

## Genotypic variation of *Agrobacterium*-mediated transformation of Italian ryegrass

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**Financial support:** This work was supported by grants from the National Institute of Animal Science (Project no. 200901OFT082558123) and the BioGreen 21 Program (Project no. 20070301-034-015), Rural Development Administration (RDA), Korea.

**Keywords:** *Agrobacterium*, forage crop, Italian ryegrass, transgenic plants.

**Abbreviations:** BA: 6-benzyladenine  
GUS:  $\beta$ -glucuronidase

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HPT: hygromycin phosphotransferase  
 X-gluc: 5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronide

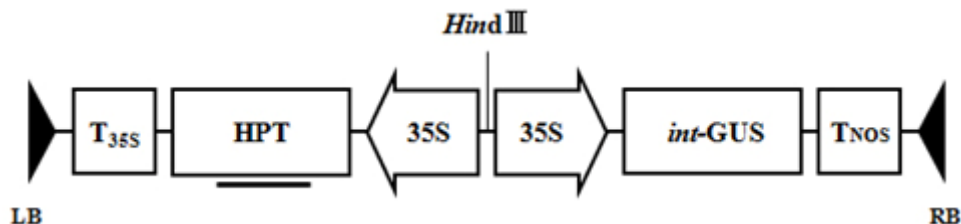
**In the present study, genotypic variation of *Agrobacterium*-mediated transformation of Korean Italian ryegrass has been evaluated. Mature seed-derived calli of seven cultivars were inoculated and co-cultured with *Agrobacterium tumefaciens* carrying the binary vector pCAMBIA1301, which contains a reporter gene (*gus*) and a plant selectable marker gene conferring resistance to hygromycin (*hpt*) in the T-DNA region. The effects of several factors such as callus type and callus age on transformation effectiveness and the expression of the GUS gene were investigated. The highest transformation effectiveness (6.7%) was obtained with the Hwasan 101 cultivar when 9-week-old calli (type-I) were inoculated with *Agrobacterium*. The overall transformation rates of the examined cultivars ranged from 0.4% to 6.7%. GUS histochemical assays, PCR, and southern analysis of transgenic plants demonstrated that transgenes were successfully integrated into the genome of Italian ryegrass. Thus, evaluation of transformation effectiveness and selection of a suitable cultivar of Italian ryegrass may improve molecular breeding of this species.**

The plant genetic transformation system is one of the most crucial technologies in plant molecular breeding. A transgenic approach to genetic improvement of traditional Italian ryegrass (*Lolium multiflorum* Lam) cultivars would promote desirable traits such as disease, insect, and pest resistance, tolerance to several abiotic stresses, forage quality, and production efficiency (Ye et al. 1997). Italian ryegrass is one of the fastest growing forage grass species, and it provides an excellent high quality feed for most classes of livestock. It is regarded as a versatile grass species that can be used for pasture, hay, green chop or silage (Hides et al. 1993; Isselstein et al. 1993), and it is widely used for forage. Improvement of the cold tolerance of Italian ryegrass is an important breeding objective for enlargement of the cultivation area in Korea (Choi et al. 2008).

The susceptibility of Italian ryegrass to gene manipulation has been well established by direct gene transfer methods such as particle bombardment (Ye et al. 1997; Dalton et al. 1999; Takahashi et al. 2002) or by silicon-carbide fiber-mediated transformation (Dalton et al. 1998). For the first time, Ye et al. (1997) generated transgenic Italian ryegrass using embryogenic suspension cells as target for particle bombardment-mediated transformation system. Transformed Italian ryegrass expressing kanamycin-resistant and  $\beta$ -glucuronidase gene have also been obtained using microprojectile bombardment wherein protoplasts were used as target cells. Although these studies generated higher number of transgenic Italian ryegrass; unfortunately, cultivars from which transgenic plants were regenerated

were not mentioned. Dalton et al. (1998) also produced transgenic Italian ryegrass using silicon-carbide-fiber-mediated transformation system which is considered as simpler and less expensive comparative to microprojectile bombardment, however; in terms of efficiency of resources the microprojectile bombardment method is preferable since larger volume of cells can be treated at one time (Dalton et al. 1998; Dalton et al. 1999). Although direct gene transfer technology has been used successfully to produce transgenic Italian ryegrass, it has been claimed in several studies that transgenic plants obtained by direct gene transfer methods through protoplast or particle bombardment showed multiple copy insertions of the transgenes (Dalton et al. 1995; Spangenberg et al. 1995). In addition, it has been hypothesized that transgenic plants obtained from particle bombardment might be an increased rate of truncated transgene insertions and/or transgene silencing, probably as a consequence of DNA fragmentation during particle coating and penetration into the target cells (Gao et al. 2008). Moreover, protoplast and cell suspension cultures used for direct gene transformation system involved laborious steps and have been difficult to reproduce in terms of initiation of and maintenance of cell suspension cultures, and also in isolation of protoplast. Recently Gao et al. (2008) revealed that transgene expression in transgenic plants was quite higher (53%) in *Agrobacterium*-mediated transformation than that of the particle bombardment method (23%), suggested that for efficient production of transgenic plants with stable and predictable transgene expression, *Agrobacterium*-mediated method offers considerable advantages over particle bombardment.

*Agrobacterium*-mediation of genetic transformation systems is a commonly used technique for genetic improvement. Advantages of *Agrobacterium*-mediation of genetic transformation, including the ability to transfer large pieces of DNA with minimal rearrangement, low experimental costs, and integration of relatively low numbers of transgene copies, have been described elsewhere (Hiei et al. 1994). To the best our knowledge, there have only been a few reports regarding *Agrobacterium*-mediated transformation (Bettany et al. 2003), which recovered a total of eight transgenic plants wherein only one showed GUS expression. In addition, up to now, there was no comparative evaluation of the responses of Italian ryegrass cultivars in *Agrobacterium*-mediated transformation system. A wide range of factors, however, such as plant genotype, explants type and age, co-cultivation media and period, can influence the gene transfer efficiency from *Agrobacterium* to plant cells (Uranbey et al. 2005; Ahsan et al. 2007). Although, *Agrobacterium*-mediated transformation in Italian ryegrass was introduced in 2003 (Bettany et al. 2003), it still does



**Figure 1. Plasmid construction of the pCambia1301 binary vector used for transformation of Italian ryegrass plants.** HPT, hygromycin phosphotransferase; *int-GUS*,  $\beta$ -glucuronidase containing intron; 35S, cauliflower mosaic virus 35S promoter; T35S, polyA cauliflower mosaic virus 35S terminator; TNOS, polyA nopaline synthase terminator; Bold bar indicates the region amplified by PCR. LB and RB indicates leaf and right border, respectively.

not yield consistent results in respect of genotypic response and transformation frequency. The absence of highly efficient genotype independent regeneration and transformation systems is a major obstacle to the application of gene transfer technology to economically important Italian ryegrass cultivars. Therefore, a simple, reproducible, and genotype independent efficient transformation method is still a basic demand for large-scale transgenic Italian ryegrass production.

Currently, the importance of forage grass transformation is increasing as a means of introducing better and more useful traits into many cultivars of economic relevance for integrated forage grass management (Takahashi et al. 2005; Wu et al. 2005; Lee et al. 2007; Zhao et al. 2007). Genetic transformation may be an important contribution to the improvement of forage grasses. However, the efficiency with which Italian ryegrass cultivars are transformed is very low (Bettany et al. 2003). In this study, we have reported an improved transformation protocol for seven cultivars of Korean Italian ryegrass by use of mature seed-derived calli as explants.

## MATERIALS AND METHODS

### Explant preparation and culture conditions

Italian ryegrass seeds were obtained from the Grassland and Forages Research Center, National Institute of Animal Science, Rural Development Administration, Korea. Mature seeds of seven cultivars of Korean Italian ryegrass (*Lolium multiflorum* Lam.), namely, Kogreen, Kospeed, Kowinearly, Kowinmaster, Hwasan 101, Hwasan 104 and Kowinner, were dehusked by stirring in 50% sulfuric acid for 20 min and rinsing with sterile water. Dehusked seeds were then surface-sterilized with 70% ethanol for 1 min and 30% bleach (5.25% sodium hypochlorite) for 30 min with gentle shaking, followed by three washes with sterile water. Finally, they were blotted with sterile Whatman filter paper.

The surface-sterilized seeds were placed in MS (Murashige and Skoog, 1962) medium containing 5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/L 6-benzylaminopurine (BA), 500 mg/L L-proline, 1 mg/L casein hydrolysate, 30 g/L sucrose, and 3 g/L Gelrite. The

cultures were transferred to controlled growth chambers at  $24 \pm 2^\circ\text{C}$  for three weeks in the dark. After three weeks, calluses were removed from the germinating shoots and divided into 4–6 mm-diameter pieces and sub-cultured every three weeks on the same fresh medium, and then the calli were used for transformation.

### Plant transformation

In the transformation experiments, *Agrobacterium tumefaciens* strain EHA105 containing the binary plasmid pCambia1301 (Center for the Application of Molecular Biology to the International Agriculture of Canberra, Australia) was used. The plasmid contains a reporter gene (*gus*) and a plant selectable marker gene conferring resistance to hygromycin (*hpt*), both driven by the CaMV 35S promoter (Figure 1). A single colony of bacteria was inoculated on liquid YEP medium containing 100 mg/L kanamycin and incubated in a shaker (200 rpm) at  $28^\circ\text{C}$  overnight. Afterwards, the culture was centrifuged at 2,500 g for 10 min, and the pellet was resuspended in MS medium supplemented with 30 g/L sucrose, 10 g/L glucose, and 40 mg/L L-cysteine with 200  $\mu\text{M}$  acetosyringone. Embryonic calli (90 pieces) were immersed in the bacterial suspension with *gentle shaking* for 60 min, and the excess bacterial suspension was removed by blotting on sterile tissue paper. Inoculated calli were transferred onto a co-culture medium for five days in the dark in MS with 3 mg/L 2,4-D, 0.5 mg/L BA, 1 g/L casein hydrolysate, 500 mg/L L-proline, 40 mg/L L-cysteine, 30 g/L sucrose, 200  $\mu\text{M}$  acetosyringone, and 2 g/L Gelrite.

### Selection and regeneration of transgenic plants

Five days after co-culture, the explants were washed in 500 mg/L cefotaxime to kill the surface *Agrobacteria*, and then co-cultivated calli were subcultured on the selection medium, which is MS medium supplemented with 1 mg/L 2,4-D, 5 mg/L BA, 1 g/L casein hydrolysate, 500 mg/L L-proline, 40 mg/L L-cysteine, 30 g/L sucrose, 3 g/L Gelrite, 250 mg/L cefotaxime, and 50 mg/L hygromycin. These calli were sub-cultured every 3 weeks onto fresh medium of the same composition at  $25^\circ\text{C}$  with a 16 hrs light photoperiod ( $100 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ) until the development of plantlets. Finally, regenerated putative transgenic shoots

**Table 1. Callus induction from mature seeds of seven Italian ryegrass cultivars after 6 weeks of culture.**

Cultivars	Callus type of mature seed culture (%) <sup>a</sup>		
	Type-I*	Type-II**	Total
Kogreen	35.3 ± 3.1	27.7 ± 3.5	63.0 ± 5.2
Kospeed	44.0 ± 4.6	30.0 ± 2.0	74.0 ± 3.6
Kowinearly	46.0 ± 8.6	28.7 ± 2.3	74.7 ± 9.3
Kowinmaster	30.7 ± 3.8	25.0 ± 3.8	55.7 ± 3.8
Hwasan 101	47.3 ± 4.9	33.3 ± 4.2	80.7 ± 4.0
Hwasan 104	25.0 ± 10.8	23.3 ± 4.4	48.3 ± 12.7
Kowinner	29.0 ± 4.6	24.3 ± 7.5	53.3 ± 3.2

<sup>a</sup>Data were expressed as the average of three replicates, each with 100 mature seeds cultured on callus induction medium and each value is the mean of three individual experiments. Morphological phenotypes of embryogenic calli\* and non-embryogenic calli\*\*.

were separated and transferred onto rooting medium containing half strength MS medium supplemented with 30 g/L sucrose, 2 g/L Gelrite, 250 mg/L cefotaxime, and 50 mg/L hygromycin. While, in the earlier study (Bettany et al. 2003), putative transgenic calli were selected with relatively higher concentration of hygromycin (75 mg/L), the present study we found that selection of putative transformant callus with higher than 50 mg/L hygromycin caused a severe inhibition of shoot regeneration from the Hm-resistant calli. In addition, it has been reported that continuous selection of low concentration of hygromycin produced higher number of transgenic plants without escape (Dalton et al. 1995). The transformation effectiveness of each cultivar was calculated as follows:

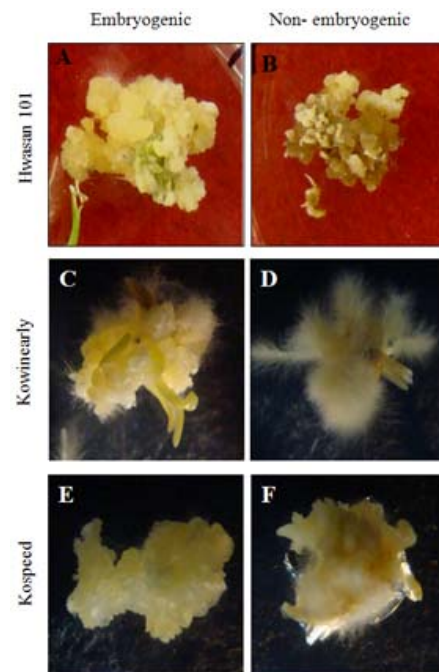
$$\frac{\text{Total number of Hm}^R \text{ calli showing regeneration} \times 100}{\text{Total number of calli inoculated in } Agrobacterium\text{-mediated transformation system}}$$

### Histochemical assay and measurement of the frequency of GUS

Following co-cultivation, the explants were subjected to a transient GUS histochemical assay according to Jefferson et al. (1987). Five days after co-cultivation, calli were incubated for 12 hrs at 37°C in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl-glucuronide) and subsequently bleached with 70% ethanol for 24 hrs to remove chlorophyll. Transient *gus* expression was measured after co-cultivation by counting the *gus*-positive calli appearing in blue zones (1 mm or more in diameter) as described previously (Lee et al. 2006), and each value is the mean of three individual experiments.

### Molecular analysis of transgenic plants

Confirmation of stable integration of the transgene gene in to the putatively transgenic Korean Italian ryegrass genome



**Figure 2. Different types of callus of Italian ryegrass. A, C and E represents the embryogenic and B, D and F represents the non-embryogenic type of callus of Hwasan 101, Kowinearly and Kospeed cultivars, respectively.**

**Table 2. Effect of callus age on GUS activity of Italian ryegrass cultivar Hwasan 101 after transformation in the co-cultivation medium.**

Age of callus (weeks)	GUS + <sup>a</sup> calli (%)			
	Experiment			Mean ± SE
	1	2	3	
3	1	2	0	1.0 ± 1.0
6	4	3	7	4.7 ± 2.1
9	5	8	9	7.3 ± 2.1
12	2	5	3	3.3 ± 1.5

<sup>a</sup>Data were taken 5 days after *Agrobacterium* inoculation and means are from 30 calli per treatment and each value is the mean of three individual experiments.

was demonstrated by polymerase chain reaction (PCR) analysis. Genomic DNA was extracted from the leaves of putative transgenic plants and non-transformed (wild type) plants by the CTAB method (Murray and Thompson, 1980). The PCR were carried out by amplifying the coding region of transgene (*hpt*) using the following set of primer: 5'-CCTGAACTCACCGCGACG-3' (forward) and 5'-AAGACCAATGCGGAGCAT AT-3' (reverse), which generate a 804 bp fragment within the *hpt* gene. The PCR reaction was conducted as previously described (Lee et al. 2006), and PCR products were separated by electrophoresis on a 1% (w/v) agarose gel. For Southern blot analysis, 20 µg of total genomic DNA was digested with *Hind*III and electrophoreted on a 1.0% (w/v) agarose gel and blotted onto a nylon membrane (Nytran-Plus, Schleicher & Schuell, Germany) according to the previously described method (Lee et al. 2006). To obtain a gene-specific probe for the *gus* gene, a 0.48-kb fragment was amplified by PCR using pCAMBIA1301 as template and the previously described primers and conditions (Lee et al. 2006). The blot was subsequently analyzed by means of autoradiography with x-ray films (Kodak, Japan).

## RESULTS AND DISCUSSION

We have investigated the cultivar-dependent *Agrobacterium*-mediated transformation effectiveness of Korean Italian ryegrass cultivars by evaluating several factors including callus type, cultivars and callus age. Transformation efficiency was determined by histochemical assays of GUS activity and hygromycin resistant regenerated plants.

### Effect of genotypes on callus type

To examine the effect of genotype on the callus induction ability of Korean Italian ryegrass, seven cultivars were

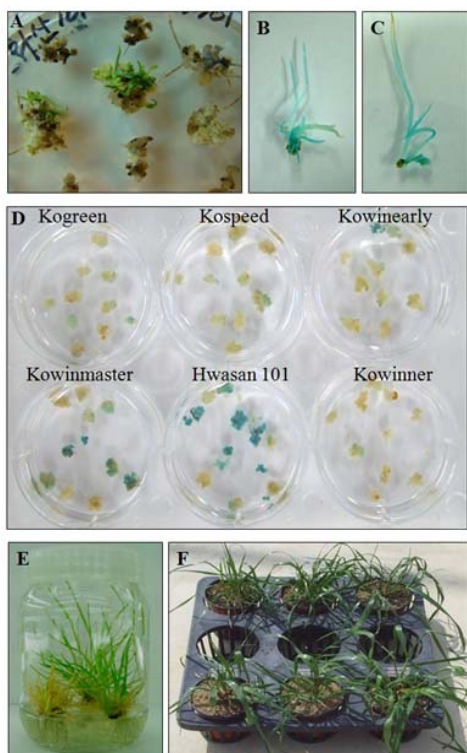
tested on callus induction medium. Calli appeared from mature seeds within 5-10 days on the callus induction medium. The primary callus induction frequency varied from 48.3 to 80.7% after a culture period of six weeks. In addition, a significant difference was found in the quality of calli induced among the seven Italian ryegrass cultivars (Table 1). For example, two types of calli, embryogenic (type-I) and non-embryogenic (type-II), were obtained. The embryogenic calli were relatively compact and white-yellow in color (Figure 2). In contrast, non-embryogenic calli were watery and somewhat brown in color and sometimes contained hairy roots (Figure 2D and Figure 2F). Hwasan 101, Kowinearly and Kospeed showed high embryogenic callus induction ability (Table 1 and Figure 2). Three weeks after callus induction, embryogenic calli only were subcultured on the same fresh callus induction medium every three weeks.

Variations in callus induction ability among the tested cultivars indicated that these differences were completely dependent on genotype. Genotypic response to callus type and regeneration frequency in *in vitro* culture systems has frequently been observed in grass species (Bai and Qu, 2000; Chaudhury and Qu, 2000).

### Effect of callus age on transformation

Based on the results presented in Table 1, we have further optimized the effect of callus age in transformation frequency using the Hwasan 101 cultivar. Although, there was no significant differences observed in callus induction frequency of Kospeed, Kowinearly and Hwasan 101 cultivars; based on the highest induction frequency Hwasan 101 was selected. Three-week-old to 12-week-old calli showed different GUS activity after 5 days co-cultivation. Results showed that transformation efficiency was greatly





**Figure 3. Regeneration of transgenic plants and histological GUS assay of putatively transgenic calli of seven Korean Italian ryegrass cultivars.**

(A) Regeneration of putatively transformed plants of Hanswan 101 after 3 weeks culture on selection medium containing 50 mg/L hygromycin.

(B) Histochemical GUS activity in leaf tissue in Hanswan 101 after 5 days co-culture with *Agrobacterium*.

(C) Histochemical GUS activity in leaf tissue in Korwinmaster after 5 days co-culture with *Agrobacterium*.

(D) Hygromycin-resistant calli showing GUS gene expression of seven Korean Italian ryegrass cultivars.

(E) Rooted transformants ready to be transferred to the greenhouse.

(F) Transgenic plants grown in pots in the green house.

influenced by callus age, wherein 9-week-old calli showed highest transformation efficiency in Hwasan 101 cultivar (Table 2). However, calli that was more than 9 weeks old turned brown and died after co-cultivation. While, the callus induction frequency of Kospeed and Kowinearly was very similar to Hwasan 101; therefore it could be expected that approximately 9 weeks old calli possibly the best explant for Korean Italian ryegrass cultivars in *Agrobacterium*-mediated genetic transformation system. Similarly, Dalton et al. (1999) noticed that young cell suspension cultures (*i.e.* less than 10 weeks after initiation) appeared to be critical for regeneration of transgenic Italian ryegrass in particle bombardment genetic transformation system. The effect of the age of explants on transgenesis and subsequent regeneration has been evaluated in many studies revealed that the response of young explants to transformation was better, however the comparatively middle aged (6-10 weeks) explants and/or cells responded

better in both transformation and regeneration (de Kathen and Jacobsen, 1995; Nandakumar et al. 2004).

### Genotypic variation of Italian ryegrass cultivars on transformation effectiveness

GUS expression was tested in the seven Korean Italian ryegrass cultivars transformed with the pCAMBIA1301 plasmid expressing the GUS reporter gene. Basal GUS expression of the pCAMBIA1301 construct with the CaMV 35S-promoter was observed in callus and leaf tissue (Figure 3). Transformed calli and leaves stained intensely with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc), while non-transformed calli and leaves did not (data not shown). The effect of cultivar on transformation effectiveness is summarized in Table 3. Transformation effectiveness ranged from 0.4 to 6.7%. Cultivar Hwasan 101 showed relatively higher transformation effectiveness (6.7%) than the other six cultivars. In contrast, cultivar Hwasan 104 showed the lowest transformation effectiveness, 0.4%, compared to the other cultivars. Based on the results, it could be concluded that significant differences existed between the cultivars in transformation, and the Hwasan 101 cultivar could be considered the most suitable cultivar for Italian ryegrass transformation. Similar to our experiment, significant genotypic variation in *Agrobacterium*-mediated genetic transformation potential has been reported in many monocotyledonous species (Kumar et al. 2005; Wu et al. 2007).

### Molecular analysis of transgenic Italian ryegrass plants

The putative T<sub>0</sub> transformant plantlets were regenerated and further grown on rooting medium for 4 weeks. Hygromycin resistant calli showed regeneration of each cultivar is shown at Table 3. A total of 46 putative transgenic hygromycin resistant plantlets were recovered. Putatively transgenic plants obtained from each cultivar were used for PCR analysis using *hpt* primer set, which yielded the expected size of 804 bp band (Figure 4). Representative PCR analysis of the hygromycin resistant plantlets of Hwasan 101, Kowinmaster, Kospeed and Kogreen cultivars revealed the transgene integration into the genome of T<sub>0</sub> transformant plantlets, whereas no band was observed in the non-transformed control plant (Figure 4A-C). Successful integration of the transgene gene into the genome of the PCR positive transgenic lines was further confirmed by Southern blot analysis using the genomic DNA digested with *Hind*III, an enzyme that has only one cutting site in the T-DNA region of the binary vector used (Figure 1). The representative Southern blot analyses of the PCR positive lines (1-6) of Hwasan 101 cultivar are shown in Figure 4D. The number of hybridizing bands reflected the number of copies of integrated transgene in the transgenic Italian ryegrass plants. Results indicated that one copy of the transgene was integrated into the genomic DNA of Italian ryegrass (Figure 5). In non-transgenic wild type plants, no hybridized band was detected. The mobilities of

Table 3. Effect of transformation effectiveness of seven Korean Italian ryegrass cultivars.

Cultivars	No. of calli inoculated <sup>a</sup> (A)	No. of Hm <sup>R</sup> calli	Hm <sup>R</sup> calli showing regeneration (B)	Transformation effectiveness (B/A, %)
Kogreen	270	9	4	1.5
Kospeed	270	11	6	2.2
Kowinearly	270	10	3	1.1
Kowinmaster	270	35	12	4.4
Hwasan 101	270	37	18	6.7
Hwasan 104	270	9	2	1.1
Kowinner	270	4	1	0.4

<sup>a</sup>Data were taken 5 days after *Agrobacterium* inoculation and means are from about 90 calli per treatment and each value is the mean of three individual experiments.

the bands differed from plant to plant, indicating independent transformation events and random integration. Although, all the tested six transgenic lines showed one copy of T-DNA insertion in to the genome of the transgenic Hwasan-101 cultivar, it is not unexpected. Similar observation has also been noticed in earlier reports on *Agrobacterium*-mediated transformation of other grass species, wherein majority of the transgenic plants showed one copy of T-DNA insertion in to the genome of transgenic plants (Han et al. 2005; Hu et al. 2005; Ge et al. 2006; Ge et al. 2007; Lee et al. 2008). In general, *Agrobacterium*-mediated transformation resulted low copy

numbers of the inserted T-DNAs ranged from one to five, whereas the number of inserted T-DNAs in the host genome was comparatively high (up to eight) in particle bombardment system (Travella et al. 2005; Gao et al. 2008).

There were no apparent phenotypic and developmental differences between the wild type and transgenic plants. Therefore, the transformation method evaluated in this study allowed to generate of a large number of transgenic Italian ryegrass. It is also demonstrated that transgenic Italian ryegrass could be produced by the use of the easily

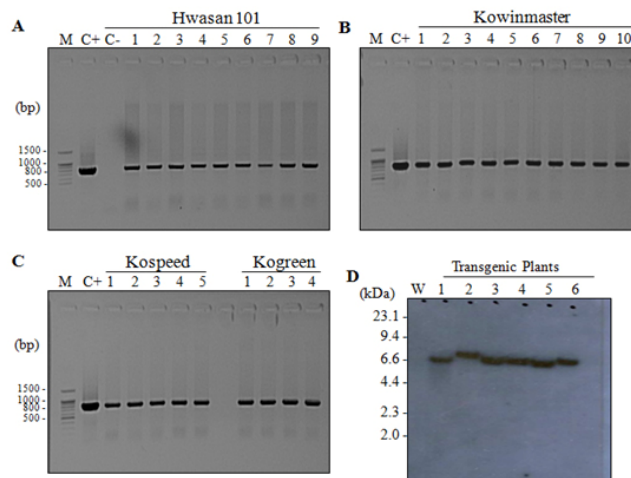


Figure 4. Molecular analyses of the putatively transformed Italian ryegrass cultivars. A-C, represents the PCR analysis, and D represents the Southern blot analysis of T<sub>0</sub> transgenic plants. M, molecular marker; C+, pCAMBIA 1301 as a positive control; C-, non-transformed plant as a negative control.

available pCAMBIA1301 vectors. The transformation efficiency was comparable with other *Agrobacterium*-mediated transformation systems in grass species such as, Italian ryegrass (Bettany et al. 2003), perennial ryegrass (Wu et al. 2005) and orchardgrass (Lee et al. 2006).

In conclusion, we have evaluated the effectiveness of *Agrobacterium*-mediated transformation of seven Korean Italian ryegrass cultivars which revealed a highly efficient ryegrass cultivar (Hwasan 101) suitable for future studies in genetic transformation system. The transient GUS expression assay was found to be an easy and reliable way of establishing optimal conditions for transformation. In addition, the present study shows that mature seeds could be a convenient source of explants for callus induction and subsequent transformation in Italian ryegrass. This is advantageous to some grass species transformation (Ye et al. 1997; Bettany et al. 2003; Ge et al. 2007; Lee et al. 2006; Lee et al. 2007). However, the uses of immature embryos, suspension cells, protoplasts culture and uses of shoot tips as explants requires continuous growth of plant and/or explants, and the timeframe for isolation of such materials is limited. Thus the present study, emphasizes that the importance in genetic transformation if Italian ryegrass depends on the explants, cultivars and callus condition.

Therefore, the *Agrobacterium*-mediated transformation system might be used to develop transgenic Italian ryegrass with agronomically important genes either to increase production or to improve forage quality.

## ACKNOWLEDGMENTS

We are very much thankful to Dr. Nagib Ahsan (National Institute of Crop Science, Tsukuba, Japan) for his valuable discussion and suggestions.

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