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Improvement of Transformation Efficiencies using *Agrobacterium*-Mediated Transformation of Korean Rice

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ABSTRACT: A reproducible transformation system via optimized regeneration media for Korean rice cultivars was established using *Agrobacterium tumefeciens* LBA4404 (pSBM-PPGN; *gusA* and *bar*). Although japonica rice genotypes were easier to produce transgenic plants compared to Tongil type cultivars, transformation efficiencies were not always correlated with regeneration efficicienies of non-transgenic callus on the control medium. Regeneration efficiencies of Donganbyeo, Ilmibyeo, and Manchubyeo were over 50% in non-transgenic control, however, transformation efficiencies were significantly low when only sucrose was added to the media as a carbon source. However, the medium, MSRK5SS-Pr (or MSRK5SM-Pr), that contains 5 mgL^{-1} kinetin, 0.5 mg L^{-1} NAA, 2% sucrose (or maltose), 3% sorbitol, and 500 mgL^{-1} proline, was the most efficient not only for regeneration of non-transgenic callus but also for regeneration of transgenic callus in the presence of L-phosphinotricin (PPT). Average transformation efficincies of 16 Korean rice cultivars were significantly enhanced by using the optimized medium from 1.5% to 5.8% in independent callus lines and from 2.9% to 19.4% in tromsgenic plants obained. Approximately 98.9% (876 out of 885) transgenic plants obtained on optimized media showed basta resistance. Stable integration, inheritance and expression of *gusA* and *bar* genes were confirmed by GUS assay and PCR and Southern analysis of the *bar* gene. With *Pst*1 digestion of genomic DNA of transgenic plants, one to five copies of T-DNA segment were observed; however, 76% (19 out of 25 transgenic plants) has low copy number of T-DNA. The transformants obtained from one callus line showed the same copy numbers with the same fractionized band patterns.

Keywords: transformation efficiency, *Agrobacterium*-mediated transformation, rice, embryogenic callus, regeneration medium

Rice (*Oryza sativa* L.) is one of the most important cereal crops and consumed as a staple food. Various biological and abiological transformation systems have been applied for genetic transformation of rice to enhance agronomically valuable traits. Aside from the choice of gene delivering system, employment of regenerable tissues as target explants is an important factor for producing the transgenic plant. A decade ago, abiological methods, PEG (Datta *et al.*, 1990) or electrophoration (Toriyama *et al.*, 1988) to the protoplast and biolistics (Christou *et al.*, 1991) to immature embryo, were applied for rice transformation because of species independence in utilization of the gene deliverying systems. And, then, embryogenic calli derived from mature seed was first employed in biolistics method by Li *et al.*, (1993).

Although the *Agrobacterium*-mediated transformation method has many advantages over abiological method, rice has been precluded in application of the system until early 90's, since this bacterium is only known as parasite to dicotyledonous plants. However, transgenic rice were successfully achieved with various tissues, especially scutella-derived embryogenic callus, of japonica rice by employment of acetosyringone, which activates *vir* genes of *Agrobacterium* to target explants, in *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994). Currently, this system is considered as one of the most reliable methods for rice transformation because of economical efficiency, manipulative effectiveness, and stable integration and expression of transgenes in host plant with low copy (Ke *et al.* 2001).

For obtaining transgenic plants, the transgenes were successfully integrated into the target explants and consequently putative transgenic calli were regenerated in high efficiencies. Thus, a number of studies have been focused on enhancing virulence of *Agrobacterium* to target explants with various *Agrobacterium* strains (Datta *et al.*, 2000), target explants (Hiei *et al.*, 1994), vector systems altering *vir* genes (Ke *et al.*, 2001), length of T-DNA (Park *et al.*, 2000), and optimized concentration of acetosyringone (Seo *et al.*, 2002). 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 & Hodges, 1996; Hashizume *et al.*, 1999; Lee *et al.*, 1999). These are mainly due to regeneration ability of scutella-derived calli of rice varied depending on the genotypes of cultivars (Cho *et al.*, 2003). Also, regeneration efficiency in each cultivar was severely influenced by *in vitro* culture medium (Cho *et al.*, 2004). Although it has been generally known that

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japonica rice is more regenerative than indica rice, regeneration efficiencies of Korean rice cultivars critically varied even within japonica types (Cho *et al.*, 2003) and were significantly improved by selected utilization of the optimized regeneration medium for each cultivar (Cho *et al.*, 2004).

The conventional breeding in combination with genetic transformation could be used for introducing target gene into recalcitrant cultivar, however, direct transformation to elite cultivar is very ideal. Therefore, efficient regeneration system has been a prerequisite for producing fertile transformed plants thereby developing the new cultivars. Although hygromycine has been widely used as a selection agent, PPT known as a glutamin synthetase inhibitor is also used to select putative transgenic callus transformed with *bar* gene because consumers are concerned the safety of genetic modified food using antibiotics resistant genes as a selection marker.

The present study was conducted with two objectives as follows; (1) for producing basta herbicide resistant rice and (2) for improving transformation efficiencies in independent events with various rice cultivars via optimized regeneration medium for each cultivar supplemented with different carbon sources in combination with different concentrations of kinetin.

MATERIALS AND METHODS

Agrobacterium strain and vector

Agrobacterium tumefeciens strain LBA4404 carrying the plasmid pSBM-PPGN including *gusA* and *bar* gene was kindly supplied by Dr. M.H. Lim, National Institue of Agricultural Biotechnology (NAIST), Rural Development Administration (RDA). As for pSBM-PPGN, *gusA* and *bar* genes were horbored in pSB11 as shown in Fig. 1. Stress inducible PAL (penylalanine ammonialyase) promoter and pinII terminator were used for *gusA* gene and 35S promoter and Tnos terminator for *bar* gene, respectively. MAR (matrix attachment region) were included at both end of right and left border in the construction. *Agrobacterium* was cultured freshly on AB-ST medium at 28 in the dark for 2-3 days (Table 1).

Plant materials and media

Compositions of all media used for the procedures were described in Table 1. Sixteen accessions; thirteen japonica rice cultivars include four color rice, two Tongil type rice,

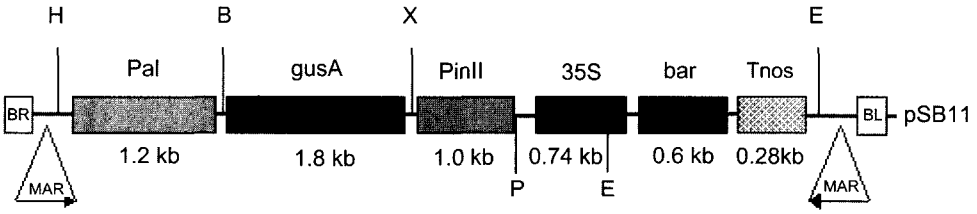

Fig. 1. Schematic map of pSBM-PPGN. H : *Hind*III, B : *Bam*HI, X : *Xue*I, P : *Pst*I, and E : *Eco*RV.

Table 1. Compositions of the media for *Agrobacterium*-mediated transformation of scutella derived calli of rice.

Selection trait	Medium	Composition
Callus induction and proliferation	2N6	Modified N6† salts and vitamins, 300 mgL^{-1} casamino acid, 500 mgL^{-1} proline, 500 mgL^{-1} glutamin, 30 gL^{-1} sucrose, 2 mgL^{-1} 2,4-D, 2.5 gL^{-1} phytagel, pH 5.8
Agrobacterium culture	AB-ST	AB‡ buffer and salt, 5 gL^{-1} glucose, 15 gL^{-1} Bacto-agar, 10 mgL^{-1} spectinomycine, 50 mgL^{-1} tetracycline, pH 7.2
Agrobacterium suspension	AAM	AA§ macro and micro salt, Amino acid stock, MS vitamines, 500 mgL^{-1} casamino acid, 68.5 gL^{-1} sucrose, 36 gL^{-1} glucose, 100 uML^{-1} (19.6 mgL^{-1}) acetosyringone, pH 5.2
Co-cultivation	2N6-AS	2N6 medium supplemented with 100 uML^{-1} (19.6 mgL^{-1}) acetosyringone, pH 5.2
Transgenic callus selection	2N6-CP	2N6 medium supplemented with 250 mgL^{-1} cefotaxime, 6 mgL^{-1} L-phosphinotricine (PPT)
Shoot regeneration#	MSRK2S	MS¶ salts and vitamins, 0.5 mgL^{-1} NAA, 2 mgL^{-1} kinetine, 30 gL^{-1} sucrose, phytagel 4 g L^{-1}, pH 5.8
	MSRK2M	Sucrose in MSRK2S was substituted with maltose
	MSRK10M	Sucrose and 2 mg L^{-1} kinetin in MSRK2S was substituted with maltose 2 mg L^{-1}kinetin
	MSRK5SS-Pr	MS salts and vitamins, 20 gL^{-1} sucrose (or maltose), 30 gL^{-1} sorbitol, 5 mgL^{-1} kinetin, 0.5 mgL^{-1} NAA, 500 mgL^{-1} proline, 4 gL^{-1} phytagel, pH 5.8
	MSRK5SM-Pr	Sucrose in MSRK5SS-Pr was substituted with maltose
Root induction	MS0	MS salts and vitamins, sucrose 30 gL^{-1}, 4 gL^{-1} phytagel, pH 5.8

†Chu *et al.*, 1975 with minor modifications; $Na_2MoO_4 \cdot 2H_2O$ (0.25 mgL^{-1}), $CuSO_4 \cdot 5H_2O$ (0.025 mgL^{-1}) and $CoCl_2 \cdot 6H_2O$ (0.025 mgL^{-1}) were added to N6 micro salts and 100 mgL^{-1} myo-inositol was added to N6 vitamins, respectively.
‡Chilton *et al.*, 1974; §Hiei *et al.*, 1994; ¶Murashinge & Skoog, 1962.
MSRK2S was used as control medium for shoot regeneration and all media were supplemented with 250 mgL^{-1} cefotaxime and 3 mgL^{-1} PPT for regeneration from transformed calli.

one japonica × Tongil hybrid line were used for the experiment as shown in Table 2.

Production of target explants and transformation

Sterilization of the mature hulled rice seeds and embryogenic callus induction were followed by Cho *et al.* (2004). Yellow-compact type of embryogenic callus (1-2 mm in diameter) was selected from scutella-derived callus induced on 2N6 medium (Table 1) and sub-cultured for 4 days under the same conditions as callus induction prior to *Agrobacterium* infection. Embryogenic calli were immersed in *Agro-*

Table 2. Comparison of transformation efficiencies of various Korean cultivars.

	Cultivars	No. of calli transformed (A)	No. of calli regenerated (B)	No. of plants produced (C)	No. (or %) of basta® plant		TEC† (B/A,%)	TEP‡ (C/A,%)	REC§(%) of non-transgenic calli		
Japonica	Daesanbyeo	692	25	37	35	(94.6)	3.6	5.3	MSRK2S	7.9	
		260	22	84	84	(100)	8.5	32.3	MSRK5SM-pr	52.6	
	Donganbyeo	125	0	0	0	-	0.0	0.0	MSRK2S	71.9	
		160	14	50	49	(98.0)	8.8	31.3	MSRK5SM-pr	90.9	
	Dongjinbyeo	250	6	23	21	(91.3)	2.4	9.2	MSRK2S	18.3	
		320	21	61	61	(100)	6.6	19.1	MSRK5SS-pr	61.1	
	Ilmibyeo	800	7	13	12	(91.6)	0.9	1.6	MSRK2S	68.9	
		230	30	109	109	(100)	13.0	47.4	MSRK5SS-pr	94.3	
	Ilpumbyeo	350	1	2	2	(100)	0.3	0.6	MSRK2S	11.8	
		300	4	10	10	(100)	1.3	3.3	MSRK2M	9.8	nd¶
	Jongnambyeo	230	4	8	8	(100)	1.7	3.5	MSRK2S	65.2	
		220	3	18	18	(100)	1.4	8.2	MSRK5SS-pr	93.3	
	Manchubyeo	300	0	0	0	-	0.0	0.0	MSRK2S	79.2	
		255	15	33	31	(93.9)	5.9	12.9	MSRK2M	90.0	
	Nagdongbyeo	494	18	36	35	(97.2)	3.6	7.3	MSRK2S	67.4	
		460	28	88	87	(98.9)	6.1	19.1	MSRK5SM-pr	96.5	
	Shindongjinbyeo	995	1	1	1	(100)	0.1	0.1	MSRK2S	10.0	
		125	1	4	4	(100)	0.8	3.2	MSRK10M	21.3	
(Color Rice)	Heugnambyeo	875	24	32	32	(100)	2.7	3.7	MSRK2S	25.0	
		420	39	104	104	(100)	9.3	24.8	MSRK5SM-pr	47.8	
	Sanghaehanghyulla	230	7	26	26	(100)	3.0	11.3	MSRK2S	26.7	
		410	40	146	143	(97.9)	9.8	35.6	MSRK5SM-pr	80.0	
	Suwon477	150	0	0	0	-	0.0	0.0	MSRK2S	10.0	
		177	11	46	46	(100)	6.2	26.0	MSRK5SS-pr	73.3	
	SR19685-5-3-1-1-1	200	0	0	0	-	0.0	0.0	MSRK2S	40.0	
		240	13	84	84	(100)	5.4	35.0	MSRK5SS-pr	95.0	
Tongil	Andabyeo	300	0	0	0	-	0.0	0.0	MSRK2S	0.0	
		430	0	0	0	-	0.0	0.0	MSRK10M	10.0	
	Dasanbyeo	350	0	0	0	-	0.0	0.0	MSRK2S	13.6	
		330	0	0	0	-	0.0	0.0	MSRK5SM-pr	17.8	nd
J×T #	SC line	370	8	15	15	(100)	2.2	4.1	MSRK2S	65.7	
		420	23	48	46	(95.8)	5.5	11.4	MSRK5SM-pr	87.0	
	Sub total	6711	101	193	187	(96.9%)	1.5	2.9	MSRK2S control medium		
		4557	264	885	876	(98.9%)	5.8	19.4	Optimized media		
	Total	11268	365	1078	1063	(98.6%)	3.2	9.6			

†TEC: transformation efficiency calculated based on the number callus
‡TEP: transformation efficiency calculated based on the number plant
§REC : Regeneration efficiencies calculated based on the number non-transgenic callus
¶nd ; not significantly different compared to control
#J×T ; Somatic culture line from japonica (Milyang 23)/indica (Gihobyeo) hybrid

bacterium suspension (OD_{600}=1.7) diluted with liquid AAM medium containing 100 mM acetosyringone by gentle agitation for 10 minutes and then co-cultivated on 2N6-AS medium containing 100 mM acetosyringone at 25°C in dark for 3 days. To eliminate overgrown *Agrobacterium*, the callus was washed thoroughly in sterilized water containing 250 mgL^{-1} cefotaixme and dried between two sheets of sterilized filter papers in a petri-dish with shaking. The putative transgenic callus were selected twice by culturing on 2N6-CP selection medium containing 250 mgL^{-1} cefotaxime and 6 mgL^{-1} PPT at 27°C for two weeks in the dark.

Regeneration of transgenic plants

The calli survived on 2N6-CP selection medium were cultured on MS basal regeneration medium containing 250 mgL^{-1} cefotaxime and 3 mgL^{-1} PPT at 25 under the light (16 hours of day length) by refreshing the medium every 2 weeks. MSRK2S-CP containing 3% (w/v) sucrose and 2 mgL^{-1} kinetin was used as a control medium for screening transformation efficiencies of the rice cultivars. For improving transformation efficiency in each cultivar or line, various MS basal regeneration media that optimized with different concentrations of kinetin (2, 5, or 10 mgL^{-1}) and various carbon sources such as sucrose (or maltose) alone or in combination with sorbitol were selectively used instead of MSRK2S-CP (Table 2). Especially, MSRK5SS-Pr (or MSRK5SM-Pr) was prepared with MS basal medium supplemented with 5 mgL^{-1} kinetin, 0.5 mgL^{-1} NAA, 500 mgL^{-1} proline, 4 gL^{-1} phytagel. As a carbon source, 3% (w/v) sorbitol combined with 2% (w/v) sucrose (or maltose) were employed for the medium. The roots of regenerated plants were induced on the hormone free MS basal medium (MS0) and the plants were acclimatized in 0.1% hyponex solution (pH 5.4) at 25°C prior to transplanting to pot in the greenhouse.

Transformation efficiencies were calculated based on two aspects; one is the transformation efficiency based on callus number (TEC (%)=Number of calli regenerated/number of calli independently inoculated×100) and the other is the transformation efficiency based on plant number (TEP (%)=Number of plants obtained/number of calli independently inoculated×100).

Basta and GUS assay

T0 plants regenerated in the presence of 3 mgL^{-1} PPT and fourteen days old T1 seedlings were sprayed twice with 0.3% (v/v) basta herbicide solution from the top with three days interval. Ten days after treatment, basta herbicide resistance of T0 plants and T1 seedlings were compared with non-transgenic control plants.

Expressions of GUS in transgenic rice tissues were assayed according to the method described by Jefferson *et al.* (1987). The PPT resistant callus, leaf segment of basta resistant T0 plants, and T1 seedlings were used for GUS assay. Each tissue was placed under the UV-light for 30 minutes prior to incubation in GUS assay buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.05% (w/v) 5-bromo-4-chloro-3-indoyl-β-D-Glucuronic acid, 0.06% (v/v) Triton X-100, 0.2 M sodium phosphate, pH 7.0) at 37°C overnight. For chlorophyll extraction, all tissues were incubated in ethanol : glacial acetic acid (3 : 1) solution for 1-2 hours at 70°C with exchanging solutions.

PCR and Southern analysis

Total genomic DNA was extracted from the leaves of the basta herbicide resistant plants by the cetymethyl-ammonium bromide (CTAB) method according to Murray & Thompson (1980) with minor modification. PCR was conducted with two sets of primers for *bar* gene, 5'-ATGAGCCCAGAACGAC-3' (Forward) and 3'-TCAGATCTCGGTGACGG-5' (Reverse). The amplification reaction was conducted as follows: predenaturation at 94 for 4 minutes, 30 cycles of denaturation at 94 for 60 seconds, anealing at 65°C for 40 seconds, extension at 72°C for 60 seconds, and the last extension at 72°C for 5 minutes.

For Southern analysis, a total of 5 μg of genomic DNA of each plant was digested with 30 units of *Eco*RV or *Pst*I for 12 hours followed by electrophoresis on 0.8% (w/v) agarose gel. 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 Co.). The membrane bound DNA was hybridized and detected with ECL labeled *bar* gene probe.

RESULTS

Production of transgenic plant

The primary embryogenic callus induced from mature seed was used for *Agrobacterium*-mediated transformation of various rice cultivars (Fig. 2A and B, Table 2). After 3-days of co-cultivation, most of the calli transferred to selection medium containing 6 mg^{-1} PPT grew slowly and turned brown during the first selection for two weeks. However, from the third week, the calli that seemed to be PPT resistant grew on the surface of the brown calli on the new selection medium (Fig. 2C).

Shoot regeneration from PPT resistant callus on regeneration media containing 3 mgL^{-1} PPT was rather slow com-

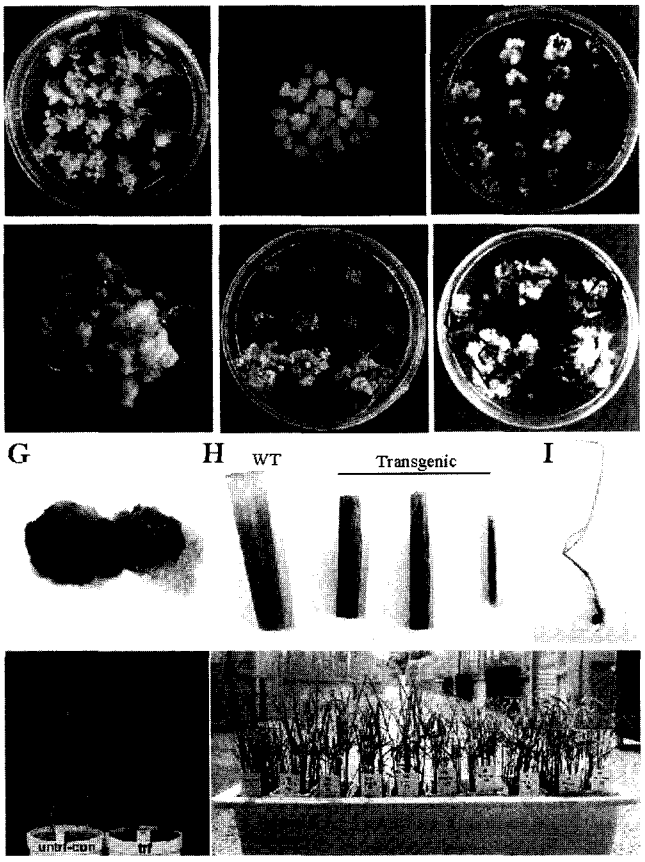

Fig. 2. Procedures of *Agrobacterium*-mediated transformations in japonica rice. A: callus induction at 4 weeks, B: embryogenic calli for the transformation, C: L-phosphinotricine (PPT) resistant calli on selection medium, D: Green tissue formation from PPT resistant calli, E and F: Shoot regeneration from transformed calli on MS basal regeneration medium containing 3mgL^{-1} PPT, G, H, and I: GUS expression of the transformed calli, leaves of T0 transgenic plant, and seedling of T1, respectively, J and K: 0.3% basta herbicide assay (WT: wild type non-transgenic control (left) and T0 transgenic plant (right), and T1 seedlings of transgenic plant. Arrows indicate non transgenic wild type plant of rice as control.

pared to that from non-transgenic callus on the same medium without PPT. In general, green tissue were observed after three weeks of cultivation on the medium containing 3 mgL^{-1} PPT (Fig. 2D) followed by shoots were initiated from the PPT resistant callus during the fourth week (Fig. 2E). On the control medium, 193 transgenic plants were regenerated from 101 independent callus lines in 10 out of 16 rice cultivars or lines tested within 14 weeks (Table 2).

Screening and improving transformation efficiencies

Aside from the regeneration efficiencies of non-transgenic calli, transformation efficiencies in independent events varied depending on the genetic background of the cultivar that ranged from 0-3.6% on the control medium. Japonica rice cultivars were easier to regenerate, but Tongil types were harder (Table 2). Nevertheless, transformation efficiencies were not always correlated with regeneration efficicienies of non-transgenic callus. Nagdongbyeo was the most widely used cultivar for rice transformation because of high regeneration ability. However, it is difficult to obtain the transgenic plants from Donganbyeo, Ilmibyeo, Jongnambyeo and Manchubyeo, even though they were showing over 50% regeneration efficiencies in non-transgenic control. Medium level of regeneration ability ranging from 7.9% to 25.0% was shown in Dasanbyeo, Dongjinbyeo, and Heugnambyeo; however, the transformation efficiencies of these cultivars were comparable to that of Nagdongbyeo. Ilpumbyeo, Shindongjinbyeo, and Suwon 477 (color rice) in japonica cultivars were not easy to get transformants on the control medium and regeneration of putative transgenic calli were similar to Tongil rice rather than japonica rice.

It was shown that regeneration efficienies in most of the cultivars could be improved by selected choice of the medium supplemented with maltose alone or sucrose in combination with sorbitol. With the media, transformation efficincy based on independent callus lines and plants produced were increased to 5.8% and 19.4% on average. Especially, transformation efficiencies based on both number of the callus and plants in Donganbyeo, Ilmibyeo, and Manchubyeo were dramatically enhanced to 8.8-31.3%, 13.0-47.4%, and 5.9-12.9%, respectively. However, since transformation efficiencies in some cultivars were decisively determined by the regeneration ability, the efficiencies were critically low in Ilpumbyeo (1.3-3.3%) and Shindongjinbyeo (0.8-3.2%) in japonica types. And, furthermore, no transformants could be obtained from all Tongil types with all media used.

Basta assay

To identify the stable integration and inheritance of *bar* gene, 0.3% (v/v) basta herbicide solution was sprayed on T0 and T1 plants. Three days after treatment, leaves of the non-transgenic control plants became withered and eventually whole plants were dead after ten days, but transgenic plants survived (Fig. 2J and K). Approximately 98.6 % T0 plants regenerated from the PPT resistant calli showed basta resistance with some exceptions (Table 2). Furthermore, 14 days old T1 seedlings also showed basta herbicide resistance (Fig. 4) but the segregation ratio varied depending on the independent plant lines (data not shown).

GUS expression

To confirm the stable expression of *gusA* gene, GUS activ-

ity on PPT resistant calli, leaf and stem segments of T0 plants, and T1 seedlings were compared to those of non-transgenic plants (Fig. 2G, H, and I). *GusA* gene was expressed in all the tissues tested. Although non-transgenic leaf segment did not show GUS expressions (Fig. 2H), independent transgenic calli selected on 2N6-CP medium and PPT resistant plants of both T0 and T1 showed GUS staining.

PCR and Southern analysis

PCR analysis of genomic DNA extracted from several transgenic rice plants obtained from Nagdongbyeo, Dongjinbyeo, and Daesanbyeo amplified a 0.6 kb band which was the size of the *bar* gene (Fig. 1) as amplified in the positive controls (Fig. 3). However, no band was observed in non-transgenic control. To confirm the successful integration of T-DNA, genomic DNA was digested with *Eco*RV and *Pst*I. As shown in Fig. 1, restriction sites of *Eco*RV were located on both right and left side of *bar* gene, however, *Pst*I cleaved at unique site within T-DNA. We confirmed the *bar* gene integration through hybridization of 0.6 kb band with *bar* gene probe by *Eco*RV digestion of genomic DNA (Fig. 4). However, it was very hard to identity if the homogeneity exists among the transgenic plant lines. Since the T-DNA of pSBM-PPGN has a single *Pst*I site (Fig. 1), the number of hybridizing bands reflected the number of copies of integrated genes in the plants unless multiple copies of the T-DNA repeats had been integrated. All of the detected bands represented fragments of more than 0.6 kb, which was the minimum size of hybridizing fragments expected from the map of pSBM-PPGN (Fig. 1 and 5). Although one to five copies of T-DNA segment were observed, low copy numbers showing one or two bands were identified in total 19 out of 25 transgenic plants (76%). Transgenic plants showing higher copy number had diverse band sizes; the transgenic plants regenerated from one callus showed same copy number with same fractionized band patterns (Fig. 5).

DISCUSSION

Introduction of target genes into the genome of elite cultivars and regeneration of transgenic plants with high efficiency are the most desirable strategies for production of transgenic plants in rice. However, many reports demonstrated that regeneration from putative transgenic callus

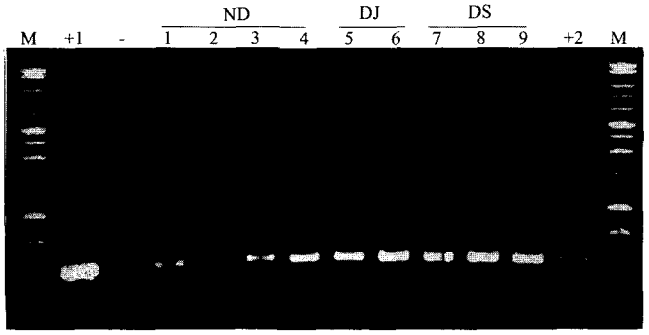

Fig. 3. PCR amplification of the *bar* gene in transgenic plants. Lanes +1 and +2: positive control of plasmid pSBM-PPGN and pKS-*bar*, respectively, -: wild type of non-transgenic rice control, Numbers 1-9: T0 transgenic plant lines, ND (1-4), DJ (5-6), DS (7-9): Nagdongbyeo, Dongjinbyeo, and Daesanbyeo, respectively. M=1 kb ladder.

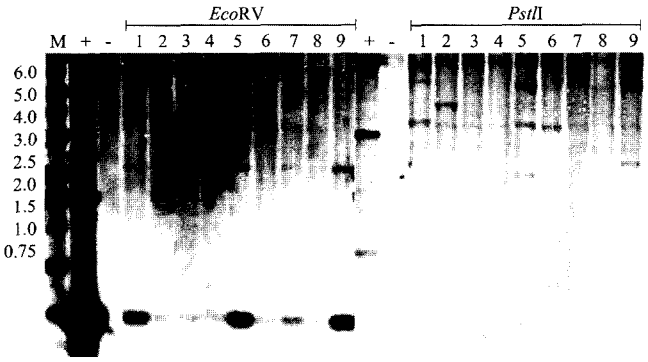

Fig. 4. Southern analysis confirming the presence of the *bar* gene in transgenic plants of Nagdongbyeo detected by ECL labeled *bar* probe. Lanes + and -: pSBM-PPGN and non-transgenic control, respectively, Numbers (1-9): lines of the transgenic plants of Nagdongbyeo. M=1 kb ladder.

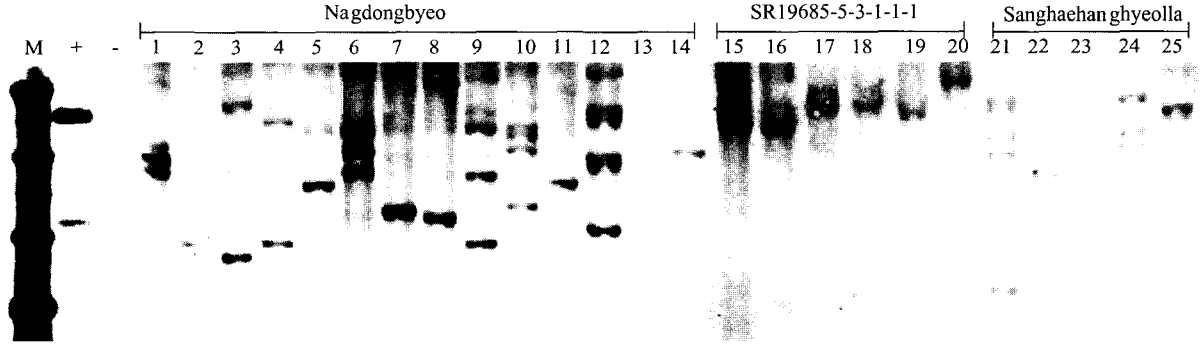

Fig. 5. Southern analysis digested with *Pst*I and detected by ECL labeled *bar* gene probe to identify the copy number of integrated gene in each transgenic plants. Lanes + and -: pSBM-PPGN and non-transgenic control, respectively. Numbers 1-14, 15-20 an 21-25 are transgenic plants of Nagdongbyeo, SR19685-5-3-1-1-1, and Sanghaehanghyella, respectively. M=1 kb ladder.

were critically lower than expected due to cultivars that showed different regeneration efficiencies depending on the media. Since reliable tissue culture system is limited for different rice cultivars, our basic strategy was to improve transformation efficiency at desirable level in an optimized culture system for each cultivar that would lead to desirable efficiencies of shoot regeneration to achieve high transformation efficiencies.

Of the various factors influencing transformation efficiencies, embryogenic callus served as the starting material and its efficient regeneration system have been most important consideration for both *Agrobacterium*-mediated (Aldemita & Hodges, 1996; Hashizume *et al.*, 1999; Hiei *et al.*, 1994; Lee et al 1999) and biolistics transformation. (Li *et al.*,1993).

Although sucrose has been most widely used as an energy source or osmotic regulator in *in vitro* culture of plants, either maltose alone or in combination with sorbitol are more effective for both callus induction and regeneration of rice (Raina & Irfan, 1998) and barley (Scott & Lyne, 1994). While sucrose was rapidly hydrolized to glucose and toxic products and accumulated within the plant cell, no toxic material was observed in the cell by supplementation of maltose. Furthermore, degradation and metabolism of maltose is slower than sucrose thereby sufficient oxygen allows cells to survive (Scott *et al.*, 1995) and could sustain the osmotic potential to the media (Raina & Irfan, 1998).

In our previous results, regeneration efficiencies of Korean rice cultivars were significantly improved by supplementation of maltose instead of sucrose, however, the medium containing 5 mgL^{-1} kinetin, 3% (w/v) sorbitol combined with 2% (w/v) sucrose (or maltose) was most effective on shoot regeneration (Cho *et al.*, 2004). Especially, this medium was very favorable to Daesanbyeo and Dongjinbyeo that showed medium level of regeneration ability and were severely influenced by type of the medium in shoot regeneration rather than other cultivars. Beneficial effects of optimized medium were reducing callus browning while stimulating green tissue formation and shoot regeneration. Although superiority of maltose to sucrose was observed in shoot regeneration of non-transgenic callus, but there was no significant difference between the efficiencies on two different media, MSRK5SS-Pr and MSRK5SM-Pr. It seems sorbitol acts as an osmotic regulator since shoot regeneration was suppressed when sorbitol was used as a sole carbon source in the media (Yang *et al.*, 1999).

Affirmative effect of proline on shoot regeneration from embryogenic callus of was reported (Yang *et al.*, 1999). However, because L-phosphinotricine (PPT) inhibits the glutamin synthetase as a selection agent while callus was proliferated and regenerated, supplementation of amino acids such as proline influences the selection effect of PPT (Dekeyser *et al.*, 1989). Although PPT in the medium suppress the regeneration of non-transformed calli, transgenic plants were successfully regenerated in this study with high efficiencies from PPT resistant callus on MSRK5SS-pr (or MSRK5SM-pr) containing 500 mgL^{-1} proline and 3 mgL^{-1} PPT with low escapes (Table 2).

Even though practical improvement of transformation efficiencies of the japonica rice was achieved with optimized regeneration media, recalcitrance to shoot regeneration in all Tongil rices, Ilpumbyeo and Shindongjinbyeo in japonica rice is still remained as a limiting factor to obtain transgenic plant. In addition, it was very hard to obtain embryogenic calli from the cultivars (Cho *et al.*, 2004). Thus, further studies should be needed to overcome this problem.

We have identified the copy numbers of T-DNA integrated in the genomic DNA of transgenic plants. Obtaining transgenic plants in independent event is very important to develop new transgenic cultivars. Many reports have demonstrated the transformation efficiencies based on the number of transgenic plants obtained (Datta *et al.*, 2000; Lee *et al.*, 1999; Seo *et al.*, 2002). However, it would be possible to regenerate transgenic plants from one callus line that might be genetically closed to each other, in turn, same copy number. Thus, transformation efficiencies in independent event could be more reasonable.

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