# Development of Transgenic Tall Fescue Plants from Mature Seed-derived Callus via *Agrobacterium*-mediated Transformation

**Sang-Hoon Lee, Dong-Gi Lee, Hyun-Sook Woo and Byung-Hyun Lee***
Major of Dairy Science, Division of Applied Life Science (BK21), College of Agriculture and Life Science
Gyeongsang National University, Jinju 660-701, Korea

**ABSTRACT :** We have achieved efficient transformation system for forage-type tall fescue plants by *Agrobacterium tumefaciens*. Mature seed-derived embryogenic calli were infected and co-cultivated with each of three *A. tumefaciens* strains, all of which harbored a standard binary vector pIG121Hm encoding the neomycin phosphotransferase II (NPTII), hygromycin phosphotransferase (HPT) and intron-containing β-glucuronidase (intron-GUS) genes in the T-DNA region. Transformation efficiency was influenced by the *A. tumefaciens* strain, addition of the phenolic compound acetosyringone and duration of vacuum treatment. Of the three *A. tumefaciens* strains tested, EHA101/pIG121Hm was found to be most effective followed by GV3101/pIG121Hm and LBA4404/pIG121Hm for transient GUS expression after 3 days co-cultivation. Inclusion of 100 μM acetosyringone in both the inoculation and co-cultivation media lead to an improvement in transient GUS expression observed in targeted calli. Vacuum treatment during infection of calli with *A. tumefaciens* strains increased transformation efficiency. The highest stable transformation efficiency of transgenic plants was obtained when mature seed-derived calli infected with *A. tumefaciens* EHA101/pIG121Hm in the presence of 100 μM acetosyringone and vacuum treatment for 30 min. Southern blot analysis indicated integration of the transgene into the genome of tall fescue. The transformation system developed in this study would be useful for *Agrobacterium*-mediated genetic transformation of tall fescue plants with genes of agronomic importance. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 10 : 1390-1394)***

**Key Words :** Tall Fescue, *Agrobacterium*, Transgenic Plants, Callus, Forage

## INTRODUCTION

Tall fescue (*Festuca arundinacea* Schreb.) is an open-pollinated, perennial, cool-season grass species widely used for forage (Buckner et al., 1979). It is commonly used for pastures, hays and lawns. Progress in breeding using conventional selection procedures is slow, since tall fescue is a polyploid (2n=6x=42), wind-pollinated species with a high degree of self-incompatibility (Barnes, 1990). Biotechnological approaches, therefore, may become an effective alternative to conventional breeding procedures in the incorporation of useful traits.

Transgenic tall fescue plants have been obtained by direct gene transfer methods such as polyethyleneglycol- or electroporation-mediated DNA uptake into protoplasts (Ha et al., 1992; Dalton et al., 1995) and microprojectile bombardment of cell suspension cultures (Spangenberg et al., 1995; Cho et al., 2000). The majority of forage grass species can now be transformed by microprojectile bombardment (Spangenberg et al., 1995). Recent reports demonstrate that *Agrobacterium*-mediated transformation offers several advantages in comparison with microprojectile bombardment method such as transfer of relatively large segments of DNA with little rearrangement and integration of low copy numbers of T-DNA to active regions of chromosome (Hiei et al., 1994). Until recently, monocotyledonous plant species were considered to be recalcitrant to *Agrobacterium*-mediated transformation. However, in the past few years successful application of the *Agrobacterium*-mediated transformation has been reported for various monocot species including rice (Hiei et al., 1994), maize (Ishida et al., 1996), sorghum (Zhao et al., 2000), barley (Trifonova et al., 2001) and wheat (Wu et al., 2003).

Although *A. tumefaciens* has been successfully used to transfer genes to a wide range of plant species, it has received little attention for transformation of forage grasses. In this study, we present here, for the first time, an efficient *Agrobacterium*-mediated transformation system for the production of large numbers of independently transformed tall fescue plants from commercially important forage-type cultivar.

* Corresponding Author: Byung-Hyun Lee. Tel: +82-55-751-5418, Fax: +82-55-751-5410, E-mail: hyun@nongae.gsnu.ac.kr
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## MATERIALS AND METHODS

### Plant materials

Dehusked mature seeds of tall fescue (*Festuca arundinacea* Schreb. cv. Kentucky-31) were surface sterilized for 30 min with 30% bleach (5.25% sodium hypochlorite) followed by three washes with sterile water. The seeds were placed on MS (Murashige and Skoog, 1962) based callus induction medium (500 mg/l L-proline, 100 mg/l myo-inositol, 3% sucrose, 0.5% gelrite, 6 mg/l 2,4-D and 0.1 mg/l BAP). Seven days after plating, germinating shoots and roots were completely removed by

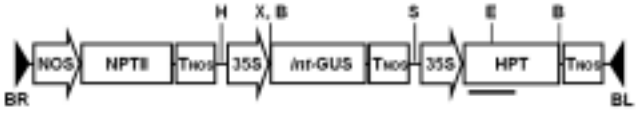


**Figure 1.** T-DNA region of pIG121Hm binary vector. BR: right border; BL: left border; NPTII: neomycin phosphotransferase; int-GUS: β-glucuronidase containing intron; HPT: hygromycin phosphotransferase; NOS: nopaline synthase promoter; 35S, 35S promoter; TNOS: 3'-signal of nopaline synthase; H: *Hin*dIII; B: *Bam*HI; X: *Xba*I; S: *Sal*I; E: *Eco*RI. Bold bar indicates the region of the probe used for Southern blot analysis, which was amplified by PCR.

manual excision. After 4 weeks incubation at 25°C in the dark, embryogenic calli were selected and subsequently cultured on the same medium for 4 weeks.

### *Agrobacterium tumefaciens* strains

Three *A. tumefaciens* strains, EHA101/pIG121Hm, GV3101/pIG121Hm and LBA4404/pIG121Hm, were used. The T-DNA region of the standard binary vector pIG121Hm (Figure 1) contained the neomycin phosphotransferase II (NPTII), hygromycin phosphotransferase (HPT) and intron-containing β-glucuronidase (intron-GUS) genes (Hiei et al., 1994). Each *A. tumefaciens* strain was inoculated into liquid YEP medium containing 50 mg/l kanamycin and 50 mg/l hygromycin and incubated for 24 h at 28°C with shaking.

### Inoculation, co-cultivation and selection

*A. tumefaciens* suspension was pelleted by centrifugation (2,500 g, 10 min) and then resuspended to $OD_{600}$ of 0.2 in liquid inoculation media which was consisted of MS medium containing 3% sucrose with or without acetosyringone. Embryogenic calli 3 to 5 mm in size were submerged into *A. tumefaciens* suspension and inoculation was carried out in the dark at 26°C for 3 h, with or without vacuum treatment, depending on the design of the experiment. Infected calli were blotted on sterile Whatman filter paper and then plated onto solid co-cultivation medium which consisted of MS medium containing 3% sucrose with or without acetosyringone. Co-cultivation was carried out in the dark at 26°C for 3 days. Following co-cultivation, a proportion of the calli were transferred to selection medium which consisted of N6 medium (Chu et al., 1975) containing 250 mg/l cefotaxime, 50 mg/l hygromycin, 1 g/l casein hydrolysate, 500 mg/l L-proline, 3 mg/l thiamine-HCl, 3% sucrose, 0.5% gelrite, 3 mg/l 2,4-D and 1 mg/l BAP. After 3 weeks, hygromycin resistant calli were selected and subsequently cultured at the same medium for 4 to 6 weeks. Regenerated shoots were transferred for rooting to one half-strength MS medium containing 50 mg/l hygromycin. After about 2 weeks, rooted plantlets were transferred to soil.

### GUS histochemical assay

Histochemical localization of GUS expression in the calli after co-cultivation and in leaves of putative transgenic plants was detected using the method of Jefferson (1987) with some modifications. Tissues were incubated for 48 h at 37°C in 50 mM sodium phosphate buffer pH 7.0 containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc). Tissues were then washed once with sterile distilled water and soaked in 70% ethanol for several days to remove chlorophyll.

### Southern blot analysis

Total genomic DNAs were isolated from leaf tissues from non-transgenic and putative transgenic plants according to the Murray and Tompson (1980). For Southern blot analysis, 30 μg of total DNA was digested with *Hin*dIII and electrophoresed on a 0.7% agarose gel and blotted onto a nylon membrane (Nytran-Plus, Schleicher and Schuell, Germany) by alkaline transfer method. To obtain a gene-specific probe for HPT gene, a DNA fragment was amplified by PCR from pIG121Hm as template. Primers used were 5'-CCTGAACTCACCGCGACG-3' and 5'-AAGACCAATGCGGAGCATATAC-3'. The PCR product was ligated into a pGEM-T vector (Promega, Madison, WI), sequenced, and used as a gene-specific probe. DNA blot hybridization and membrane washing were performed as previously described (Yeo et al., 2002).

### Statistical analysis

The significant difference among treatments was determined by Duncan's multiple range test using the Statistical Analysis System (SAS, 1988). Data are presented as means and standard errors.

## RESULTS AND DISCUSSION

### Effect of acetosyringone

Initially, the effect of the addition of acetosyringone to both inoculation and co-cultivation media were investigated. Acetosyringone is known to enhance the ability of *A. tumefaciens* to transform recalcitrant plant host (Godwin et al., 1991). We investigated four concentrations of acetosyringone (0 to 300 μM) in both inoculation and co-cultivation media. Addition of 100 μM acetosyringone to both inoculation and co-cultivation media resulted in the highest frequency of GUS expressing calli, and the differences were significant between this concentration and the other concentrations of acetosyringone (Table 1). Acetosyringone is a phenolic compound produced during wounding of plant cells that induces the transcription of the virulence genes of *A. tumefaciens*. Its beneficial role has been demonstrated in the genetic transformation of some monocotyledonous species, such as rice (Hiei et al., 1994),

**Table 1.** Effect of acetosyringone (AS) on transient GUS expression in mature seed-derived calli

| Concentration of AS (μM) | Number of calli inoculated | Number of calli expressing GUS | % of calli expressing GUS |
|---|---|---|---|
| 0 | 70 | 18.7±2.5[b] | 26.7±3.6[b] |
| 100 | 70 | 40.3±5.9[a] | 57.6±8.4[a] |
| 200 | 70 | 25.7±2.1[b] | 36.7±3.0[b] |
| 300 | 70 | 22.0±4.6[b] | 31.4±6.5[b] |

Data are recorded after 3 days of co-cultivation with *A. tumefaciens* strain EHA101/pIG121Hm.
Values represent the mean of three independent experiments. Acetosyringone was supplemented to both the inoculation and co-cultivation media. Different superscripts of same row indicate significant differences at $p<0.05$.

**Table 2.** Effects of *Agrobacterium tumefaciens* strain on transient GUS expression in mature seed-derived calli

| *A. tumefaciens* strain | Number of calli inoculated | Number of calli expressing GUS | % of calli expressing GUS |
|---|---|---|---|
| EHA101/pIG121Hm | 70 | 41.0±4.6[a] | 58.6±6.5[a] |
| GV3101/pIG121Hm | 70 | 31.7±3.1[b] | 45.2±4.4[b] |
| LBA4404/pIG121Hm | 70 | 22.0±2.6[c] | 31.4±3.8[c] |

Data are recorded after 3 days of co-cultivation. Values represent the mean of three independent experiments. Both the inoculation and co-cultivation media were supplemented with 100 μM acetosyringone. Different superscripts of same row indicate significant differences at $p<0.05$.

**Table 3.** Effects of duration of vacuum infiltration on transient GUS expression in mature seed-derived calli

| Duration of vacuum (h) | Number of calli inoculated | Number of calli expressing GUS | % of calli expressing GUS |
|---|---|---|---|
| 0 | 60 | 35.3±4.5[b] | 58.9±7.5[b] |
| 0.5 | 60 | 46.7±3.5[a] | 77.8±5.9[a] |
| 1 | 60 | 39.0±4.6[b] | 65.0±7.6[b] |
| 2 | 60 | 37.3±3.2[b] | 62.2±5.4[b] |

Data are recorded after 3 days of co-cultivation with *A. tumefaciens* strain EHA101/pIG121Hm. Values represent the mean of three independent experiments. Both the inoculation and co-cultivation media were supplemented with 100 μM acetosyringone. Vacuum applied at 600 mmHg. Different superscripts of same row indicate significant differences at $p<0.05$.

maize (Ishida et al., 1996), barley (Trifonova et al., 2001) and wheat (Wu et al., 2003). We have shown here that mature seed-derived calli of tall fescue plants responded positively to the presence of 100 μM acetosyringone both in inoculation and co-cultivation media. Acetosyringone was thus used at 100 μM in all subsequent experiments.

**Effects of *Agrobacterium* strain and vacuum infiltration**

To test the ability of different *A. tumefaciens* strains in transformation of mature seed-derived calli, we used three *A. tumefaciens* strains EHA101/pIG121Hm, GV3101/pIG121Hm and LBA4404/pIG121Hm as inoculums. Of the three *A. tumefaciens* strains tested, EHA101/pIG121Hm was found to be most effective followed by GV3101/pIG121Hm and LBA4404/pIG121Hm for transient GUS expression after 3 days co-cultivation (Table 2). Transformation efficiency by the EHA101/pIG121Hm strain was about twice that of LBA4404/pIG121Hm. Different combinations of bacterial strains and vectors have great effects on transformation efficiency in other monocotyledonous plants. In rice, Hiei et al. (1994) tested combinations of two strains and two vectors, showing that LBA4404/pTOK233 was more effective than EHA101/pIG121Hm and LBA4404/pIG121Hm. In wheat and barley, EHA101/pIG121Hm was much more efficient than LBA4404/pTOK233 and GV3101/pPCV6NFHGusInt (Guo et al., 1998). These results suggest that virulence of *A. tumefaciens* strains varies widely among plant hosts and is important for the transformation of recalcitrant monocotyledonous species. Our results indicate that EHA 101/pIG121Hm may be more suitable for transformation of tall fescue plants.

We investigated the effect of vacuum treatment during inoculation period on transformation efficiency in mature seed-derived calli of tall fescue. Vacuum treatments have been used as an aid in inoculation to infiltrate tissues with *A. tumefaciens* in *Arabidopsis* and other plant species (Clough and Bent, 1998; Amoah et al., 2001). Vacuum infiltration up to 30 min increased frequency of GUS expression in mature seed-derived calli (Table 3). Vacuum in excess 30 min duration, however, resulted in reduced frequency of GUS expression, even though the spots obtained were more intense. These results suggest that vacuum infiltration increases the number of *A. tumefaciens* coming into contact with plant cells, and then subsequently increases the number of cells receiving the T-DNA.

**Stable transformation efficiency**

To test the effect of *A. tumefaciens* strain on stable transformation, mature seed-derived calli (Figure 2A) were co-cultivated with EHA101/pIG121Hm for 3 days and then transferred to the selection medium containing 50 mg/l hygromycin. Beginning 3 weeks after co-cultivation, hygromycin-resistant calli were identified by their sustained growth on the selection medium, and were histochemically analyzed for stable expression of the GUS (Figure 2B).

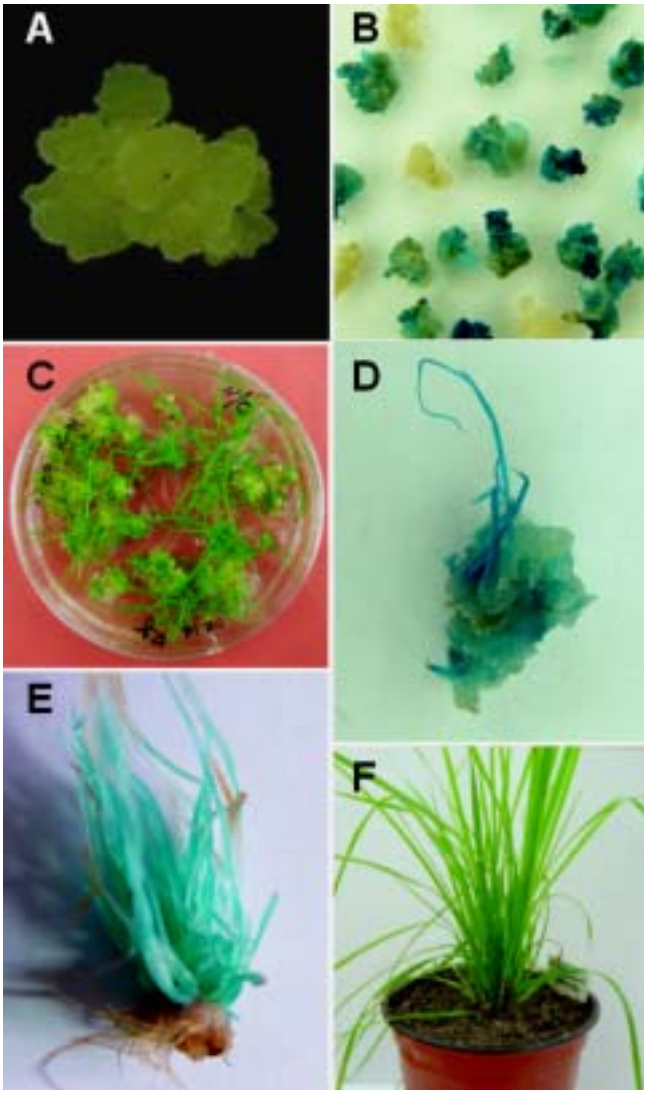


**Figure 2.** Tall fescue transformation mediated by *Agrobacterium tumefaciens*. (A) Mature seed-derived embryogenic callus used for *A. tumefaciens* co-cultivation; (B) GUS staining of hygromycin-resistant calli; (C) Regeneration of hygromycin-resistant tall fescue plantlets on the selection medium; (D) GUS staining of a shoot regenerated from hygromycin-resistant callus; (E) GUS staining of whole plant of transgenic tall fescue; (F) A transgenic plant established in the green house.

Results from 3 independent experiments are summarized in Table 4. Stable transformation efficiency in these experiments averaged 21.6% and ranged 18% and 24.6%. GUS histochemical assay of regenerated whole plant resulted in blue staining of entire tissues in most case (Figure 2D and E). The plantlets regenerated from hygromycin resistant calli (Figure 2C) were grown in the green house and all of them were normal in development and morphology (Figure 2F).

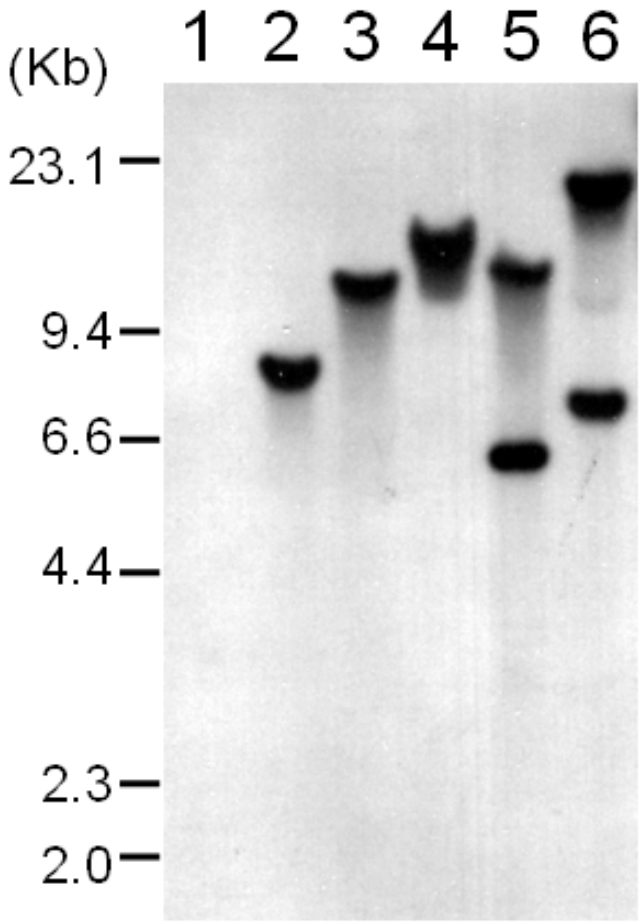


**Figure 3.** Southern blot analysis of transgenic tall fescue plants. *Hin*dIII-digested DNA samples were hybridized with HPT gene probe. Lane 1: non-transgenic plant; lanes 2-6, transgenic plants obtained with *A. tumefaciens* strain EHA101/pIG121Hm.

Southern blot analysis using the HPT gene probe was carried out on several regenerated plants (Figure 3). Since the T-DNA of pIG121Hm has only one *Hin*dIII site (Figure 1), the number of hybridizing bands reflected the number of copies of integrated transgene in transgenic tall fescue plants. Copy numbers of the transgene are varied among different plants: either one copy was integrated into the

**Table 4.** Stable transformation efficiency of tall fescue by *A. tumefaciens* strain EHA101/pIG121Hm

| Experiment | No. of calli inoculated (A) | No. of $Hyg^R$ calli | No. of regenerated $Hyg^R$ +GUS plants (B) | Transformation frequency (B/A, %) |
|---|---|---|---|---|
| 1 | 61 | 46 | 15 | 24.6 |
| 2 | 50 | 36 | 9 | 18.0 |
| 3 | 54 | 41 | 12 | 22.2 |
| Mean | 55 | 41±5.0 | 12±3.0 | 21.6±3.3 |

Both the inoculation and co-cultivation media were supplemented with 100 μM acetosyringone.
Vacuum applied at 600 mmHg for 30 min during inoculation.

genomic DNA of tall fescue (Figure 3, lanes 2, 3 and 4) or two copies were integrated (Figure 3, lanes 5 and 6). In non-transgenic plants, no hybridized band was detected (Figure 3, lane 1). All of the hybridized bands represented the fragments of more than 6 kb. The mobilities of the bands differed from plant to plant, indicating independent transformation events and random integration.

In conclusion, we have established a stable transformation system mediated by *A. tumefaciens* in commercial cultivar of tall fescue, Kentucky-31, using GUS as a reporter gene and HPT as a selectable marker with higher transformation efficiency. The transformation efficiency was 18 to 24.6% based on the experiments that produced the transgenic plants. To the best of our knowledge, this is the first report of *Agrobacterium*-mediated transformation of commercial cultivar of tall fescue using mature seed-derived calli as target tissue. Acetosyringone, vacuum infiltration and *A. tumefaciens* strain have a significant influence on transformation efficiency. The transformation system developed in this study will thus be useful for *Agrobacterium*-mediated genetic transformation of tall fescue plants with genes of agronomic importance.

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