*, 2001).

Among the various factors influencing transformation efficiencies, embryogenic callus served as a starting material and its efficient regeneration system have been most important consideration in *Agrobacterium*-mediated gene transformation (Aldemita and Hodges, 1996; Hashizume *et al.*, 1999; Hiei *et al.*, 1994; Lee *et al.*, 1999; Rashid *et al.*, 1996). For obtaining transgenic plants, the transgenes were successfully integrated into the target explants and consequently putative transgenic calli were regenerated in high efficiencies. Thus, many studies have been focused on interaction between target explants and *Agrobacterium* using various target explants and *Agrobacterium* strains carrying different vector systems (Datta 2000, Hiei *et al.*, 1994, Ke *et al.*, 2001). However, many results demonstrated that regeneration efficiencies from transformed calli selected by various selection agents such as L-phosphinotricine (PPT) or hygromycine were critically lower than expected (Aldemita and Hodges, 1996; Hashizume *et al.*, 1999; Lee *et al.*, 1999; Rashid et al., 1996). These are mainly due to regeneration ability of scutella-derived callus of rice critically varied depending on the genotypes of rice cultivars (Cho *et al.*, 2004a; Lee *et al.*, 1999). It was reported that the regeneration efficiency in each cultivar was severely influenced by selected choices of carbohydrates and balances of cytokinin in combination with auxin in *in vitro* culture medium (Cho *et al.* 2004; Lee *et al.*, 2002).

The most desirable strategy in producing transgenic rice would be that target genes were directly induced into the elite cultivar and transgenic plants were regenerated with high efficiencies in independent events. Our strategy for improving transformation efficiency at desirable level is that optimized culture system will lead to desirable efficiency of shoot regeneration, thereby high transformation efficiency could be achieved.

Thus, in present study, we conducted with two objectives; one is for improving transformation efficiencies via optimized regeneration media and the other is for demonstrating the importance of transformation efficiencies in independent event by comparing the Southern analysis with 44 transgenic plants regenerated from various putative transgenic callus lines.

MATERIALS AND METHODS

Plant materials and *Agrobacterium* strain

Five japonica type of Korean elite rice cultivars were used for the experiment as follows; Nagdongbyeo, Daesanbyeo, Donganbyeo, Dongjinbyeo, and Ilmibyeo. Yellow-compact type of embryogenic callus induced from mature seeds on N6 basal callus induction medium (Hiei *et al.*, 1994) was used as a target materials for *Agrobacterium*-mediated transformation using *Agrobacterium tumefeciens* LBA4404 (pSBM-PPGN; *gusA* - *bar*) (Fig. 1).

Production of basta herbicide resistant plant

Agrobacterium-mediated transformation was

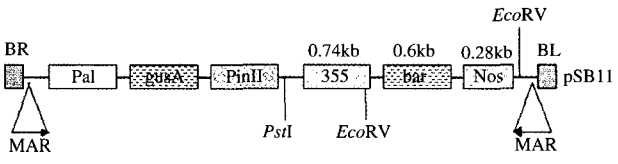

Fig.1. Schematic map of pSBM-PPGN.

Table 1. Compositions of regeneration media for producing basta resistant plant of rice from PPT resistant callus.

Experiment	Medium	Composition
EXP 1	MSRK2S	MS salts and vitamins, 2mgL^{-1} kinetine, 0.5 mgL^{-1} NAA, 30 gL^{-1} sucrose, 4 gL^{-1} phytagel, pH 5.8
EXP 2	MSRK5SS-Pr	MS salts and vitamins, 5 mgL^{-1} kinetin, 0.5 mgL^{-1} NAA, 20 gL^{-1} sucrose, 30 gL^{-1} sorbitol, 500 mgL^{-1} proline, 4 gL^{-1} phytagel, pH 5.8

All regeneration media were supplemented with 250 mgL^{-1} cefotaxime for eliminating *Agrobacterium* and 3 mgL^{-1} PPT for regeneration from transgenic calli.

conducted followed by Cho *et al.* (2004b). *Agrobacterium* was cultured on AB-ST medium at 28℃ dark place for 2-3 days. The calli were inoculated with *Agrobacterium* suspension (OD_{600}=1.7) diluted in liquid AAM medium containing 19.6 mgL^{-1} (100 mM) acetosyringone by gentle agitation for 10 minutes followed by eliminating the suspension from the calli with sterilized filterpaper. After three days of co-cultivation on 2N6-AS medium containing 100 mM acetosyringone (AS) at 25℃ dark place, the putative transgenic callus was selected with two rounds of selection by culturing on 2N6-CP medium containing 250 mgL^{-1} cefotaxime and 6 mgL^{-1} L-phosphinotricine at 27℃ for each two weeks in dark place.

For regeneration of transgenic plant of rice, two different MS (Murashinge and Skoog, 1962) basal regeneration media, MSRK2S and MSRK5SS-Pr, were used as shown in Table 1. The PPT resistant calli survived on selection medium were transferred onto the both MS basal regeneration a containing 250 mgL^{-1} cefotaxime and 3 mgL^{-1} PPT at 25℃ under the light (16 hours of day length). Transformation efficiencies were calculated as follows; one is the number the independent calli producing transgenic plants / the number of callus transformed × 100 (%) and the other is the number of the transgenic plants obtained / the number of callus transformed × 100 (%).

Transgenic plants regenerated in the presence of 3 mgL^{-1} PPT were sprayed twice with 0.3% (v/v) basta herbicide solution from the top with three days interval. After ten days of basta spraying to leaves, the resistance of the plants was compared with that of non-transgenic plants.

Molecular cytogenetic analysis

Probe labeling

Clone pTa71, containing a 9.1 kb fragment of 18S-5.8S-25S rRNA genes (45S rDNA) of *Triticum aestivum* L. and plasmid pSBM-PPGN were labeled with biotin16-dUTP and digoxigenin-11-dUTP using Nick Translation System (GibCO BRL).

Fluorescence *in situ* hybridization

The meiotic chromosome preparation and FISH analysis were performed according to the method reported by Koo *et al.* (2002, 2004). The probes were detected with avidin-FITC and Cy™ 3-conjugated IgG Fraction Monoclonal Mouse Anti-Digoxin (Jackon Immuno Research). The chromosomes were counterstained with 1 ㎍/ml DAPI (Sigma). The signals were detected with a cooled CCD camera (CoolSNAP, Photometrics). The images were recorded with software (Meta imaging series™ 4.6) using a Leica epi-fluorescence microscope equipped with FITC-Rhodamine-DAPI three-way filter sets (Leica, Japan).

PCR and Southern analysis

PCR and Southern analysis were carried out to

identify the stable integration and copy number of transgene in the genomic DNA of transgenic plants. Total genomic DNA was extracted from the leaves of the basta herbicide resistant plants by the cetymethyl-ammonium bromide (CTAB) method according to Murray and Thompson (1980).

PCR amplification reaction was conducted with two sets of primers for *bar* gene, 5'-ATGAGCCCAGAACGAC-3' (forward) and 5'-GGCAGTGGCTCTAGACT-3' (reverse) as follows: predenaturation at 94℃ for 4 minutes, 30 cycles of denaturation at 94℃ for 60 seconds, annealing at 65℃ for 40 seconds, extension at 72℃ for 60 seconds, and the final extension at 72℃ for 5 minutes.

For Southern analysis, a total of 5 ug genomic DNA of each plant was digested with 30 units of *Pst*I, which digests one unique site within the construction (Fig. 1), followed by conducting electrophoresis on 0.8% agarose gel at 25 volt for 18 hours. The DNA was transferred onto a H^+-bond nylon membrane (Amersham Phamacia Co.) followed by Southern blotting was conducted with ECL (Enhanced Chemi-luminescence) kit according to the manufacturer's instruction (Amersham Phamacia Co.). The membrane bound DNA was hybridized and detected with ECL labeled *bar* gene probe. The fractionized band patterns were compared among the transgenic plants which are obtained from various independent PPT resistant callus lines.

RESULTS AND DISCUSSION

Effect of regeneration media on transformation efficiencies

Transformed calli transferred on to selection medium containing 6 mg^{-1} PPT were grown slowly and turned brown during the selection. However, the calli which seemed to be PPT resistant were grown on the surface of the brown color calli (Fig. 2A and B). Selection ratios of PPT resistant calli were not significantly different between two independent experiments; 38.3% and 41.9%. However, transformation efficiencies varied depending on the regeneration media used (Table 2, Fig. 2C and D). With MSRK2S control regeneration medium containing 3 mg^{-1} PPT, transformation efficiencies based on the number of callus regenerated and the number of the transgenic plants obtained were 3.7% and 7.3%, respectively. However, on MSRK5SS-Pr which supplemented with 2% (w/v) sucrose and 3% (w/v) sorbitol as carbohydrate sources and 500mgL^{-1} proline under 5 mgL^{-1} kinetin, both transformation efficiencies were greatly enhanced up to 6.3% in independent transformation events based on callus number and 19.7% in number of the transgenic plants

Table 2. Comparison of transformation efficiencies in Nagdongbyeo between two different regeneration media.

Experiments[1)]	No. of calli transformed (A)	No. (%) of calli selected by PPT	No. of calli regenerated (B)	No. of plants produced (C)	No. (%) of basta® plant	TEC[2)] (B/A,%)	TEP[3)] (C/A,%)
Exp 1	300	115 (38.3)	11	22	22 (100)	3.7	7.3
Exp 2	320	134 (41.9)	20	63	62 (98.4)	6.3	19.7

[1)]After selecting putative transgenic calli, two different regeneration media such as MSRK2S (EXP 1) and MSRK5SS-Pr (EXP 2) were used in each experiment.

[2)]TEC: transformation efficiency calculated based on the number of callus

TEC (%) = (Number of callus regenerated / number of callus independently transformed) × 100

[3)]TEP: transformation efficiency calculated based on the number of plant

TEP (%) = (Number of plant obtained / number of callus independently transformed) × 100.

obtained. It was shown that regeneration efficiencies could be improved by additional supplementation of sorbitol and proline under 5 mgL^{-1} kinetin, even though frequencies of selected calli were not significantly different between two experiments (Table 2).

Cho *et al.* (2004a) reported that regeneration efficiencies of non-transgenic callus of various rice cultivars were enhanced by employment of optimized concentration of phytohormone and selected utilization of carbohydrate source. Although sucrose has been most widely used as a carbohydrate source in *in vitro* culture of various plants, either maltose alone or in combination with sorbitol was more effective for both callus induction and regeneration of rice (Cho *et al.*, 2004a; Raina and Irfan, 1998) and barley (Scott and Lyne, 1994). Sorbitol is a major factor for embryogenic callus formation in the monocot plant such as maize (Swedlund and Locy, 1993), proliferation and regeneration of rice callus were suppressed by supplementation of sorbitol as a sole carbohydrate source (Yang *et al.*, 1999). Additionally, the types and concentrations of cytokinins combined with NAA have been considered as important factors on shoot regeneration of both indica (Xue and Earle, 1995) and japonica rice (Lee *et al.*, 2002).

In our previous study, however, the regeneration efficiencies of Korean rice cultivars were significantly improved by supplementation of 3% (w/v) sorbitol in combination with 2% (w/v) sucrose (or maltose) instead of 3% (w/v) sucrose alone under 5 mgL^{-1} kinetin and 0.5 mgL^{-1} NAA (Cho *et al.*, 2004a).

Although Dekeyser *et al.* (1989) reported that proline influenced the selection efficiency of PPT and regeneration from the putative transgenic calli, transformation efficiencies were significantly improved with the medium containing 500 mgL^{-1} proline in the presence of 3 mgL^{-1} PPT with low escapes (Table 2). *Bar gene* was stably expressed in 62 out of 63 (98.4%) transgenic plants regenerated from the PPT resistant calli on MSRK5SS-Pr in the presence of PPT. Non-transgenic control plants became withered and eventually whole plants died by spraying 0.3% (v/v) basta herbicide solution, but transgenic plants were not (Fig. 2E).

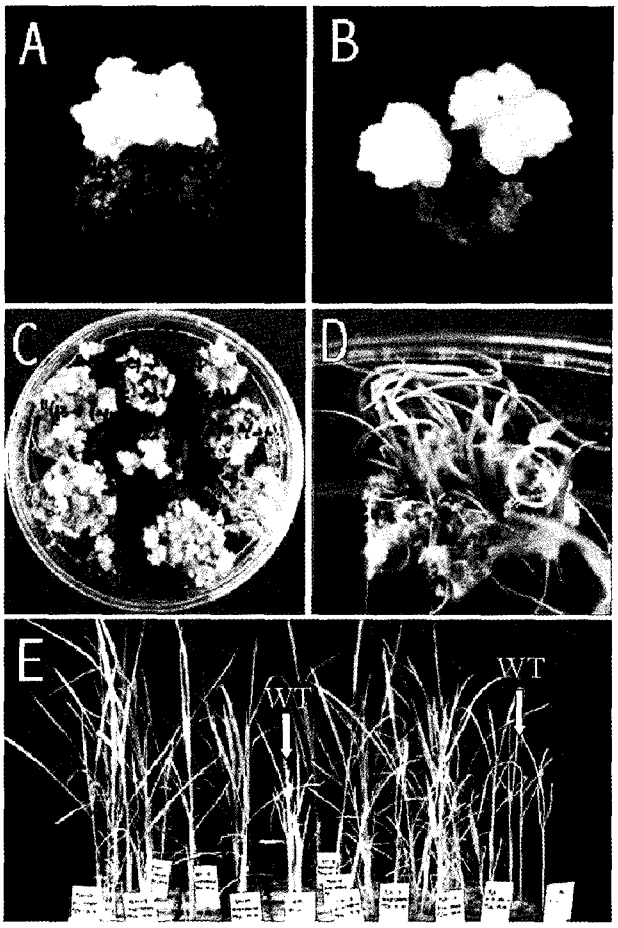

Fig. 2. Procedures of *Agrobacterium*-mediated transformation of rice.
A and B: L-phosphinotricine (PPT) resistant calli on selection medium, C: Shoot regeneration from transformed calli on MS basal regeneration medium containing 3mgL^{-1} PPT, D: Multiple shoots regenerated from one independent PPT resistant callus, E: 0.3% basta herbicide assay of transgenic plant (T_0). Arrows indicate nontransgenic wild type plant of rice as negative control.

FISH, PCR, and Southern analysis

Transgenes integrated in the metaphase chromosome

Fig. 3. FISH analysis using meiotic metaphase chromosomes of transgenic plant.
Fluorescence *in situ* hybridization with the 45S rDNA and pSBM-PPGN diakinesis in the meiotic prophase I in transgenic rice plant. The arrow indicates the transgene (pSBM-PPGN) in chromosome and the arrowheads indicate 45S rDNA in chromosome 9 in transgenic plant of rice.

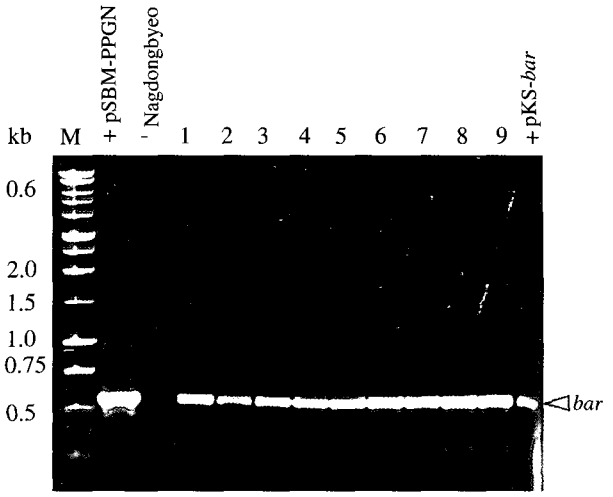

Fig. 4. PCR amplification of the *bar* gene in transgenic plants of Nagdongbyeo.
For *bar* gene detection, pSBM-PPGN and pKS-*bar* were used as positive control and genomic DNA of non-transgenic plant of Nagdongbyeo was used as negative control, respectively. M : 1 kb ladder, Numbers (1, 2, 3, and etc) : T_0 plant lines of Nagdongbyeo. Arrow indicated the expected size of *bar* gene.

of the transgenic plants was observed by Fluorescence *in situ* hybridization (FISH) analysis using pSBM-PPGN as a probe (Fig. 3). While the copy number and

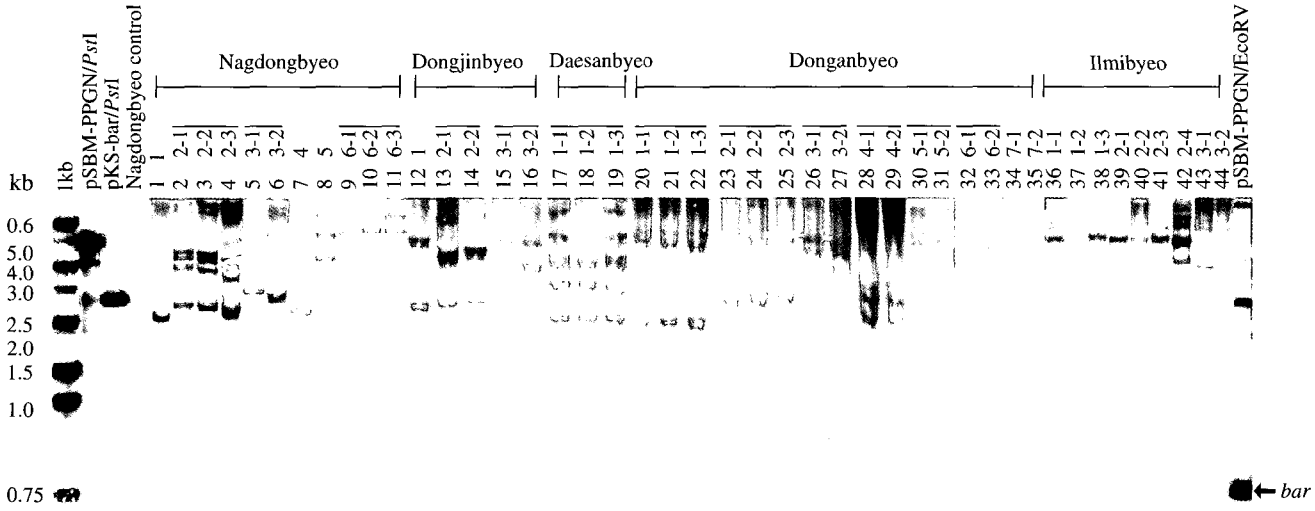

Fig. 5. Southern analysis digested with *Pst*I and detected by ECL labeled bar gene to identify the copy number of integrated gene in each transgenic plants.
pSBM-PPGN digested with *Pst*I (left side) and *Eco*RV (right end) were used as positive control for bar gene and genomic DNA of non-transgenic plant of Nagdongbyeo was used as negative control. 1 kb: size marker, Lower numbers (1, 2, 3, and etc.) indicates the plant number in each lane and uppers (1, 2-1, 2-2, and etc.) were independent transgenic callus line for each plant. The arrow indicates *bar* gene.

structural information of stably inherited transgenes are traditionally analyzed by Southern blot analysis, the location of the transgenes could be determined at the chromosome level by FISH analysis.

PCR was conducted using genomic DNA extracted from transgenic rices of Nagdongbyeo. As expected, 0.6 kb bands which is desirable size of *bar* gene (Fig. 1) were successfully amplified from the genomic DNA of transgenic plants of rice together with both two positive controls, pSBM-PPGN and pKS-*bar*. However, no band was observed on non-transgenic control (Fig. 4).

To identify the copy numbers of T-DNA integrated in genomic DNA in each transgenic plants obtained from various independent callus line, the genomic DNA was digested with *Pst*I that cleaved the unique site within T-DNA in the pSBM-PPGN (Fig. 1). Since *Eco*RV digestion produces the same sizes of transgene segments of *bar* gene which stacks same location on the gel and shows single band, it is very hard to identify whether transformants obtained from one callus line have same genetic background representing copy number and its fractionized band pattern (Cho *et al.*, 2004b). However, diverse bands in size were produced by *Pst*I digestion with various transgenic plants (Fig. 5). The number of hybridizing bands produced by the restriction enzyme which cleave the unique site within T-DNA reflected the number of copies of integrated genes in the plants unless multiple copies of the T-DNA repeats had been integrated (Hiei *et al.* 1994). All of the represented bands showed more than 1.62 kb, which is the minimum size of hybridizing fragments expected from the map of pSBM-PPGN (Figs. 1 and 5).

Neverthless, similar genetic insertion was shown among the plants regenerated from one callus line (Fig. 5). Total 39 out of 44 (88%) transgenic plants regenerated from 20 independent callus lines showed the same copy numbers with the same fractionized band patterns within the plants obtained from one callus as shown in lane 2-4 and 5-6 in Fig 5. These seemed to be due to that the plants were regenerated from one putative transgenic callus line transformed by one independent transformation event (Fig. 2A). However, in some cases, two or more PPT resistant calli were grown from different location in one callus (Fig. 2B). In this case, even though transgenic plants were obtained from one callus, these plants showed genetically different patterns as shown in Fig. 4 (lane 9-11). This seemed to be that independent transformation events were occurred in different cells of the one callus, consequently, different transformants were independently differentiated from these cells.

Many reports demonstrated the transformation efficiencies based on the number of plants obtained to the number of the calli transformed (Datta *et al.*, 2000; Lee *et al.*, 1999). However, it is possible that the transgenic plants regenerated from one callus line might be genetically similar, in turn, same copy numbers with resemblance of fractionized band pattern. Therefore, improvement of transformation efficiencies in independent event could be very important for producing transgenic plant.

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