GENETIC TRANSFORMATION OF ELITE TURF-TYPE CULTIVARS OF TALL FESCUE

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ABSTRACT

Biotechnology approaches can supplement conventional breeding efforts for turfgrass improvement. The objective of this study was to develop reliable transformation and selection schemes for elite turf-type tall fescue cultivars. Transgenic tall fescue (Festuca arundinacea Schreb.) plants were obtained by microprojectile bombardment of embryogenic suspension cells of two elite turf-type cultivars, ‘Coronado’ and ‘Virtue’. Two selection systems were successfully used in the transformation. A bialaphos-based selection used plasmid pAHC25 that contains phosphinothricin acetyltransferase (bar) and β-glucuronidase (GUS) genes both driven by the maize ubi1 promoter. A two-step solid medium selection with increasing bialaphos concentration was used to obtain resistant callus. Alternatively, a hygromycin B (hyg B)-based selection strategy was employed using plasmid pAcH1 containing a hygromycin phosphotransferase (hph) gene driven by the rice Act 1 gene promoter. A three-step liquid medium or a two-step solid medium selection was used to obtain resistant callus. Additional selection in the rooting medium eliminated escapes. Expression of the bar and GUS genes was demonstrated by resistance to the herbicide ‘Finale’ and by GUS activity assays, respectively. The transgenic nature of the resistant plants was confirmed by Southern hybridization analysis.

INTRODUCTION

Tall fescue is an open-pollinated, perennial, cool-season grass species widely used for forage and turf [Buckner et al., 1979]. Many new cultivars have been developed [National Turfgrass Evaluation Program (NTEP), 1996]. Further improvements on traits such as disease resistance would be helpful for tall fescue as a turfgrass species.

Successful transformation of tall fescue was initially accomplished by using the protoplast method. DNA was introduced using either electroporation [Wang et al., 1992; Dalton et al., 1995] or polyethylene glycol (PEG) [Ha et al., 1992]. The procedures involved in protoplast preparation and regeneration from protoplasts were cumbersome and technically difficult. Transformation methods for suspension cells included microprojectile bombardment [Spangenberg et al., 1995] and silicon carbide whiskers [Dalton et al., 1998]. Microprojectile bombardment was also used for callus transformation [Cho et al., 1999].

All tall fescue transformation work reported so far has employed hygromycin B (hyg B) as the selection agent and the hygromycin phosphotransferase (hph) gene as the means to confer resistance to this antibiotic. One report also used the bar gene, coding for phosphinothricin acetyltransferase, to confer resistance to phosphinothricin [Wang et al., 1992], an analogue of L-glutamic acid, which is a potent inhibitor of glutamine synthetase [De Block et al., 1987]. This selectable marker also confers resistance to the herbicide bialaphos, which is a tripeptide containing phosphinothricin with two alanine residues. Transgenic tall fescue plants selected with bialaphos has not been reported.

Intensive breeding efforts in the past decades have resulted in a number of elite turf-type tall fescue cultivars [e.g., NTEP, 1996]. The ability to directly transform these cultivars could accelerate the application of biotechnology approaches in tall fescue breeding. In an evaluation of tissue culture responses of 23 elite turf-type tall fescue cultivars [Bai and Qu, 2000], two cultivars, ‘Coronado’ [Fricker et al., 1999] and ‘Virtue’[C. Fricker, pers. comm.], performed well in their regeneration abilities. The objective of this study was to develop a reliable transformation protocol for these elite turf-type tall fescue cultivars using microprojectile bombardment [Klein et al., 1987; Sanford, 1988] and two selective agents, hyg B and bialaphos.

MATERIALS AND METHODS

Establishment and maintenance of suspension cultures

Approximately 5 g mature seeds of the cultivars ‘Coronado’ or ‘Virtue’ were dehusked by stirring in 500 ml L⁻¹ sulfuric acid for 20 min [Lowe and Conger, 1979]. The dehusked seeds were rinsed with distilled water followed by 700 ml L⁻¹ ethanol, then surface-sterilized, with stirring, in full strength Clorox® (containing 52.5 g L⁻¹ sodium hypochlorite) plus approximately 1 ml L⁻¹ of Tween-20 detergent (Fisher Chemical, Fairlawn, NJ) for
20 min. After five times rinsing with sterile distilled water, the seeds were plated on a callus induction medium which contains a MS (Murashige and Skoog, 1962) basal medium components supplemented with 30 g L\(^{-1}\) sucrose, 5 mg L\(^{-1}\) 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 mg L\(^{-1}\) BAP (6-benzylaminopurine) and 3 g L\(^{-1}\) phytagel. Cultures were kept in the dark at 25°C for callus induction. After four weeks, induced calli were subcultured on the same medium except that the 2,4-D concentration was lowered to 2 mg L\(^{-1}\). Four weeks later, white, knobby, very compact embryogenic calli were selected to initiate suspension cultures. Approximately ten pieces of embryogenic calli, each derived from a single seed, were transferred to a 125-ml Erlenmeyer flask containing AA liquid medium [Müller and Grafe, 1978] supplemented with 2 mg L\(^{-1}\) 2,4-D, 20 g L\(^{-1}\) sucrose and 30 g L\(^{-1}\) D-sorbitol. The suspension cultures were maintained in the dark at 25°C on an orbital shaker at 120 rpm. Once a week, the culture medium was replaced with fresh medium.

Suspension cells took 4 to 8 weeks to multiply substantially to become established. Suspension cells established in a flask was considered a line. The regeneration ability of a suspension line was tested by transferring cells to a regeneration medium containing MS basal medium, 30 g L\(^{-1}\) sucrose, 2 mg L\(^{-1}\) 2,4-D, and 3 g L\(^{-1}\) phytagel; and 2.5 mg L\(^{-1}\) BAP. Environmental conditions in the incubator (CU-32L, Percival, Boone, IA, USA) used for regeneration included a 16-hr light /8-hr dark cycle with a constant 25°C temperature. All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. Cultured cells of suspension lines with good regeneration ability were split and transferred into other flasks to multiply. These lines were subsequently used for transformation experiments. A suspension line was terminated if its cell clusters failed to regenerate on regeneration medium.

**Transformation**

The plasmid used to introduce the bar gene and GUS (β-glucuronidase) reporter gene was pAHC25; both genes on this plasmid were under the transcriptional control of the maize ubi1 promoter [Christensen and Quail, 1996] (Fig. 1A). Plasmid pAcH1 [Spangenberg et al., 1995] carrying a hph gene under the control of the rice act1 5' regulatory sequence was used in experiments designed to confer hyg B resistance (Fig. 1B).

The transformation experiments were performed using a PDS-1000/He Biolistic® particle delivery system (Bio-Rad, Hercules, CA, USA). Based on preliminary experiments using transient GUS gene expression levels as an indicator, 1550 psi (pound per square inch) helium pressure, two shots per target plate, and 1.0 µm gold particles were chosen for all bombardment experiments. Gold particles were coated with plasmid DNA based on the manufacturer’s recommendation (Bio-Rad). A 42.5 mm Ø filter paper disk (Whatman, Maidstone, England) was placed in the middle of a plate containing osmotic medium (MS basal salts, 0.6 M D-mannitol, 30 g L\(^{-1}\) sucrose, 2 mg L\(^{-1}\) 2,4-D, and 3 g L\(^{-1}\) phytagel; Vain et al, 1993). Suspension cells, approximately 2 ml of packed cell volume, were transferred onto the filter paper four hours prior to bombardment and were left on the same medium for 3 to 4 days before transfer to the selection medium.

**Selection of stable transformed colonies and recovery of transgenic plants**

For the gene construct containing the bar gene, bombarded cells were transferred onto a solid subculture medium (MS basal medium, 30 g L\(^{-1}\) sucrose, 2 mg
L-2, 4-D, 0.1 mg L-1 BAP, and 3 g L-1 phytagel) containing 3 mg L-1 bialaphos (Meiji Seika Kaisha Ltd., Tokyo, Japan). After four weeks selection in the dark at 25°C, surviving calli were transferred to a fresh selection medium that contained 5 mg L-1 bialaphos for another four weeks. After the second round selection, actively growing, bialaphos-resistant calli were transferred either to a pre-regeneration medium that was a MS basal medium supplemented with 1 mg L-1 NAA (α-naphthalene acetic acid), 1 mg L-1 BAP, and 5 mg L-1 ABA (abscisic acid) for 2 to 3 weeks [L. Li, pers. comm.] or were plated directly on the regeneration medium (MS basal medium, 30 g L-1 sucrose, 2.5 mg L-1 BAP, and 3 g L-1 phytagel) that contained 2 mg L-1 bialaphos. Regenerated shoots were transferred for rooting onto a half-strength MS, hormone-free rooting medium containing 4 mg L-1 4-D, 0.1 mg L-1 BAP, and 0.5 mg L-1 IAA (α-naphthalene acetic acid) for another four weeks. For 'solid medium selection', the bombarded suspension cells were transferred to the MS subculture medium containing 50 mg L-1 bialaphos for four weeks. For 'liquid medium selection' and 'solid medium selection' schemes were evaluated. With the 'liquid medium selection' scheme, bombarded cells were transferred into liquid AA medium containing 50 mg L-1 hyg B (Roche, Indianapolis, IN, USA) in 6-cm culture containers (190 mL, Greiner GmbH, Nürtingen, Germany) and were kept at 25°C in the dark on an orbital shaker at 60 rpm for two weeks. After an additional two weeks selection in AA medium with 100 mg L-1 hyg B, the suspension cells were transferred onto a solid MS selection medium containing 250 mg L-1 hyg B for four weeks. For 'solid medium selection', the bombarded suspension cells were transferred to the MS subculture medium containing 100 mg L-1 hyg B for selection in the dark at 25°C for four weeks. Surviving calli were transferred to the same medium containing 250 mg L-1 hyg B for an additional four weeks selection. Actively growing hyg B-resistant calli were selected and transferred either to the pre-regeneration medium for 2 to 3 weeks or directly to the regeneration medium containing 50 mg L-1 hyg B. Regenerated shoots were transferred to rooting medium containing 50 mg L-1 hyg B in a Magenta box. After about four weeks, rooted plants were transplanted to the potting soil as described above.

All plantlets derived from a single resistant callus and survived the rooting medium selection were considered a putative transgenic plant.

Southern hybridization analysis of putative transgenic plants

Genomic DNA was extracted from leaf tissues of non-transformed and putative transgenic tall fescue plants based on the protocol of Dellaporta et al. [1983] with the addition of an RNase A treatment (0.5 mg mL-1, 37°C, 30 min). Twenty five μg genomic DNA from each sample, undigested or digested with restriction endonucleases BamHI or PstI (GIBCO-BRL, Rockville, MD, USA), was separated by electrophoresis in a 0.8% agarose gel. Plasmid DNAs equivalent to one copy in a 2C tall fescue genome [Arumuganathan et al., 1999] were used as positive controls (34.5 pg for pAHC25 or 17.3 pg for pACH1, respectively, together with 4.9 μg Herring sperm DNA as carrier). The fractionated DNA was transferred to a GeneScreen™ hybridization transfer membrane (NEN Research Products, Boston, MA, USA) according to instructions of the manufacturer. Hybridization was carried out following standard protocols [Sambrook et al., 1989]. The hph probe was a full-length coding region of the gene within a 1.1 kb SmaI fragment of the plasmid pMON410 (map not shown). The bar gene probe was a 0.6 kb PstI fragment of pAHC25, that covers the full length coding region of the gene, while the GUS gene probe was a 1.9 kb fragment of pAHC25 digested with BamHI and SstI, which covers the full length coding region of the gene. The probes were labeled with [32P]dCTP (NEN) by random hexamer priming with Amersham Multiprime DNA Labeling System (Amersham Pharmacia Biotech., Little Chalfont, England) according to the manufacturer's instructions.

Histochemical GUS activity assay of transgenic plants

GUS assay buffer was prepared and filter sterilized according to Jefferson [1987]. Leaves of putative transgenic plants transformed with plasmid pAHC25 were immersed in the GUS buffer overnight and examined under a stereo microscope for the blue 'staining' of leaf tissue as a result of the GUS activity.

Bialaphos resistance testing of transgenic plants

Cotton swabs were dipped in a freshly-prepared 1/500 dilution of a commercial herbicide Finale® (AgrEvo USA, Montvale, NJ) and used to paint marked leaf segments of tall fescue plants of non-transgenic or plants transformed with the bar gene. Finale® contains 11% glufosinate (phosphinothricin), which is the active ingredient. The leaf responses to the herbicide application were recorded a week later.

RESULTS AND DISCUSSION

Isolation of resistant calli and regeneration of putative transgenic plants

A total of 140 plates were bombarded in 12 separate transformation experiments. Various number of plates from six ‘Virtue’ and 23 ‘Coronado’ suspension cell lines were used in these experiments (Table 1). In eight transformation experiments, the hph gene was used as the selectable marker and the bar gene was used for the remaining four experiments. Resistant calli were...
obtained from four 'Virtue' and seven 'Coronado' suspension lines used in the experiments. A total of 115 resistant calli were recovered, among which 54 regenerated into green plantlets and 7 albino ones (Table 1). Of the 61 putative transgenic plants, 6 plants (4 green, 2 albino) were transformed with the \textit{bar} gene, and 55 (50 green, 5 albino) with the \textit{hph} gene. By cultivars, 5 transgenic plants (2 green, 3 albino) were obtained from 'Virtue' and 56 (52 green, 4 albino) were 'Coronado'-derived.

A 'solid medium selection' scheme was used for the four \textit{bar} gene transformation experiments and four of the eight \textit{hph} gene transformation experiments. After four weeks selection in the dark, most of the cultured cells were totally inhibited in growth and turned dark brown. Surviving calli were transferred to a second selection medium for an additional four weeks selection. Some of the calli that survived the first selection were eliminated during the second cycle of selection as they turned dark in color and stopped growing.

The results of these experiments indicated that selection using bialaphos was quite efficient and could be carried out with much lower bialaphos concentrations when compared to the selection using phosphinothricin (100 mg L\textsuperscript{-1}) [Wang et al., 1992].

A 'liquid medium selection' scheme was used for the remaining four \textit{hph} gene transformation experiments. After four weeks selection, the suspension cells looked dark in color and were inhibited in growth. After an additional four weeks selection on the solid medium, the transformed cells grew into a white or light yellow colored callus, and were easily distinguished from the background of dead tissues in the plate (Fig. 2).

Two distinct groups of resistant calli were recognized from both selection schemes. The first group of calli had solid, compact structures. The second type of calli was soft in texture. Calli from the latter group were first transferred to a pre-regeneration medium for 2 to 3 weeks before the transfer to the regeneration medium. The combination of ABA, NAA, and BAP in the pre-regeneration medium exhibited a remarkable effect on

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<th>Table 1. Transformation results of tall fescue suspension cells using bialaphos and hyg B selection</th>
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† Eighteen other suspension lines (2 of 'Virtue' and 16 of 'Coronado') were used for transformation without recovery of resistant calli.
‡ Cells were transformed with plasmid pAHC25 containing the \textit{bar} gene.
§ Cells were transformed with plasmid pAcH1 containing the \textit{hph} gene.

Figure 2. Growing resistant calli four weeks after being transferred to the solid selection medium containing 250 mg L\textsuperscript{-1} hyg B after liquid selection (cv. 'Coronado').
enhancing the soft calli to develop compact structures.

Inclusion of an appropriate concentration of selective agents in the rooting medium proved to be a critical step in distinguishing the true transgenic plants from the non-transgenic escapes for both bar and hph selection schemes. Both leaves and roots of the resistant plants grew well in the selective rooting medium. Preliminary experiments showed that a small percentage of non-transformed cells could survive the 5 mg L\(^{-1}\) bialaphos or 250 mg L\(^{-1}\) hyg B secondary selection and regenerate into shoots. When these shoots were transferred to rooting medium containing the corresponding selective agents, however, they either could not develop roots at all or merely developed a few roots without root hairs. Their leaves turned yellow (Fig. 3) and the plants eventually died. The development of root hairs was very sensitive to the presence of the selective agents and was a good indicator for identifying transgenic plants. Thus, the well-developed root system, root hairs, and full leaf expansion could be used as reliable criteria in selecting transgenic plants.

In addition to the variable regeneration abilities of transformed calli among the suspension lines, a considerable difference was observed in transformation competence among the individual suspension cell lines (Table 1). The most successful line, 8231, averaged 1.9 resistant calli per plate whereas 18 other lines yielded none. Since tall fescue cultivars are synthetics, the observed variation may be related to the genotypic differences within a cultivar.

All of the green putative transgenic plants survived the transplantation to the soil. Most of them appeared normal and could not be distinguished from wild type plants. Three of the putative transgenic plants, however, grew poorly in soil, which may be the result of somaclonal variation or deleterious transgene insertion events.

**Southern analyses of transgenic plants**

Southern hybridization analyses were performed to confirm the transgenic nature of the hyg B- or bialaphos-resistant plants. The results of five individual
plants transformed with the *hph* gene are shown in Fig. 4. In analysis using non-digested genomic DNA (Fig. 4A), the hybridization of *hph* probes with high molecular weight genomic DNA of these plants suggested integration of the transgene into the plant genome. When hybridization using restriction enzyme-digested genomic DNA was analyzed (Fig. 4B), the five plants displayed various hybridization patterns and transgene copy numbers, confirming that they were derived from independent transformation events. Each transgenic plant displayed an expected 1.3 kb *BamHI* fragment when hybridized to the probes. This fragment contained a full-length *hph* coding region and a segment of rice *act1* gene intron 1 (Fig. 1B).

The hybridization results of a representative transgenic plant, transformed with pAHC25 and selected with bialaphos, are shown in Fig. 5. Genomic DNA of the transgenic plant digested with *BamH* I hybridized with both *bar* gene probe (Fig. 5A) and *GUS* gene probe (Fig. 5B).

**Resistance of bialaphos-selected transgenic plants to the herbicide Finale®**

Leaves of a 'Coronado' plant transformed with the *bar* gene and a non-transformed 'Coronado' plant were treated with 1/500 dilution of Finale®. As shown in Fig. 6, while the painted leaves of the non-transformed control turned completely yellow a week after the application, no sign of herbicide damage was observed on the transgenic plant leaves, indicating full resistance to the herbicide.
GUS gene expression in transgenic plants

Among the six plants transformed with pAHC25 and selected with bialaphos, three displayed GUS activity, giving a 50% co-expression frequency for the linked, non-selectable reporter gene. GUS assay results of a transgenic plant and a non-transgenic plant are shown in Fig. 7.

CONCLUSION

Genetic transformation of two elite turf-type tall fescue cultivars, ‘Coronado’ and ‘Virtue’, was achieved. Bialaphos was an efficient selection agent for recovering tall fescue transgenic plants when the bar gene was used as a selectable marker. Plants resistant to the selection agent hyg B were also obtained. Southern analysis confirmed the transgenic nature of the resistant plants. Plants transformed with the bar gene displayed resistance to the corresponding herbicide, Finale®. GUS activity was observed in plants when the GUS reporter gene was introduced. Selection at the stage of root induction was critical in eliminating escapes. Variation in transformation competence among suspension cell lines was observed. This research demonstrated the feasibility to transform elite cultivars for turf-type tall fescue improvement, and advanced one step closer to commercialization of genetically enhanced tall fescue cultivars.

ACKNOWLEDGMENTS

The authors are grateful to Drs. Z. Y. Wang, L. Li and M.-J. Cho for helpful discussions, Dr. Wang for the plasmid pAcH1, and Dr. P. Quail for plasmid pAHC25. We thank Pure Seed Testing, Inc. and Pennington Seed Co. for providing breeder/foundation stock seeds of the cultivars ‘Coronado’ and ‘Virtue’, respectively, and AgrEvo USA for the herbicide Finale®. We also like to thank Drs. R. Dewey and L. Li for critical reading of the manuscript. This work was, in part, supported by a grant from Pure Seed Testing, Inc. (Hubbard, OR, USA).

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