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# *Agrobacterium*–mediated High-efficiency Transformation of Creeping Bentgrass with Herbicide Resistance

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**We performed *Agrobacterium*-mediated genetic transformation of creeping bentgrass (*Agrostis stolonifera* L.) and produced herbicide-resistant transformants from commercial cultivars Crenshaw and Penncross. Seed-derived embryogenic calli were infected with *A. tumefaciens* EHA105 harboring pCAMBIA 3301, which includes an intron-containing *gus* reporter and a *bar* selection marker. To establish a stable system, we examined various factors that could potentially influence transformation efficiency during the pre-culture, infection, and co-cultivation steps. The addition of kinetin to the callus pre-culture media increased efficiency about three-fold. Once the optimum infection and co-cultivation conditions were identified, this protocol was used successfully to bulk-produce herbicide-resistant transgenic plants whose herbicide resistance was confirmed using the BASTA® resistance test. Southern blot analysis demonstrated integration and low copy numbers of the integrated transgenes, and northern blot analysis verified their expression. Thus, we have established an efficient genetic transformation system for creeping bentgrass and confirmed a high frequency of single-copy transgene integration and functional gene expression.**

*Keywords*: *Agrobacterium*, bentgrass, herbicide, resistance, transformation, turfgrass

Creeping bentgrass (*Agrostis stolonifera* L.) is economically important as the principal turfgrass species for golf course greens and fairways in temperate climates around the world (Bonos et al., 2000; Warnke, 2003). For vigorous growth, creeping bentgrass often requires daily irrigation and weekly spraying with pesticides or fungicides. Both practices are expensive and the latter may cause environmental pollution. Because this crop is also highly susceptible to weed competition, genetics approaches have been taken to develop herbicide-resistant cultivars that reduce maintenance costs (Asano et al., 1997; Toyama et al., 2003; Wang et al., 2003). Although conventional breeding has been used to improve turfgrass traits, its success has been limited by barriers to sexual reproduction and the relatively long time periods required for such research programs. More recently, genetic transformation methods have been adopted for more efficient introduction of useful traits from a broader range of sources and within an economically viable time frame (Lee, 1996; Chai and Stichlen, 1998; Wang et al., 2001; Zilinskas and Wang, 2004; Wang and Ge, 2006).

Transgenic turfgrass plants have been produced via microprojectile bombardment of DNA-coated particles into callus cells, and by *Agrobacterium*-mediated transformation (Zhong et al., 1993; Asano et al., 1997; Yu et al., 2000; Chai et al., 2003, 2004; ; Luo et al., 2004; Dong and Qu, 2005; Fu et al., 2005a, b, 2007; Han et al., 2005; Wang and Ge, 2005; Ge et al., 2006, 2007). In addition to the considerable time and expense required, the former method has certain disadvantages, including a low frequency of success and the insertion of multiple gene copies, which is thought to correlate with gene silencing. The latter approach overcomes some of these disadvantages, such as transgene copy number and cost (Dai et al., 2001). Transformation is an important tool for improving the performance of crop species and effective techniques should allow for the incorporation of foreign genes that reduce crop requirements for water and chemical sprays. The first transgenic creeping bentgrass was generated by microprojectile bombardment of the embryogenic callus (Zhong et al., 1993; Hartman et al., 1994). Since then, its transformation has been achieved via both biolistics and DNA uptake by protoplasts (reviewed by Wang et al., 2001). Because previous attempts at *Agrobacterium*-mediated transformation of this crop resulted in only a limited number of independent lines (Yu et al., 2000; Chai et al., 2003; Fu et al., 2005a), it remained necessary to develop a more efficient method that would generate a large number of transgenic plants.

The objective of our study was to develop an *Agrobacterium*–mediated high-efficiency transformation system and to produce herbicide-resistant creeping bentgrass plants from two commercial cultivars through the use of *A. tumefaciens* co-cultures and seed-derived embryogenic calli. Herbicide-resistant cultivars developed in this study would be useful for controlling unwanted weeds and allowing easier maintenance of golf courses and lawns, thereby reducing the number and amount of agrochemicals required and decreasing the environmental pollution.

## MATERIALS AND METHODS

### Plant Materials

Seeds for two commercial cultivars ('Penncross' and 'Crenshaw') of creeping bentgrass (*Agrostis stolonifera* L.)

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**Figure 1.** T-DNA region of binary vector plasmid pCAMBIA 3301. RB, right border; LB, left border; 35S, CaMV 35S promoter; intron/GUS, GUS coding region with catalase intron insertion; *bar*, phosphinotricin acetyltransferase gene coding region; T1, CaMV 35S terminator; T2, *A. tumefaciens nos* gene terminator. Arrows indicate directions of transcription. *Eco*RI and *Bam*HI restriction sites used in Southern analysis are indicated.

were purchased from KVBio Inc., and stored at 4°C before use.

### *Agrobacterium* Strain and Plasmid used for Transformation

*Agrobacterium tumefaciens* EHA105 containing the binary vector pCAMBIA 3301 (www.cambia.org) was used for transformation of the creeping bentgrass cultivars. pCAMBIA 3301 carries the *bar* gene for herbicide resistance as a selectable marker, as well as an intron-containing β-glucuronidase gene (*intron-gus*) as a reporter; both genes are under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1). The *intron-gus* reporter gene contains a catalase intron within the coding sequence, which ensures that *gus* expression is derived from eukaryotic cells and not from residual *A. tumefaciens*. The *bar* gene encodes a phosphinotricin acetyltransferase that confers resistance to the herbicides phosphinotricin (PPT, or glufosinate) and bialaphos (phosphinotricyl-alanyl-alanine) by inhibiting glutamine synthase, thus, interfering with amino acid synthesis. We used the freeze-thaw method to introduce binary vector DNA into *A. tumefaciens* EHA105 (Chen et al., 1994).

### Conditions for the Induction of Embryogenic Calli

Mature seeds were de-husked and surface-sterilized in 70% (v/v) ethanol for 10 min, then in 2% (w/v) sodium hypochlorite (NaOCl) for 20 min with shaking. After rinsing in sterile distilled water five times, the seeds were placed on a callus induction medium (CIM) containing MS basal salts and vitamins (Murashige and Skoog, 1962), and which was supplemented with 30 g $L^{-1}$ sucrose or maltose, 2 mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma, USA), and 3 g $L^{-1}$ Gelrite. To test the effect of phytohormones on the embryogenic callus in some experiments, we substituted 6.6 mM Dicamba (Duchefa, The Netherlands) for 2,4-D in the CIM. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Culture plates were maintained in the dark at 25°C for 4 weeks, and induced calli were sub-cultured to fresh media every 2 weeks. Embryogenic calli were selected visually and pre-cultured on CIM, with or without 0.5 mg $L^{-1}$ kinetin, for 1 week prior to *Agrobacterium*-mediated transformation. To test their potential, we transferred the induced calli onto a plant regeneration medium (MS basal salts supplemented with 3% (w/v) sucrose and solidified with 0.3 % (w/v) Gelrite). To determine transformation efficiencies, we performed GUS assays and calculated the percentage of calli with blue spots.

### *Agrobacterium*-mediated Transformation and Optimization

*A. tumefaciens* EHA105 harboring pCAMBIA 3301 was used for transformation of induced calli. The bacterial cultures were grown at 28°C in a liquid Luria-Bertani (LB) medium (Difco, USA) with shaking (200 rpm). At $OD_{600}$ = 0.5, cells were harvested by centrifugation at 3000 rpm for 15 min, then re-suspended in an equal volume of infection medium for 4 h. Different media were examined (AAM, MS, or 1/2 MS) along with a variety of pH levels (5.2, 5.5, or 5.8) and carbon sources (3% sucrose or 2% sucrose plus 1% glucose), plus vitamins and 100 μM acetosyringone. The treated embryogenic calli were then immersed in *Agrobacterium* suspensions and incubated for 10 min with gentle shaking, followed by the removal of excess bacteria and air-drying on filter paper. Infected calli were then transferred to a co-cultivation medium and incubated in the dark at 25°C for 3 d. This medium also was optimized through tests of MS vs. 1/2 MS, pH (5.2 vs. 5.8), and carbon source (3% sucrose vs. 2% sucrose plus 1% glucose), in addition to the vitamins, 2 mg $L^{-1}$ 2,4-D, and 100 μM acetosyringone.

After 3 d of co-cultivation, the infected calli were transferred onto a CIM supplemented with 5 mg $L^{-1}$ phosphinotricin (Duchefa) and 250 mg $L^{-1}$ cefotaxime, before being cultured under darkness for 2 weeks. For plant regeneration, pieces of embryogenic calli were cultured on the same medium for 8~10 weeks under appropriate lighting (100 $mmol \cdot m^{-2} \cdot s^{-1}$). PPT-resistant green shoots were then transferred to plastic vessels containing a hormone-free MS medium, 10 mg $L^{-1}$ PPT, and 250 mg $L^{-1}$ cefotaxime. Plantlets with well-developed roots were placed in soil and grown under greenhouse conditions.

### Staining for GUS Activity

β-Glucuronidase (GUS) activity in the transformed calli was assayed by histochemical staining with 2 mM 5-bromo-4-chloro-3-indolyl-3-glucuronic acid (X-Gluc), as described previously (Jefferson, 1987). Following their selection, the PPT-resistant calli were incubated in a GUS assay solution at 37°C overnight. This solution was then discarded and the calli were bleached sequentially with 50, 70, and 100% ethyl alcohol, followed by incubation at 4°C. After the pigments were removed, GUS-positive signals (blue spots) were counted manually using a light microscope.

### Herbicide Resistance Test

To screen our transgenic bentgrass plants, we grew them in soil for 2 weeks in the greenhouse prior to spraying them with BASTA®. Herbicide resistance was determined after another 1 week. These trials covered a range of concentrations -- 0.4 to 6.0% BASTA® -- that was equivalent to a normally recommended 1× application (0.4% BASTA) to a 15× concentration, respectively.

### Genomic PCR and Southern Blot Analysis

Total genomic DNA was isolated from the leaves of greenhouse-grown plants, as described previously (Prince et al., 1993). The coding regions for the *bar* transgenes were PCR-

amplified from either genomic DNA or a positive-control vector, using the following sets of oligonucleotide primers: *bar*, 5-TACATCGAGACAAGCACGGTCAACTT-3 (forward) and 5-TGCCAGAAACCCACGTCATGCCAGTT-3 (reverse). Two rice actin primers, 5-AACTGGGATGATATGGAGAA-3 (forward) and 5-CCTCCAATCCAGACACTGTA-3 (reverse), served as loading controls for the genomic DNA. PCR products encoding *bar* and *actin* were expected to be 421 and 1046 bp, respectively. The reaction mixtures (50 μL) contained 200 ng genomic DNA or 5 ng pCAMBIA 3301 plasmid DNA (positive control), 400 μM dNTPs, 200 μM of each primer, and 2 units EX Taq DNA polymerase (Takara, Japan). Reactions began with a hot start at 94°C for 7 min; then 30 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min. The amplification products were analyzed by electrophoresis on 0.8% agarose/ethidium bromide gels.

For Southern blot analysis, genomic DNA was digested with either *Eco*RI or *Bam*HI, which were single cutting restriction enzymes for the specific binary vector (Fig. 1). DNA (20 μg per lane) was separated using 0.8% agarose gel electrophoresis and transferred to Hybond $N^+$ membranes (Amersham Biosciences, UK) according to standard protocols (Sambrook and Russell, 2001). For probes, the *bar* gene was isolated from pCAMBIA 3301 by restriction digestion and then labeled with [$\alpha^{32}$P] dCTP using the Radiprime™ II Random Prime Labeling System (Amersham Biosciences, UK). Unincorporated nucleotides were removed with Microspin™ G-50 columns (Amersham Biosciences, UK). Southern hybridizations were performed via Quick Hyb® (Stratagene, USA), diluted in 5 × SSC at a 1:1 ratio. Pre-hybridization, hybridization, and washing steps were carried out at 65°C. After hybridization, the membranes were washed twice (5 min each) in washing solution I (2× SSC, 0.1% SDS), followed by 5-min washes with washing solution II (1× SSC, 0.1% SDS) and washing buffer III (0.5× SSC, 0.1% SDS). The final wash was performed in 0.1× SSC and 0.1% SDS for 15 min with agitation. Afterward, the membranes were exposed at –70°C to X-ray film (AGFA, Belgium) for 2 to 3 d.

### Northern Blot Analysis

Total RNA was extracted from plant leaves using Trizol® reagent, according to the manufacturer's instructions (Invitrogen, USA). Total RNA (10 μg) was separated on 1.2% denaturing agarose gels in the presence of formaldehyde. Blotting, hybridization, and washing of positively charged nylon membranes (Hybond $N^+$) were conducted according to the manufacturer's instructions. The same [$\alpha^{32}$P] dCTP-labeled *bar* probe was used for both Southern and northern hybridizations.

## RESULTS AND DISCUSSION

### Induction of Embryogenic Calli for Efficient Genetic Transformation

One of the most important factors in determining the success of a plant transformation and regeneration protocol is

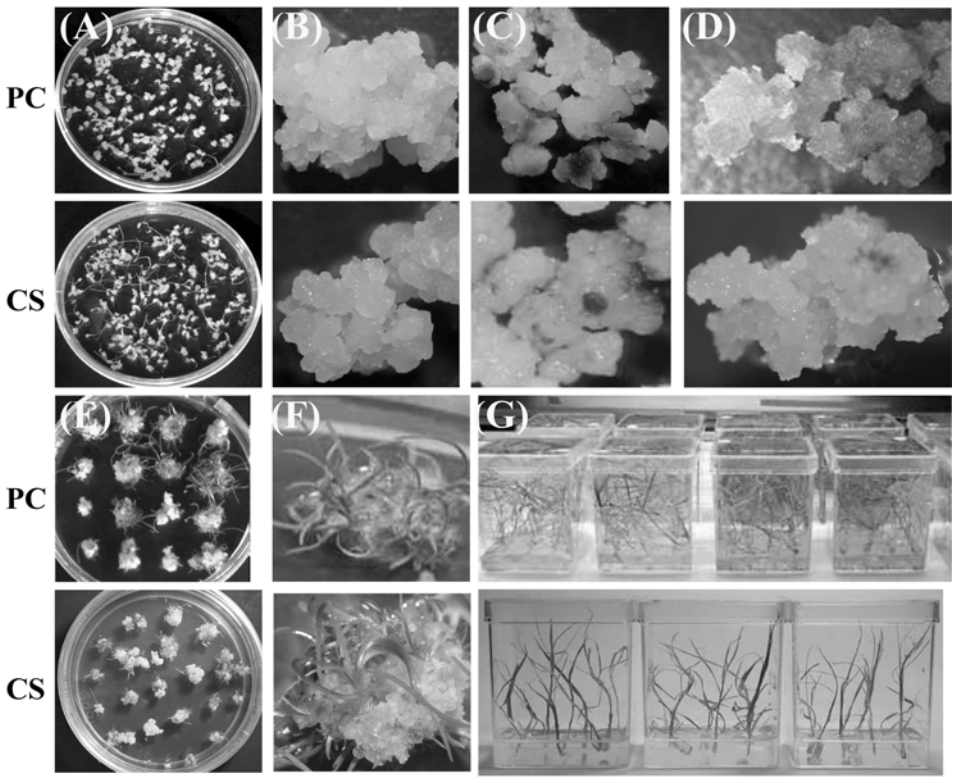


**Figure 2.** Production of transgenic creeping bentgrass cultivars, Penncross (PC) and Crenshaw (CS). **A**, Embryogenic callus induced from mature seeds on callus induction medium; **B**, Magnification of induced embryogenic callus; **C**, Transient GUS expression is visible on surfaces of early globular embryos after 3 d of *Agrobacterium* co-cultivation; **D**, PPT-resistant calli during selection with 5 mg $L^{-1}$ PPT; **E** and **F**, Shoot formation of PPT-resistant calli; **G**, Rooted transgenic plantlets selected with 10 mg $L^{-1}$ PPT.

**Table 1.** Regeneration rates of induced calli.

| Callus induction | | Regeneration rate (%) (with hormone-free media) | |
|---|---|---|---|
| Media composition | Carbon source | 'Crenshaw' | 'Penncross' |
| MS + 2 mg $L^{-1}$ 2,4-D | + 3% sucrose | 86.7 (52/60) | 93.3 (56/60) |
| MS + 2 mg $L^{-1}$ 2,4-D | + 3% maltose | 76.7 (46/60) | 83.3 (50/60) |
| MS + 6.6 mg $L^{-1}$ Dicamba + 0.5 mg $L^{-1}$ BA+ 500 mg $L^{-1}$ casein hydrolysate | + 3% sucrose | 90.0 (54/60) | 86.7 (52/60) |

**Table 2.** Effect of adding hormones and kinetin during the pre-culture step for induction of transformable embryogenic calli[a].

| Conditions[b] | No. calli infected | No. GUS-positive calli | % GUS-positive calli |
|---|---|---|---|
| 2,4-D | 75 | 10 | 13.3 |
| 2,4-D + Kinetin | 100 | 33 | 33.0 |
| Dicamba | 75 | 10 | 13.3 |
| Dicamba + Kinetin | 100 | 31 | 31.0 |

[a]The induced embryogenic calli treated with 2,4-D or Dicamba were selected visually and pre-cultured on a callus induction medium under the specified conditions for 1 week prior to *Agrobacterium*-mediated transformation.
[b]2,4-D = 2.0 mg $L^{-1}$; Dicamba = 6.6 mM; Kinetin = 0.5 mg $L^{-1}$.

the efficient induction and maintenance of embryogenic calli (Kim et al., 2001; Zilinskas and Wang, 2004; Chung and Park, 2005). Here, we were able to obtain high-quality, friable embryogenic calli from the seeds of 'Penncross' (PC) and 'Crenshaw' (CS) creeping bentgrass (Fig. 2A-C). These induced calli were transferred to a hormone-free plant regeneration medium, where they exhibited regeneration potentials of 86.7% (CS) and 93.3% (PC) (Table 1). During callus induction, neither carbon source (maltose vs. sucrose) nor hormone conditions (Dicamba or BA) led to improved regeneration rates when compared with the combination of MS supplemented with 2 mg $L^{-1}$ 2,4-D, 3% (w/v) sucrose and 0.3% (w/v) Gelrite. With these calli with high regeneration potentials, conditions for efficient transformation were then investigated.

To investigate factors that could influence transformation efficiency, GUS assays were conducted for embryogenic calli prepared under different induction conditions. The factors investigated here included the concentration of acetosyringone, cell density of *Agrobacterium*, pre-incubation of *Agrobacterium* in acetosyringone-containing media before infection, and infection time. The best results were achieved when we used 100 mM acetosyringone, an *Agrobacterium* density of $OD_{600}$ = 0.5, pre-incubation for 4 h, and an infection time of 10 min (data not shown). Therefore, we applied those criteria toward further establishing the optimum conditions for pre-culturing the callus prior to *Agrobacterium* infection, as well as conditions during infection and co-cultivation. We first monitored the effects of hormone and kinetin treatment during pre-culture, and found that, whereas both 2,4-D and Dicamba similarly induced transformable embryogenic calli, the addition of 0.5 mg $L^{-1}$ kinetin caused a *ca.* 3-fold increase in such induction (Table 2). Furthermore, the greatest transformation efficiencies were obtained with half-strength MS media, a pH of 5.2,

**Table 3.** Optimization of *Agrobacterium* infection conditions.

| | Infection conditions | No. calli infected | No. GUS-positive calli | % GUS-positive calli |
|---|---|---|---|---|
| Medium[a] | AAM | 50 | 2 | 4 |
| | MS | 50 | 4 | 8 |
| | 1/2 MS | 50 | 16 | 32 |
| pH[b] | 5.2 | 50 | 20 | 40 |
| | 5.5 | 50 | 14 | 28 |
| | 5.8 | 50 | 8 | 16 |
| Carbon source[c] | 3% sucrose | 50 | 4 | 8 |
| | 2% sucrose + 1% glucose | 50 | 18 | 36 |

[a]The infection conditions included vitamins, 2% sucrose, 1% glucose, and 100 μM acetosyringone, in the presence of different media types (pH 5.2). [b]Conditions included 1/2 MS salts, vitamins, 2% sucrose, 1% glucose, and 100 μM acetosyringone, at different pH values Conditions included 1/2 MS salts, vitamins, and 100 μM acetosyringone, in the presence of different carbon sources (pH 5.2).

**Table 4.** Optimization of co-cultivation conditions.

| | Infection conditions | No. calli infected | No. GUS-positive calli | % GUS-positive calli |
|---|---|---|---|---|
| Medium[a] | MS | 50 | 24 | 48 |
| | 1/2 MS | 50 | 16 | 32 |
| pH[b] | 5.2 | 50 | 20 | 40 |
| | 5.8 | 50 | 26 | 52 |
| Carbon source[c] | 3% sucrose | 50 | 4 | 8 |
| | 2% sucrose + 1% glucose | 50 | 20 | 40 |

[a]The infection conditions included vitamins, 2 mg $L^{-1}$ 2,4-D, 2% sucrose, 1% glucose, and 100 μM acetosyringone, in the presence of different media types (pH 5.8). [b]Conditions included MS salts, vitamins, 2 mg $L^{-1}$ 2,4-D, 2% sucrose, 1% glucose, and 100 μM acetosyringone, at different pH values. [c]Conditions included MS salts, vitamins, 2 mg $L^{-1}$ 2,4-D, and 100 μM acetosyringone, in the presence of different carbon sources (pH 5.8).

and 2% sucrose plus 1% glucose (Table 3). The highest percentage of GUS-positive calli approached 40%, which likely represents the near maximum number of transformable embryogenic calli. In tests of the optimum co-cultivation conditions, the best performance was obtained with full-strength MS media (pH 5.8) that contained 2% sucrose and 1% glucose (Table 4).

Based on the above results, we concluded that the optimum protocol for inducing embryogenic calli, as well as for *Agrobacterium* infection and co-cultivation, had to involve the following steps: 1) pre-culture prior to transformation,

**Table 5.** Optimum conditions for efficient *Agrobacterium*-mediated transformation of creeping bentgrass.

| Transformation step | Medium | Supplements |
|---|---|---|
| Callus induction | MS | 2 mg $L^{-1}$ 2,4-D, 3% sucrose, 0.3% Gelrite; pH 5.8 |
| Callus pre-culturing | MS | 2 mg $L^{-1}$ 2,4-D, 3% sucrose, 0.5 mg $L^{-1}$ kinetin, 0.3% Gelrite; pH 5.8 |
| *Agrobacterium* infection | 1/2 MS | 2 mg $L^{-1}$ 2,4-D, 2% sucrose, 1% glucose, 100 μM acetosyringone; pH 5.2 |
| Co-cultivation | MS | 2 mg $L^{-1}$ 2,4-D, 2% sucrose, 1% glucose, 100 μM acetosyringone, 0.3% Gelrite; pH 5.8 |
| PPT selection | MS | 2 mg $L^{-1}$ 2,4-D, 3% sucrose, 250 mg $L^{-1}$ cefotaxime, 5 mg $L^{-1}$ PPT, 0.3% Gelrite; pH 5.8 |
| Shoot induction | MS | 3% sucrose, 250 mg $L^{-1}$ cefotaxime, 5 mg $L^{-1}$ PPT, 0.3% Gelrite; pH 5.8 |
| Root induction | MS | 3% sucrose, 250 mg $L^{-1}$ cefotaxime, 5 mg $L^{-1}$ PPT, 0.3% Gelrite; pH 5.8 |
| Plant regeneration | MS | 3% sucrose, 250 mg $L^{-1}$ cefotaxime, 10 mg $L^{-1}$ PPT, 0.3% Gelrite; pH 5.8 |

treatment with 2 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ kinetin; 2) *Agrobacterium* infection, use of half-strength MS (pH 5.2) that contained 2% sucrose and 1% glucose; and 3) co-cultivation of calli with *Agrobacterium*, use of full-strength MS (pH 5.8) that contained 2% sucrose plus 1% glucose (Table 5). Both 'Crenshaw' and 'Penncross' exhibited similar results (Fig. 2), hence, it is likely that these conditions could be applied to the transformation of other creeping bentgrass cultivars.

## Establishment of an Efficient *Agrobacterium*-mediated Transformation System for Creeping Bentgrass

Using the above optimized conditions, we performed *Agrobacterium*-mediated genetic transformation of 'Crenshaw' and 'Penncross' (Fig. 2). Because we used the herbicide resistance gene (*bar*) as a selection marker, transformation efficiency was estimated from PPT (phosphinotricin)-resistant shoots or BASTA®-resistant transgenic plants. PPT is the active component of bialaphos (phosphinotricyl-alanyl-alanine), which is a non-selective and broad-spectrum contact herbicide also known as glufosinate or Finale™ (Wehrmann et al., 1996; Lohar et al., 2001). BASTA® is a commercial herbicide containing 18% glufosinate. Thus, bialaphos (i.e., PPT or BASTA®) resistance in turfgrass is achieved by introducing *bar*, which encodes the detoxifying enzyme phosphinotricin acetyltransferase. During incubation of *Agrobacterium*-infected calli on selection media containing 5 mg $L^{-1}$ PPT, only PPT-resistant calli grew and appeared green in color (Fig. 2D). In contrast, PPT-sensitive calli died and turned dark yellow (see Fig. 2D for 'Penncross'). On shoot induction media, multiple shoots usually emerged from PPT-resistant calli (Fig. 2E-F). After we moved them to a root induction medium containing 10 mg $L^{-1}$ PPT, we observed root development on our PPT-resistant plants (Fig. 2G). Plantlets with well-developed roots were transferred to soil, grown in the greenhouse for 2 weeks, and then tested for herbicide resistance by spraying them with BASTA®. Most plants selected during the transformation process exhibited herbicide resistance, whereas the non-transgenic controls died (Fig. 3).

The efficiency of *Agrobacterium*-mediated transformation can be defined in several ways. In most cases, it is calculated as the percentage of all infected calli that produce PPT-resistant shoots. Our results, obtained from four independent transformation trials, demonstrated efficiency rates of 18.0 to 37.2% (Table 6), which are higher than most of those previously reported (Yu et al., 2000; Chai et al., 2003, 2004; Luo et al., 2004; Fu et al., 2005a). In addition, the rate for 'Penncross' was higher than for 'Crenshaw'. However, a number of false positives may have been included in this first determination. Specifically, some shoots that emerged on the PPT-containing selection media died after they were sprayed with herbicide. Therefore, we calculated a more precise assessment of efficiency from the number of herbicide-resistant plants obtained from all infected calli. This ranged from 13.5 to 25.6%, still a level higher than previously reported (Table 6). Thus, we conclude that we have established a protocol for stable and highly efficient *Agrobacterium*-mediated transformation of creeping bentgrass, and have obtained herbicide-resistant transformants from two commercial cultivars.

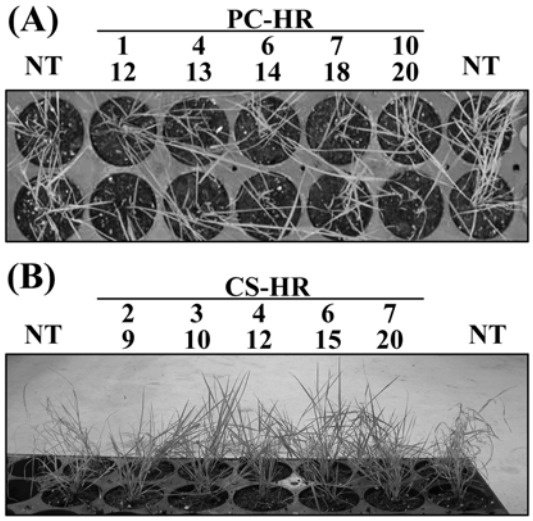


**Figure 3.** Herbicide resistance assays of putative transgenic plants from 'Penncross' (**A**) and 'Crenshaw' (**B**) that were randomly selected and sprayed directly with 0.4% BASTA®. Photos were taken 7 d after herbicide application. Numbers represent selected transgenic lines. NT, non-transgenic creeping bentgrass used as control; PC-HR, 'Penncross' with herbicide resistance; CS-HR, 'Crenshaw' with herbicide resistance.

## Herbicide Resistance in Transgenic Plants

All of our transgenic bentgrass plants appeared normal under greenhouse conditions and, with the exception of their herbicide resistance, were morphologically indistinguishable from the untransformed wild-type control plants. To verify the insertion of *bar*, we performed PCR assays on genomic DNA extracted from the leaves of representative

**Table 6.** *Agrobacterium*-mediated transformation efficiencies for creeping bentgrass.

| Cultivar | No. infected calli | No. PPT-resistant shoots (%)[a] | No. herbicide-resistant plants | Transformation efficiency (%) from PPT resistance[b] | Transformation efficiency (%) from Southern analysis[c] |
|---|---|---|---|---|---|
| Penncross | 250 | 93 (37.2%) | 64 | 25.6 | 6.40 (16 lines) |
| Penncross | 375 | 110 (29.3%) | 68 | 18.1 | 5.33 (20 lines) |
| Crenshaw | 400 | 83 (20.8%) | 54 | 13.5 | 9.50 (38 lines) |
| Crenshaw | 450 | 81 (18.0%) | 67 | 14.9 | 7.55 (34 lines) |

[a]The percentage was calculated using the number of PPT-resistant shoots obtained from all infected calli. [b]Percent transformation efficiency was calculated after BASTA®-spraying, using the number of herbicide-resistant plants obtained from all infected calli. [c]Percent transformation efficiency was calculated after Southern blot analysis using the projected number of independent lines obtained from all infected calli.

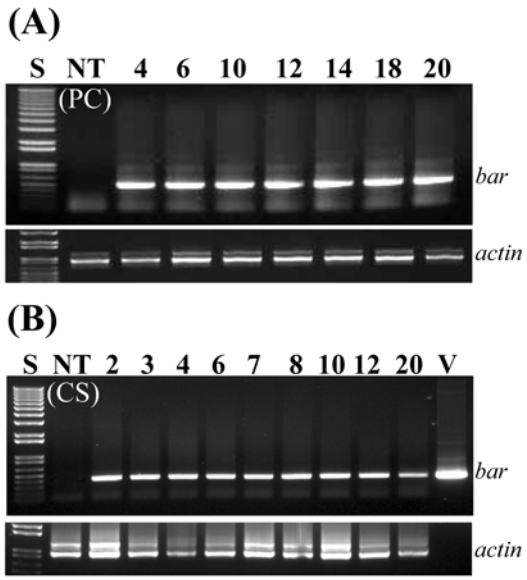


**Figure 4.** Genomic PCR analysis of putative transgenic creeping bentgrass plants. PCR-amplification of *bar* from 'Penncross' (**A**) and 'Crenshaw' (**B**). Lane S, standard DNA markers; Lane NT, non-transgenic plant as control. Numbers in lanes represent transgenic lines selected for analyses. *actin* PCR products were used as loading controls.

plants from each independent transformation event. Integration of the transgene was observed in all resistant plants (Fig. 4). Herbicide spray assays were conducted to evaluate *bar* expression, in which regenerated plants were treated with 0.4 to 6.0% BASTA®. Following treatment, the untransformed wild-type control plants died within 1 week, whereas the transformants exhibited resistance at all concentrations tested (Fig. 5). These results indicate that *bar* is expressed well in these transgenic turfgrass plants, and that it functions *in vivo* against BASTA® concentrations of up to 6%.

## Molecular Analysis of Transgenic Plants and Estimation of Transformation Efficiency

Southern blot analysis was performed to assess the stability of integration of the *bar* gene in the independent transformation events. Total DNA extracted from leaf tissue was digested with either *Eco*RI or *Bam*HI; Southern blots were prepared and then probed with the labeled *bar* gene. Hybridizing bands detected the different transgene integration sites and represented an estimate of transgene copy number in the grass genome. Southern analysis was performed on several randomly selected, independent transgenic plants (Fig. 6). Most contained one or two genomic

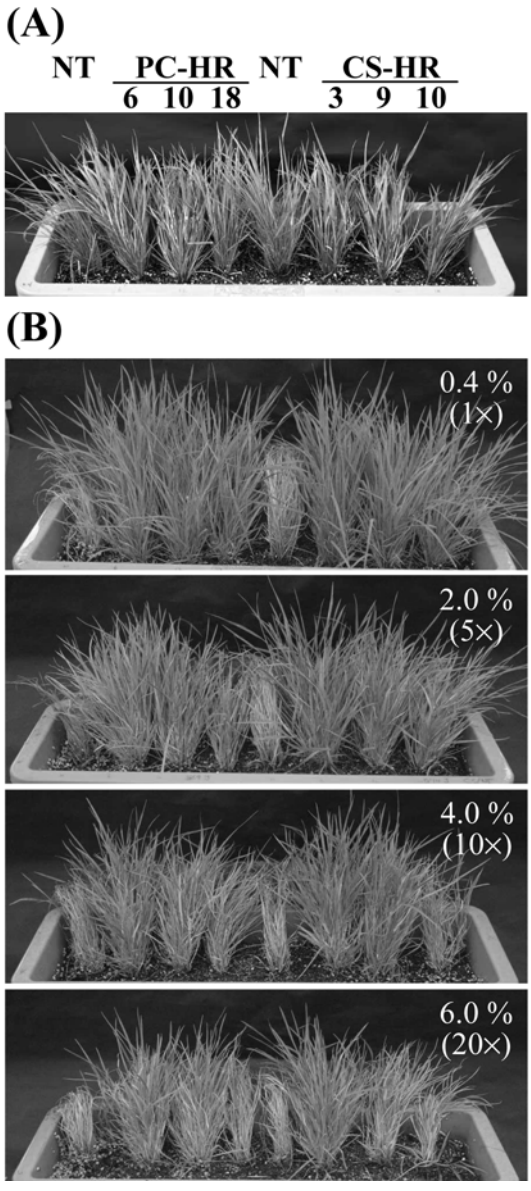


**Figure 5.** Herbicide resistance of transgenic plants grown in greenhouse, prior to treatment (**A**) and after herbicide applications (**B**). NT, non-transgenic creeping bentgrass used as control. Numbers represent selected transgenic lines. To test resistance, 0.4 to 6.0% BASTA® was applied to transgenic plants. Photos were taken 7 d after herbicide application. Recommended 1× concentration of BASTA® is 0.4%.

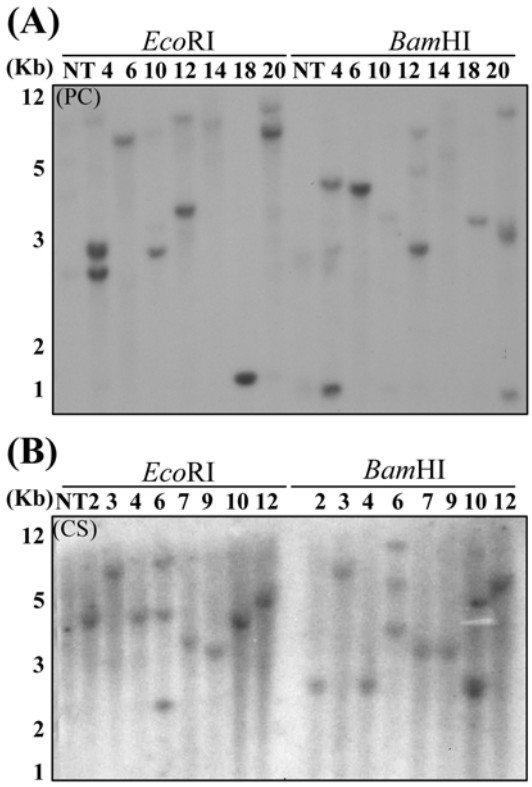


**Figure 6.** Southern blot analysis of representative transgenic plants. Genomic DNA from 'Penncross' (**A**) and 'Crenshaw' (**B**) was digested with either *Eco*RI or *Bam*HI; *bar* gene was used as probe. NT, non-transgenic control plant.

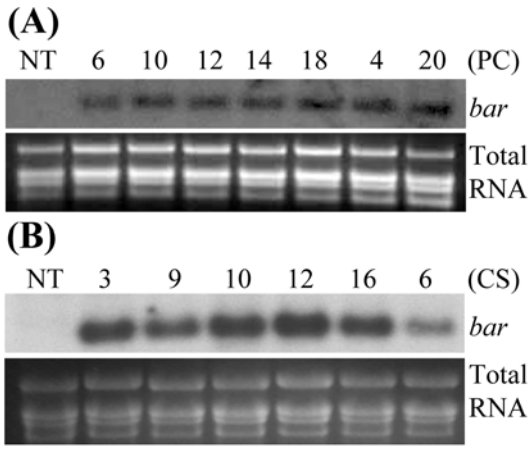


**Figure 7.** Northern blot analysis of representative transgenic plants. Total RNA was isolated from leaves of 'Penncross' (**A**) and 'Crenshaw' (**B**); *bar* gene was used as probe. NT, non-transgenic control plant.

copies, whereas no hybridization signal was detected from the non-transformed control. Many of our independent transformants had identical integration sites, probably due to multiple shoot formation during plant regeneration. Our data indicated that 20 of the 'Penncross' transgenics corresponded to 5 and 6 independent integration events, i.e., to 16 and 20 independent lines, respectively, that arose from the two transformation experiments (Table 6, last column). These results are comparable to the previous identification of 6 independent lines from 24 transgenic plants (Fu et al., 2005a). In contrast, the 20 transgenic 'Crenshaw' plants analyzed in our two transformation experiments corresponded to 14 and 10 independent integration events, which would be projected to generate 38 and 34 independent lines, respectively (Table 6). This all suggests that 36 independent 'Penncross' lines could be obtained from 132 transgenic plants (27.3%) and 72 independent 'Crenshaw' lines from 121 transgenic plants (59.5%). The difference in numbers of lines might have been because, during transformation and selection, more multiple shoots were generated from each embryogenic 'Penncross' callus than from those of 'Crenshaw', and these multiple shoots may have generated transgenic plants with identical integrations. Southern blot analysis indicated that about two-thirds of the independent lines carried a single-copy transgene insertion into the grass genome, with no apparent rearrangement in either cultivar, whereas the remaining one-third contained more than one insertion.

Previously, most bentgrass transformation efficiencies were estimated using hygromycin- or herbicide-resistant shoots (or plants) (Yu et al., 2000; Chai et al., 2003; Fu et al., 2005a). However, those calculations may have identified plants containing the same transgene insertion(s) as being independent lines, thereby resulting in the inclusion of false positives. Therefore, if we considered the number of independent lines identified via Southern blots, the actual transformation efficiency could then be calculated by dividing the number of independent lines by the number of infected calli (Table 6, last column). By doing so, this calculation removed additional plants originating from a single transformed callus (i.e., plants with the same insertion site for the *bar* gene) and provided the true rate of efficiency. Adopting this method led to transformation averages of 5.9% ('Penncross') and 8.5% ('Crenshaw'), suggesting that one real and independent transgenic creeping bentgrass plant could be obtained from every 12 to 17 calli. We placed about 40 calli on a culture plate for embryogenic callus induction. Therefore, it is clear that only a few plates of induced calli would be required for generating multiple independent lines of transgenic plants that contained the target gene(s). Although our transformation protocol is very efficient, the success of transformation was higher from 'Penncross' calli than from 'Crenshaw', with data of 33.25% vs. 19.40% and 21.85% vs. 14.20%, for PPT-resistant shoots and BASTA®-resistant plants, respectively. However, because the actual transformation efficiencies calculated from independent lines were 5.9% ('Penncross') and 8.5% ('Crenshaw'), we can conclude that the latter cultivar would be better suited for genetic manipulation.

Finally, northern blot analysis was performed to confirm the expression of *bar* and to determine any variations in its expression level. We selected lines containing one or two copies of the integrated transgene and, in general, found similar expression (Fig. 7), although lines containing two copies exhibited slightly elevated transcript levels.

In conclusion, we have developed a highly stable and efficient *Agrobacterium*-mediated genetic transformation system for creeping bentgrass, and have generated herbicide-

resistant lines in two commercial cultivars. A number of independent lines were obtained with this protocol, and molecular analysis confirmed that many of these contained only a single genomic integration of the transgene, which was expressed in a stable manner. In addition, all of the transgenic herbicide-resistant turfgrass plants were morphologically normal. The overall transformation frequency varied according to the method used for its calculation. In four independent experiments that examined the number of PPT-resistant shoots, efficiencies ranged from 18.0 to 37.2%. In contrast, when the number of herbicide-resistant plants was considered, the numbers fell to between 13.5% and 25.6%. For the first time, we have also calculated efficiency with respect to the number of independent lines produced and, using this more accurate determination, have found that efficiencies are from 5.33 to 9.50%. The development of this high-efficiency transformation system for creeping bentgrass should help in the introduction of useful traits from a broad range of sources within a relatively short time frame. Such genetic modifications will enhance the production of a high-value crop and provide a useful tool for controlling unwanted weeds, thereby allowing for easier maintenance of golf courses and lawns and reducing the number and amount of pollution-generating agrochemicals required.

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