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High-Frequency *Agrobacterium*-Mediated Genetic Transformation of Tongil Rice Varieties

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We investigated the frequency of callus induction from mature-seed scutella of 39 varieties of Tongil rice (*Oryza sativa* japonica x indica). These were divided into two groups according to condition of the callus at the time of induction. Members of the first group, which developed brown calli, were further classified into two types (B1 or B2), based on the extent of their color change and callus condition. Tissues in the second group developed yellow calli, with varieties being placed into one of three types (Y1, Y2, or Y3) according to solidity. Type-Y1 seeds generated suitably soft calli that were healthy and grew rapidly. These calli were transformed with binary vector pGA2722 via *Agrobacterium*-mediated co-cultivation. The vector contained the hygromycin-resistance selectable marker and a *gus* reporter gene with an intron that is functional in plant cells but not in *Agrobacterium*. Most varieties that formed brown calli failed to generate transformants. Likewise, several varieties with suitably soft calli also failed. Among the 16 transformable varieties, the final regeneration frequencies ranged from 1.0 to 10.7%. The highest frequency was achieved by the variety Tongil, which also had the most rapidly growing calli. DNA gel-blot analysis revealed one to three copies of the introduced *gus* gene.

Keywords: *Agrobacterium*, embryogenic callus, *gus* gene, Tongil rice, transformation

Rice (*Oryza sativa* L.) is one of the most essential staple crops, especially in developing countries (Anonymous, 1995). Now that its genome sequence has been determined (Goff et al., 2002; Yu et al., 2002), this species has become a more important research subject in plant science. One of the major goals is to develop a superior variety for increased quality and quantity. To achieve this, researchers must identify useful genes and examine their manifested patterns and interactions with other genes. These endeavors will require the development of effective transformation procedures.

Among the various methods reported (Raineri et al., 1990; Chan et al., 1992, 1993; Aldemita and Hodges, 1996; Toki, 1997; Cho et al., 1998; Ryu et al., 2001), the one presented by Hiei et al. (1994) is the most widely used for successful transformation of rice with *Agrobacterium*. However, their technique has been limited primarily to japonica rice. For indica varieties, *Agrobacterium*-mediated transformations have been described by Chan et al. (1992), Li et al. (1992), and Rashid et al. (1996). In addition, Mohanty et al. (1999) have been able to enhance transformation frequencies by varying the composition of the media. However, success with indica rice has been restricted to only certain varieties; most have been recalcitrant (Abe and Futsuhara, 1986; Bajaj and Rajam, 1995, 1996; Sohn et al., 1995; Hiei et al., 1997; Kwon and Sohn, 2000; Shoeb et al., 2001). Here, we describe the transformation of varieties of Tongil rice (i.e., hybrids between the japonica and indica varieties) that carry a number of valuable traits, including those for high yield and disease resistance. Successful transformation of this rice type would allow researchers to isolate and investigate those genes present in the indica varieties that have, to date, been difficult to transform.

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MATERIALS AND METHODS

Plant Materials and Culture Media

Seeds for 39 varieties of Tongil rice (*O. sativa* L.) were obtained from the Young-Nam Experimental Station, Rural Development Administration, Republic of Korea. All of the media components for rice tissue culture were as described by Lee et al. (1999) except

for the particular concentration of hygromycin used in culture (see below).

Vector Construction

The HindIII-SacI fragment of pIG121Hm, carrying the CaMV 35S promoter and the intron-containing *gus* gene, was connected to the nopaline synthase (*nos*) terminator, resulting in construction of pGA1725 (Fig. 1). The HindIII-ClaI fragment of pGA1725 was inserted into the multi-cloning sites of pGA1605 (Lee et al., 1999), thereby generating the binary vector pGA2722. Plasmid pGA2722 contains the hygromycin-resistance gene between the 35S promoter and the transcript 7 terminator as a selectable marker. In addition, this plasmid carries the *gus* reporter gene containing an intron, so that functional GUS protein cannot be synthesized in *Agrobacterium*.

Transformation and Regeneration

Mature seeds were manually husked and sterilized for 30 min in a solution containing 70% ethanol and 50% Clorox. The seeds were rinsed thoroughly with sterilized water, and cultured for four weeks at 27°C in darkness on a 2N6 medium [N6 medium containing 2 mg L^{-1} 2,4-D, 30 g L^{-1} sucrose, 1 g L^{-1} casamino acids, and 2 g L^{-1} Phytagel (Sigma); pH 5.6] (Lee et al., 1999). Embryogenic calli that were derived from the scutella (Fig. 2A) were then selected and subcultured on fresh 2N6 media for 4 to 7 d. Rapidly proliferating calli (yellow, 2 to 3 mm in diameter) were used for co-cultivation with *Agrobacterium* that carried binary vector pGA2722 and a helper-Ti plasmid pAL4404 (Hoekema et al., 1983). The calli (Fig. 2B) were co-cultured with *Agrobacterium* in darkness at 20°C for 3 d on a 2N6ASB medium (2N6 medium supplemented with 100 μM acetosyringone, 1 mM betaine, and 10 g L^{-1} sucrose; pH 5.2). These calli were then washed with sterilized water and cultured in darkness at 27°C on a 2N6CH35 medium (2N6 medium containing 250 mg L^{-1} cefotaxime, 35 mg L^{-1} hygromycin B, and 2 g L^{-1} Phytagel; pH 5.6). After four weeks of culture, actively-dividing calli were transferred onto a 2N6BA medium (supplemented with BA and 40 mg L^{-1} hygromycin), and were cultured for two weeks in darkness at 27°C (Fig. 2C). The proliferating hygromycin-resistant calli were then cultured on an MSR16 medium (25°C, 16-h photoperiod), where they formed shoots and roots (Fig. 2, D and E).

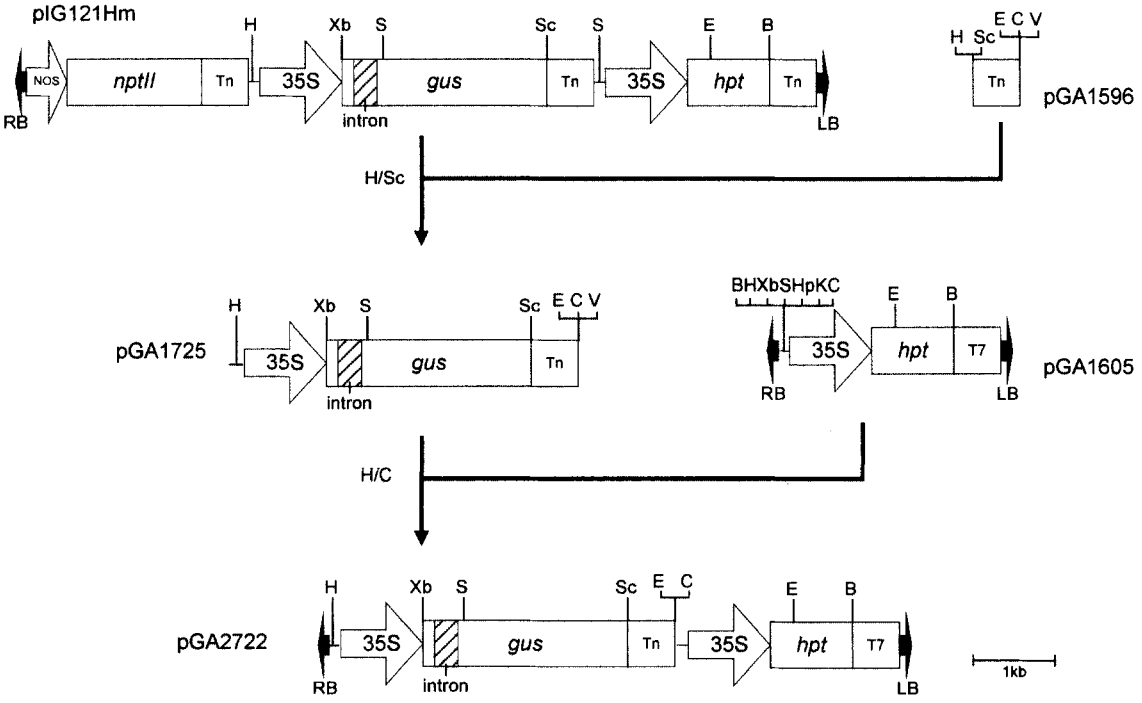

Figure 1. Schematic diagram for construction of the binary vector pGA2722. RB, right border; LB, left border; *nptII*, neomycin phosphotransferase; *gus*, β-glucuronidase; *hpt*, hygromycin phosphotransferase; NOS, nopaline synthase promoter; 35S, the CaMV 35S promoter; Tn, nopaline synthase terminator; T7, transcription termination region of Gene 7 of the pTiA6; B, BamHI, C, ClaI; E, EcoRI; H, HindIII; Hp, HpaI; K, KpnI; S, SalI; Sc, SacI; V, EcoRV; Xb, XbaI.

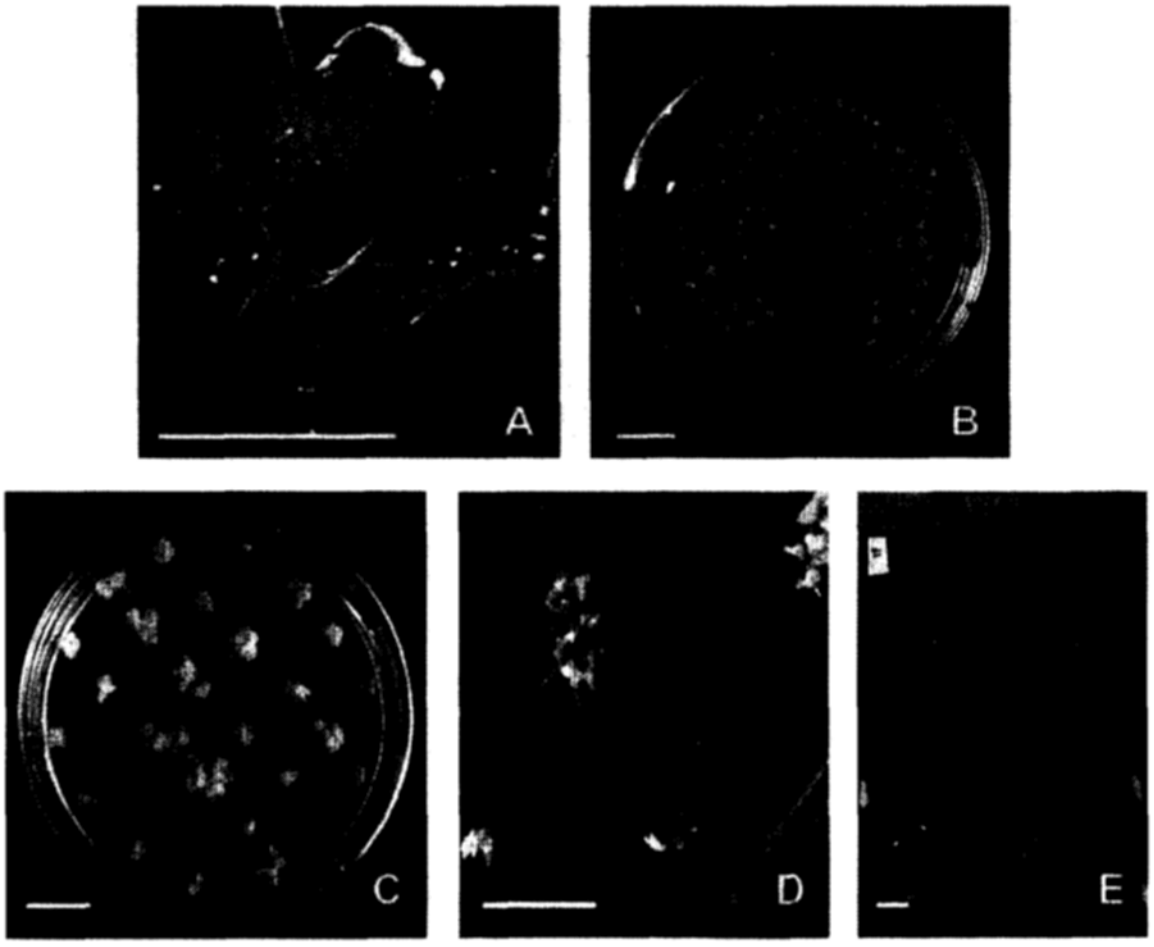

Figure 2. Production of transgenic rice plants by *Agrobacterium*-mediated transformation. **A.** Induction of calli from scutella of rice seeds on the 2N6 medium. **B.** Co-cultivation of *Agrobacterium* with calli. **C.** Selection of transgenic calli on the 2N6CH35 medium after co-cultivation. **D.** Plants regenerating from the transformed calli on the MSR16 medium. **E.** Plants growing in soil. Bars = 1 cm.

GUS Analysis

Calli and regenerated plants were incubated for 24 h at 37°C in a GUS solution comprising 100 mM sodium phosphate buffer, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.5% Triton X-100, 10 mM EDTA, 2% DMSO, 0.1% x-gluc, and 20% MeOH (Jefferson, 1987).

DNA Gel-Blot Analysis

Total genomic DNA was isolated by the CTAB (cetyltrimethyl-ammonium bromide) method from mature leaves of the transformants (Chen and Ronald, 1999). Three micrograms of total DNA were digested with EcoRI, separated on a 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled *gus* probe for 16 h at 60°C in a solution containing 20% SDS, 1M Na_2HPO_4, and 0.5M EDTA. The *gus* probe (sequence 209-704) was amplified by PCR. After hybridization, the blot was washed with a solution containing 0.2× SSC and 0.1% SDS for 30 min at 60°C.

RESULTS AND DISCUSSION

Frequency of Callus Formation

Calli were initiated from the scutellar tissue of mature seeds on a 2N6 medium supplemented with 2,4-D. Formation frequencies varied significantly among the varieties, ranging from 9.2 to 93.9% (Table 1). Varieties with frequencies >60% included Geumgang (63.3%), Milyang 42 (75.0%), Namcheon (69.9%), Namyeong

(62.3%), Samseong (61.5%), and Tongil (93.9%).

The condition of the callus was defined by its color change and solidity. Ten varieties formed calli that turned brown. These were divided into two types (B1 and B2). For those of Type B1 (Anda, Gaya, Jangseong, Naegyeong, and Tongilchalbyeo), the embryogenic calli were juicy and minimally developed (Table 1). In addition, the mature seeds of their explants turned brown during the callus-induction period. For Type-B2 varieties (Dasan, Hangangchalbyeo, Jungwon, Seogouang, and Taebaek), the embryogenic calli, which partially turned brown, were so watery that separation of a single callus was difficult.

The remaining varieties formed healthy, yellow calli, and were subdivided into three types according to the hardness of their calli (Table 1). Chilseong, Geumgang, Manseok, Milyang 42, Pungsan, S450, and Tongil were classified as Type Y1. Their calli were soft, and their

Table 1. Formation frequencies and condition of calli from Tongil rice varieties.

Group[a]	Variety	Number of seed	Callus formation frequency (%)	Callus condition	
				Color[b]	Solidity[c]
Type B1	Anda	504	23.1	B	−−
	Gaya	509	18.2	B	−−
	Jangseong	570	27.1	B	−−
	Naegyeong	432	9.2	B	−−
	Tongilchalbyeo	636	40.7	B	−−
Type B2	Dasan	456	35.2	B	−
	Hangangchalbyeo	612	48.5	B	−−
	Jungwon	396	32.6	B	−−
	Seogouang	864	40.5	B	−
	Taebaek	480	33.5	B	−−
Type Y1	Chilseong	372	41.5	Y	−
	Geumgang	804	63.3	Y	−
	Manseok	936	49.0	Y	−
	Milyang 42	552	75.0	Y	−
	Pungsan	552	27.1	Y	−
	S450	804	39.9	Y	−
	Tongil	336	93.9	Y	−
Type Y2	Baekyang	420	40.6	Y	+
	Cheongcheong	696	38.2	Y	+
	Chilseong 23	252	53.9	Y	+
	Chupung	444	31.5	Y	+
	Honamjosaeng	312	25.7	Y	+
	Josaengtongil	768	46.0	Y	+
	Milyang 23	528	34.7	Y	+
	Namcheon	432	69.9	Y	+
	Nampung	1008	48.7	Y	+
	Namyeong	624	62.3	Y	+
	Nopung	900	17.9	Y	+
	Saetbyeol	732	40.3	Y	+
	Samgangbyeo	444	38.1	Y	+
	Samseong	456	61.5	Y	+
	Sujeong	540	24.9	Y	+
	Wonpung	732	51.7	Y	+
	Yeongnamjosaeng	516	28.0	Y	+
	Yeongpung	420	38.0	Y	+
	Yongju	588	42.2	Y	+
	Yongmun	528	47.8	Y	+
Type Y3	Baekunchalbyeo	528	55.5	Y	++
	Yushin	480	46.2	Y	++

[a]Types B1 and B2 were classified according to the extent of color change and the condition of their calli. Types Y1, Y2, and Y3 were classified according to the solidity of their calli.
[b]B, brown; Y, yellow.
[c]+, adequately solid; ++, very solid; −, adequately soft; −−, very soft.

Table 2. Transformation and regeneration efficiencies by *Agrobacterium* for Tongil rice varieties in Groups 1 and 2.

Group	Variety	Co-cultivated calli (A)	First selected Hm^R calli (B)	Second selected Hm^R calli (C)	Transformation Frequency (C/A, %)	Calli having plantlet (D)	Regeneration frequency (D/C, %)	Final regeneration frequency (D/A, %)
1	Anda	35	0	–	–	–	–	–
	Chupung	26	0	–	–	–	–	–
	Gaya	4	0	–	–	–	–	–
	Jangseong	25	0	–	–	–	–	–
	Naegyeong	16	0	–	–	–	–	–
	Chilseong 23	23	1	0	0	–	–	–
	Josaengtongil	256	79	0	0	–	–	–
	Saetbyeol	433	208	0	0	–	–	–
	Yeongpung	15	2	0	0	–	–	–
2	Baekunchalbyeo	125	58	23	18.4	0	0	0
	Chilseong	70	24	3	4.3	0	0	0
	Dasan	211	65	3	1.4	0	0	0
	Hangangchalbyeo	64	16	3	4.7	0	0	0
	Honamjosaeng	53	45	19	35.8	0	0	0
	Pungsan	140	14	10	7.1	0	0	0
	S450	253	42	5	2.0	0	0	0
	Samseong	51	14	10	19.6	0	0	0
	Seogouang	284	79	29	10.2	0	0	0
	Sujeong	108	44	39	36.1	0	0	0
	Taebaek	78	8	5	6.4	0	0	0
	Tongilchalbyeo	23	2	1	4.3	0	0	0
	Yeongnamjosaeng	238	25	12	5.0	0	0	0
	Yushin	91	47	9	9.9	0	0	0

Table 3. Transformation and regeneration efficiencies by *Agrobacterium* for Tongil rice varieties in Group 3.

Group	Variety	Co-cultivated calli (A)	First selected HmR calli (B)	Second selected HmR calli (C)	Transformation frequency (C/A, %)	Calli having plantlet (D)	Regeneration frequency (D/C, %)	Final regeneration frequency (D/A, %)
3	Baekyang	594	313	156	26.3	10	6.4	1.7
	Cheongcheong	610	181	67	11.0	15	22.4	2.5
	Geumgang	38	14	2	5.3	1	50.0	2.6
	Jungwon	353	172	38	10.8	21	55.3	5.9
	Manseok	348	96	17	4.9	10	58.8	2.9
	Milyang 23	64	31	3	4.7	2	66.7	3.1
	Milyang 42	411	271	24	5.8	4	16.7	1.0
	Namcheon	195	81	29	14.9	6	20.7	3.1
	Nampung	243	124	67	27.6	15	22.4	6.2
	Namyeong	417	32	14	3.4	5	35.7	1.2
	Nopung	54	43	20	37.0	1	5.0	1.9
	Samgangbyeo	16	1	1	6.3	1	100.0	6.3
	Tongil	1153	577	267	23.2	123	46.1	10.7
	Wonpung	141	15	9	6.4	2	22.2	1.4
	Yongju	745	283	72	9.7	15	20.8	2.0
	Yongmun	574	233	49	8.5	16	32.7	2.8

embryogenic tissue could be easily isolated. In contrast, Baekunchalbyeo and Yushin (Type Y3) formed solid calli that were difficult to separate. The remaining 20 varieties (Type Y2) developed calli that were intermediate in hardness.

We observed that the solidity of the calli was an important factor when determining transformation success. In general, soft calli grew well, but as solidity increased, growth rates were reduced. This growth rate during subculture is another important factor in transforma-

tion (Toki, 1997). Binns and Thomashow (1988) have also demonstrated a distinct correlation between the activity of cell division and the competence of such cells to be transformed by *Agrobacterium tumefaciens*. They have proposed that processes related to DNA synthesis and cell division are required for incorporating foreign DNA into a host genome. Evidence consistent with this hypothesis has also been presented by Wullems et al. (1981), Kudirka et al. (1986), Valvekens et al. (1988), and Iida et al. (1991).

Frequency of Transformation

Embryonic calli were transformed with pGA2722 via co-cultivation with *Agrobacterium* carrying the binary vector. The first selection was on a 2N6 medium containing hygromycin; the second selection, on a 2N6 medium with BA and hygromycin. Nine varieties (see Group 1 in Table 2) did not produce transformed calli during these selections. Five of the varieties formed brown calli, while four developed hard, yellow calli. These transformation failures probably resulted from the poor quality of the calli, which did not actively proliferate.

In Group 2, 14 varieties produced transformed calli, but failed to regenerate, again a likely result of poor calli quality (Table 2). Five of these (Dasan, Hangangchalbyeo, Seogouang, Taebaek, and Tongilchalbyeo) had previously formed brown calli. Another six (Baekunchalbyeo, Honamjosaeng, Samseong, Sujeong, Yushin, and Yeongnamjosaeng) had generated hard, yellow calli. Unexpectedly, three other varieties (Chilseong, Pungsan,

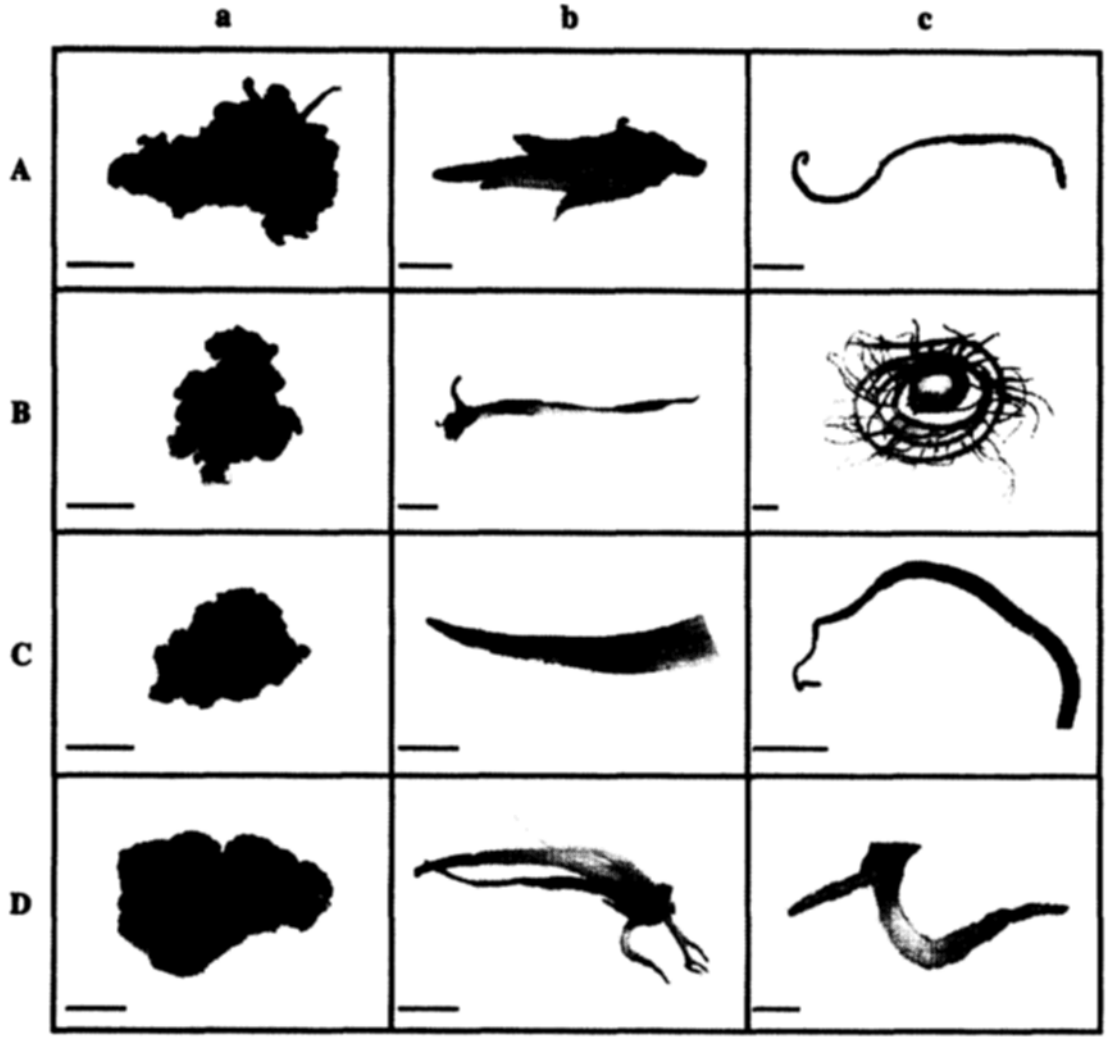

Figure 3. Histochemical GUS analysis in transformed calli (a), shoots (b), and roots (c). A, Geumgang; B, Milyang 42; C, Tongil, D, Control. Bars = 0.1 cm.

and S450), which had earlier produced soft, yellow calli, nevertheless failed to produce regenerants. These results demonstrate, therefore, that the condition of the callus is not the only component that determines success.

Several factors influence transformation efficiency. First, individual varieties may differ in their susceptibility to *Agrobacterium* (Donaldson and Simmonds, 2000). Second, varietal differences exist for hygromycin susceptibility (i.e., resistance). In this study, we did not optimize the concentrations of hygromycin or other ingredients when choosing the media for culturing each variety. Whereas Tongil generated an actively proliferating callus mass on the selection media, other varieties did not form any masses. These results suggest that the culture medium was suitable for Tongil but not for others, so choice of culture media and hygromycin concentration should be carefully analyzed in the future.

Finally, the starting condition of the culture materials also can affect transformation success. When vigorously growing calli are used, the efficiency of agroinfection increases (Vijayachandra et al., 1995; Hiei et al., 1997). In this study, we observed that, of the 39 varieties examined, growth of the Tongil calli was most rapid. Moreover, 16 varieties (Group 3) produced regenerated plants within two to four weeks, although frequencies varied significantly, ranging from 1.0 to 10.7% (Table 3). Among these, 11 varieties (Baekyang, Cheongcheong, Milyang 23, Namcheon, Nampung, Namyeong, Nopung, Samgangbyeo, Yongmun, Yongju, and Wonpung) had previously produced hard, yellow calli, 4 (Geumgang, Manseok, Milyang 42, and Tongil) generated soft, yellow calli, while only Jungwon had produced brown calli. We therefore conclude that callus conditions are not absolute determinants of efficiency. For example, although the calli appeared to be equally suitable from both Milyang 42 and Tongil, the former did not regenerate well, whereas the latter had the best frequency (10.7%). These results suggest the possibility that the concentrations of plant growth regulator used in this study may not have been optimal for certain varieties.

We conducted GUS assays on the calli that grew on the second selection media (Fig. 3). Regenerated shoots and roots were also examined. All three tissue types were GUS-positive, indicating that the regenerated plants were, indeed, true transformants. We also performed DNA gel-blot analysis to confirm the integration of T-DNA into the rice genome. The results showed that one to three copies of T-DNA were present in the transformants (Fig. 4).

In summary, we have demonstrated here that the variety Tongil can be used for high-frequency transformation of this important type of rice. Considering that indica varieties are difficult to transform, hybrid varieties may be useful for functional analysis of those indica genes that are present in these Tongil varieties.

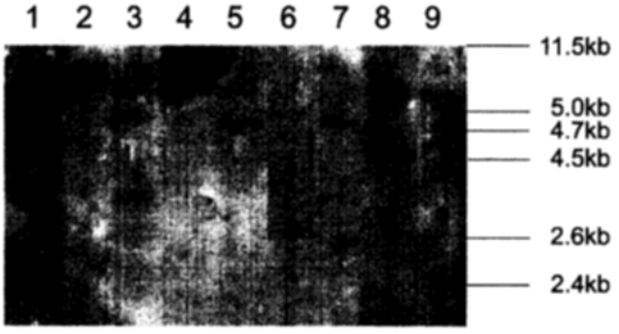

Figure 4. DNA gel-blot analysis of transgenic plants. DNA was digested with EcoRI and hybridized with the *gus* probe (sequence 209-704 from ATG). Numbers indicate the independently transformed lines. Size markers are indicated on the right of the figure.

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