

# High efficiency transformation of tall fescue with *Agrobacterium tumefaciens*

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## Abstract

An efficient genetic transformation system for tall fescue (*Festuca arundinacea* Schreb.), using *Agrobacterium tumefaciens*-mediated T-DNA delivery, is described. Seed-derived embryogenic calli were infected with *Agrobacterium tumefaciens* strain EHA105 harboring plasmids pTOK47 and pCAMBIA1301. Infected calli were selected at 250 mg L<sup>-1</sup> hyg B and the regenerated plantlets at 50 mg L<sup>-1</sup>. Using the protocol developed, 34% of the calli infected were hyg B resistant, and the overall plant transformation frequency (number of independently transformed plants over number of calli infected) was 8%. Stable integration of transgene into plant genome and GUS reporter gene expression were confirmed. Low transgene copies (1 or 2 as estimated) were observed in all the transgenic plants analyzed, and the transgene was transmitted to the progenies. Further experiments suggest an elevated 2,4-D concentration (5 mg L<sup>-1</sup>) used during callus culture and co-cultivation was partially attributable to the observed high transformation efficiency, while the inclusion of plasmid pTOK47 in *Agrobacterium* was not. The whole process from callus induction to transplanting green plantlets to the soil takes about six months, significantly shorter than the suspension culture approach often used in tall fescue transformation.

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**Keywords:** 2,4-D; *Agrobacterium tumefaciens*; Genetic engineering; Tall fescue; Transgenic plants

## 1. Introduction

Tall fescue is a widely used open-pollinated, perennial, cool-season turf and forage grass species [1]. Improvement on important agronomic traits, such as disease resistance and abiotic stress tolerance, would be very helpful for tall fescue as a grass crop. Genetic engineering has opened new avenues to the modification of turf and forage grasses, and provides us an alternative approach to meet specific breeding goals [2–4]. Establishment of high frequency transformation system would greatly facilitate the efforts for grass improvement via genetic engineering.

First attempts to introduce foreign DNA into tall fescue were through the protoplast [5–7]. Microprojectile bom-

bardment of embryogenic calli or suspensions was later used [8–10]. A recent correspondence reported *Agrobacterium tumefaciens*-mediated transformation of tall fescue [11]. In the report, the authors used the ‘super-binary’ vector system to infect tall fescue suspension cells and recovered two transgenic plants.

Plant transformation mediated by the soil-borne pathogen *Agrobacterium tumefaciens* was first reported in the 80’s [12]. Since then *Agrobacterium tumefaciens*-mediated transformation has been the standard method to genetically modify dicotyledonous plants. Repeatable and efficient *Agrobacterium*-mediated transformation of monocotyledonous plants was first demonstrated in rice a decade ago [13]. The key factors in the method were a ‘super-binary’ vector and the addition of acetosyringone to the co-cultivation medium. Subsequently, *Agrobacterium*-mediated transformation of maize [14,15], wheat [16], barley [17], sorghum [18], creeping bentgrass [19], and Italian ryegrass [11] were reported using similar approaches. *Agrobacterium*-mediated transformation is often preferred over other plant transfor-

**Abbreviations:** GUS,  $\beta$ -glucuronidase; hph, hygromycin phosphotransferase gene; hyg B, hygromycin B; X-Gluc, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide; 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; NAA,  $\alpha$ -naphthalene acetic acid; ABA, abscisic acid

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mation systems because of the simplicity, the low cost, and lower transgene copies integrated into plant genome [14]. Although in some cases, relatively high transformation frequencies were reported [13,15,16,18], *Agrobacterium*-mediated transformation of monocot plants often suffers from its inefficiency.

In this correspondence, we report establishment of an efficient *Agrobacterium*-mediated transformation system for tall fescue. In our experiments, average frequency of stable callus transformation was 34%, and the overall plant transformation frequency (number of independently transformed plants over number of calli infected) was 8%.

## 2. Materials and methods

### 2.1. Plant materials and tissue culture conditions

Approximately 5 g mature seeds of tall fescue cultivar “Matador” [20] or “Coronado” [21] were dehusked by stirring in 50% sulfuric acid for 30 min [22]. The dehusked seeds were rinsed with distilled water followed by 95% ethanol, and then surface-sterilized with stirring in full strength Clorox<sup>®</sup> (containing 6% sodium hypochlorite, Clorox, Oakland, CA) plus 0.1% of Tween-20 (Fisher, Fairlawn, NJ) for 30 min. After 10 times rinsing with distilled water, the seeds were sliced longitudinally and plated on callus induction medium which contains MS basal medium ingredients (Caisson Laboratories, Sugar City, Idaho) supplemented with 30 g L<sup>-1</sup> sucrose, 5 mg L<sup>-1</sup> 2,4-D, 0.05 mg L<sup>-1</sup> BAP, and 3.2 g L<sup>-1</sup> phytigel [23]. After 4 weeks, the induced calli were sub-cultured on the same medium. Four weeks later, light yellowish and compact embryogenic calli were chosen for *Agrobacterium tumefaciens*-mediated transformation.

### 2.2. *Agrobacterium* strains and vectors

The binary vector pCAMBIA1301 (<http://www.cambia.org.au>) was used in the transformation experiment. The T-DNA of pCAMBIA1301 includes a selectable marker gene construct for hyg B resistance and a construct of a GUS reporter gene containing the first intron of castor bean CAT-1 gene in GUS coding region [24] (Fig. 1A). The freeze-thaw method [25] was used to mobilize pCAMBIA1301 and another plasmid, pTOK47, into *Agrobacterium* strain EHA105 [25a]. Plasmid pTOK47 carries a 20 kb *Kpn*I fragment [26] of Ti plasmid from pTiBo542, which contains *virB*, *virC*, and *virG* virulence genes [27]. The resulted *Agrobacterium* strain, EHA105 (pTOK47, pCAMBIA1301), designated as ET1301, was grown in 50 ml YEP medium [25] in the presence of 20 mg L<sup>-1</sup> rifampicin, 5 mg L<sup>-1</sup> tetracycline, and 50 mg L<sup>-1</sup> kanamycin until OD<sub>595</sub> reached above 1.0. The bacteria were diluted with liquid MS medium to OD<sub>595</sub> = 0.4 for co-cultivation with tall fescue calli.

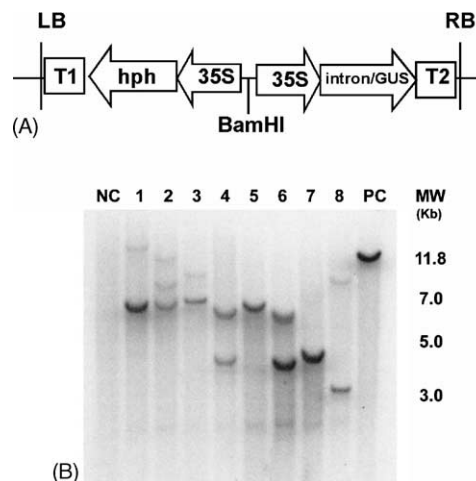


Fig. 1. Map of plant expression vector and southern blot analysis of transgenic plants (A) map of the T-DNA region containing intron-*gus* and *hph* plant expression cassettes in pCAMBIA1301. RB: right border, LB: left border, 35S: CaMV 35S promoter, intron/GUS: GUS coding region with intron insertion, *hph*: hygromycin phosphotransferase gene coding region, T1: CaMV35S terminator, T2: *Agrobacterium tumefaciens nos* gene terminator. Arrows indicate direction of transcription. The location of restriction site *Bam*HI used in Southern analysis is also indicated. (B) Southern blot analysis of transgenic plants using *hph* coding sequence as the probes. Genomic DNA was digested with *Bam*HI. NC, negative control, DNA from a non-transformed tall fescue plant; 1–8, DNA from eight putative transgenic plants, PC, positive control and reconstruction: plasmid DNA equivalent to one copy of *hph* gene in a 2C tall fescue genome [30], mixed with 25  $\mu$ g non-transgenic plant DNA; MW, molecular markers in kb.

### 2.3. Transformation procedure

Embryogenic calli were immersed in *Agrobacterium* suspension for 15 min, then transferred to callus induction medium supplemented with 100  $\mu$ M acetosyringone (Aldrich, Milwaukee, WI), in the dark at 25 °C for 3-day co-cultivation. The calli were then cultured on the “resting medium” (callus induction medium containing 200 mg L<sup>-1</sup> carbenicillin (Apollo Scientific, Stockport, UK) and 150 mg L<sup>-1</sup> Timentin (GlaxoSmithKline, Research Triangle Park, NC)) for a week at 25 °C in the dark to inhibit *Agrobacterium* growth as well as to have the calli recovered from co-cultivation “shock” [15]. The calli were then incubated on selection medium containing 150 mg L<sup>-1</sup> hyg B in dark at 25 °C for 4 weeks. Surviving calli were transferred to the same medium containing 250 mg L<sup>-1</sup> hyg B [9] for two more rounds of selection for a total of 4 additional weeks. Selection at 250 mg L<sup>-1</sup> hyg B is very tight for tall fescue and no escapes were obtained [9]. Hyg B resistant calli were then cultured on pre-regeneration medium, which is the basal MS medium supplemented with 1 mg L<sup>-1</sup> NAA, 1 mg L<sup>-1</sup> BAP, 5 mg L<sup>-1</sup> ABA, 30 g L<sup>-1</sup> sucrose, and 3.2 g L<sup>-1</sup> phytigel, for 1 to 2 weeks [31]. The calli were then transferred onto selective regeneration medium (MS basal medium, 30 g L<sup>-1</sup> maltose (Spectrum Chemical, Gardena, CA), 2.5 mg L<sup>-1</sup> BAP, 7.5 g L<sup>-1</sup> phytagar (GIBCO, Langley, OK) and 50 mg L<sup>-1</sup>

1 hyg B) in a lighted growth chamber at 25 °C under a 16/8 h (day/night) photoperiod (140  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of cool white fluorescent light). Regenerated shoots were transferred for rooting onto a half strength MS rooting medium containing 50 mg L<sup>-1</sup> hyg B and 0.5 mg L<sup>-1</sup> NAA in Magenta boxes (Magenta Corp., Chicago, IL). About 4 weeks later, rooted plants were transplanted into potting soil (Metro-Mix 200, Scotts, Marysville, OH), covered for the first few days with transparent lids for fast recovery. The plants were subsequently grown in a greenhouse at 25 °C. All the chemicals used in the experiments were purchased from Sigma (St. Louis, MO) unless otherwise specified.

#### 2.4. GUS histochemical assay and Southern analysis

GUS gene expression was confirmed by histochemical assays with X-gluc [28] as the substrate for the enzyme. Putative transgenic calli and leaves were immersed in the GUS assay buffer overnight at 37 °C and examined under a dissection microscope. For Southern analysis, genomic DNA were isolated from plant leaves based on the protocol of Dellaporta et al. [29] with the addition of DNase-free RNase A treatment (Sigma, 0.5 mg mL<sup>-1</sup>, 37 °C, 10 min). Twenty-five micrograms of genomic DNA from each sample was digested with *Bam*HI (Promega, Madison, WI) overnight and was subjected to electrophoresis in a 0.8% agarose gel. Plasmid DNA equivalent to one copy of *hph* gene in a 2C tall fescue genome [30], mixed with 25  $\mu\text{g}$  non-transgenic plant DNA was used as a transgene reconstruction/positive control. The fractionated DNA was transferred to a Hybond N<sup>+</sup> hybridization membrane (Amersham, Little Chalfont, Bucks, UK) according to the manufacturer's instructions. A 1.1 kb *Xho*I fragment of the pCAMBIA1301 containing the full-length coding region of the *hph* gene was used as a probe for Southern hybridization. The probe was labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP (Amersham) using the Primer-It<sup>®</sup> II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX) and hybridized with the blotted membrane in MiracleHyb<sup>™</sup> hybridization solution (Stratagene) according to the manufacturer's instructions.

#### 2.5. Inheritance study of the transgenic plant

Transgenic plant ETR3-6 was naturally vernalized in the field during winter and the flowers were pollinated with pollen grains from non-transgenic 'Coronado' plants in the following summer inside a greenhouse. The seeds from the

crossing were collected from ETR3-6 and germinated in half MS medium without addition of phytohormones. To test the hyg B resistant phenotype, the plantlets were transferred to the same medium containing hyg B (50 mg L<sup>-1</sup>).

In PCR assays of *hph* gene, genomic DNA was extracted from the offspring seedlings based on the protocol of Dellaporta et al. [29]. The two primers used were as previously described [31] and the expected size of the amplified fragment is 592 bp. HotM Taq DNA polymerase (Eppendorf, Westbury, NY) was used in a standard 35-cycle reaction with annealing temperature of 58 °C. The positive control for PCR used 1 ng plasmid CAMBIA1301 as the template while the negative control used genomic DNA extracted from a non-transgenic plant.

### 3. Results and discussion

#### 3.1. Establishment of an efficient *Agrobacterium*-mediated transformation system of tall fescue

Two-month-old, light yellowish, compact calli were used as explants for transformation experiments. A total of 300 pieces of calli of cv. "Matador" was co-cultivated with *Agrobacterium* strain ET1301 in the three transformation experiments reported here (Table 1). Spotted transient GUS expression was observed on calli after co-cultivation (Fig. 2A). Since "intron-GUS" gene is used in the construct, the observed expression indicated the T-DNA delivery into the plant cells. During the selection period on hyg B (150 mg L<sup>-1</sup>), a majority of calli gradually turned brown whereas some yellowish hyg B resistant calli were observed after three to four wks selection (Fig. 2B). These calli were subjected to a higher-level selection (250 mg L<sup>-1</sup> hyg B). The resistant calli were then subjected to the regeneration process. In each of the three transformation experiments, 30–40% calli showed resistance to the hyg B selection (Table 1). A total of 24 such resistant calli regenerated into green plantlets (Fig. 2D, Table 1). No albino plantlets were observed in the experiments. All the plantlets developed good root system in the selective rooting medium (Fig. 2E) and survived transplantation (Fig. 2G).

#### 3.2. Analyses of transgenic plants

GUS expression was observed from resistant calli and plants (Fig. 2C and F). All the 24 resistant plants were GUS

Table 1  
Results of three experiments of *Agrobacterium*-mediated tall fescue transformation (cv. "Matador")

Experiment	Total no. of callus infected	Hyg B resistant callus (%)	Regenerated plants	GUS+ plants	Overall transformation frequency (%)
1	120	33.30	7	7	5.83
2	90	33.30	1	1	1.10
3	90	36.70	16	16	17.78
Total	300	34.30	24	24	8.00

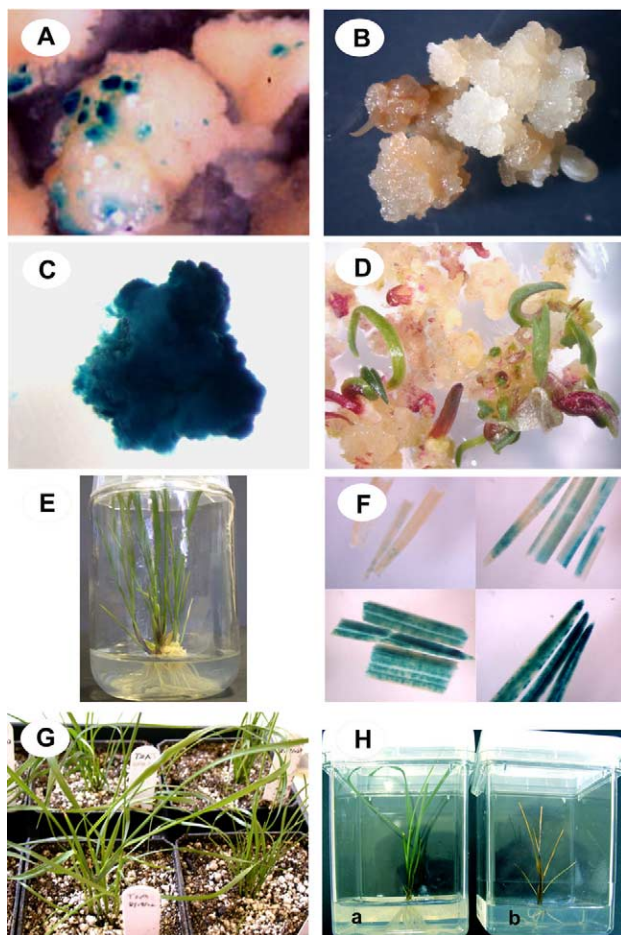


Fig. 2. *Agrobacterium tumefaciens*-mediated transformation of tall fescue with hyg B selection and inheritance of hyg B resistance to T1 offspring plants. (A) Transient GUS expression in the infected cells; (B) a resistant callus growing on hyg B selection; (C) stable GUS gene expression in an hyg B resistant callus; (D) plantlet regeneration under hyg B selection; (E) root development of a hyg B resistant plantlet in the selection rooting medium; (F) various levels of GUS gene expression in different transgenic plants; (G) transgenic plants growing in soil; (H) response of progenies of transgenic plant ETR3-6 to hyg B (a, an offspring plant resistant to hyg B; b, an offspring plant susceptible to hyg B); the photo was taken after two-month selection.

positive (Table 1) although the GUS expression levels varied among the plants (Fig. 2F).

Southern analysis was performed on 23 putative transgenic plants and the transgenic nature of these plants was confirmed, suggesting the tightness of the selection. The hybridization results of eight such plants were shown in Fig. 1B. The various positions of the hybridized *hph* gene among the analyzed plants indicated stable integration of the transgene into plant genome. Comparing to the transgenic tall fescue plants obtained from microprojectile bombardment [9], transgenic plants recovered from *Agrobacterium*-mediated transformation had simpler hybridization patterns and were estimated to have one or two transgene copies. Because of this, it is sometimes difficult to judge whether two plants with similar hybridization patterns were from the same or different transformation events, such as in the case

of lanes 4 and 6. To solve the confusion, the same blot was re-hybridized with GUS gene probe. Distinct hybridization patterns between the two lanes were observed suggesting the two plants derive from two independent transformation events (data not shown).

*Agrobacterium*-mediated transformation in monocots is often genotype dependent [14]. To see whether our protocol also applies to other cultivars, we used the protocol to transform cultivar ‘Coronado’. Among 1846 pieces of calli infected, 31.5% developed into resistant calli under  $250 \text{ mg L}^{-1}$  hyg B selection, indicating the high transformation efficiency was also achieved for ‘Coronado’, and the protocol may apply to other cultivars.

In the inheritance study, among the ten germinated plants from the offspring seeds obtained from crossing on transgenic plant ETR3-6, six were resistant to hyg B (Fig. 2H(a)) and GUS positive (data not shown), while the other four were hyg B sensitive (Fig. 2H(b)) and GUS negative. The observation and PCR analysis of the offspring plants for *hph* gene (data not shown), suggest the transgene be inherited to the offspring plants. The segregation ratio was near 1:1, an indication that the transgene(s) was inserted at a single genetic locus.

### 3.3. Analyses of factors that may affect tall fescue transformation efficiency

Plasmid pTOK47, containing an extra copy of *virB*, *virC*, and *virG* virulence genes [27], respectively, was shown to enhance *Agrobacterium*-mediated transformation in white spruce [27] and loblolly pine [32]. Although the *Agrobacterium* strain used in the experiments contains pTOK47, its role in the observed high transformation frequency was not clear. To address this question, the strain EHA105 (pCambia1301), designated as E1301, was constructed. E1301 is the same as ET1301 except that it does not contain pTOK47. The two were compared in an experiment for transformation efficiency, where 240 pieces of calli (cv. ‘Matador’) were infected by each strain, respectively. Almost identical numbers of hyg B resistant calli were obtained from the two treatments (102 from E1301 versus 101 from ET1301), suggesting that pTOK47 do not play a substantial role in the observed high transformation frequency, and E1301 is sufficient and equally efficient to ET1301 in tall fescue transformation. However, inclusion of pTOK47 does not have a detrimental effect either.

In addition, experiments were performed to evaluate the role of 2,4-D concentration on the observed high transformation efficiency. In the replicated experiments, 647 pieces of callus (cv. ‘Matador’) were cultured, co-cultivated, and selected on media containing  $5 \text{ mg L}^{-1}$  2,4-D, while 640 pieces of callus were on media containing  $2 \text{ mg L}^{-1}$  2,4-D throughout. In  $5 \text{ mg L}^{-1}$  treatment, 25.2% calli were resistant to hyg B whereas only 17.3% were hyg B-resistant for the  $2 \text{ mg L}^{-1}$  treatment. ANOVA and *F* test [33] indicated that the difference between the two treatments was

significant ( $p = 0.0363$ ), suggesting that 2,4-D concentration in the medium play an important role in the efficient transformation. 2,4-D stimulates cell division and is the most commonly used growth regulator to induce callus and maintain callus growth in grass tissue culture [34]. It is possible that at  $5 \text{ mg L}^{-1}$  2,4-D, more cells are actively dividing and are thus more competent for *Agrobacterium* infection [35]. Alternatively, elevated 2,4-D may somehow make the host cells more competent for transformation [36], other than cell division. The possibility that the elevated 2,4-D may help the transformed cells recover [37], or even facilitate the infection process by *Agrobacterium*, cannot be excluded either. To our knowledge, this is the first report regarding an effect of 2,4-D concentration on *Agrobacterium*-mediated transformation efficiency. It remains to be seen whether this approach can be applied to other plant species to improve their transformation efficiencies. Since long-term exposure to high level of 2,4-D may have adverse effects on callus regeneration, the period of high-level 2,4-D treatment will need to be optimized.

It seems the callus transformation frequency was more consistent from batch to batch using this protocol than the plant transformation frequency in the experiments. Thus, more attention will be needed to maintain the callus regeneration ability during selection. Overall, our system yields 34% hyg B resistant calli and has 8% overall transformation efficiency. That is comparable to the rice transformation system [13] and is among the best of *Agrobacterium*-mediated transformation systems in monocot species. The whole process, spared from the need of time-consuming establishment of suspension cultures, only takes approximately six months from callus induction from mature seeds to transplantation of transgenic plantlets to the soil.

In conclusion, this correspondence reports an efficient and reliable *Agrobacterium*-mediated transformation system using two elite tall fescue cultivars. Southern analysis confirmed that the transgenes were integrated into the genome of the hyg B-resistant plants, and that the individual plants were from independent transformation events. This system has significant advantages over the previous reported ones on tall fescue transformation, including high transformation efficiency, low transgene copy number in plants, simple procedure, and a shorter period to recover transgenic plants. The low transgene copies could help reduce the possibility of gene silencing and increase the stability of the transgenes [38]. The efficiency and reliability of the reported transformation system make it possible to generate a large number of transgenic tall fescue plants in a relatively short period, and to test the performance of various transgenes for tall fescue improvement.

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