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Ploidy variation among herbicide-resistant bermudagrass plants of cv. TifEagle transformed with the *bar* gene

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Abstract A protocol was developed for biolistic transformation of hybrid bermudagrass cv. TifEagle using the *bar* gene. TifEagle is an ultradwarf used exclusively on golf greens. Herbicide resistance should serve as a useful management tool, especially if methyl-bromide is unavailable for fumigation prior to plant establishment. Hybrid bermudagrass is completely sterile, which should limit the chance of gene escape via out-crossing. Sliced nodes were used to initiate embryogenic tissue cultures on MS medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.01 mg/l 6-benzylaminopurine (BA). Embryogenic tissue was bombarded with the *bar* gene, and herbicide-resistant tissue was selected in the dark on medium supplemented with 0.75 mg/l 2,4-D, 0.01 mg/l BA and 5–15 mg/l phosphinothricin (PPT). Resistant somatic embryos were induced to germinate in the light on MS medium supplemented with 0.13 mg/l 2,4-D and 0.5 mg/l BA. Plants were transferred to the greenhouse after rooting in the presence of 10–15 mg/l PPT and testing positive in a chlorophenol red assay. A total of 89 herbicide-resistant plants were recovered from at least nine independent events from six separate bombardments, although the number of independent transformation events was not confirmed for the entire group. Flow cytometry indicated that most of the plants (82/89) were hexaploid and that the remaining seven plants were triploid. The hexaploid plants were a darker green than the triploids or TifEagle control. Other variation, present only in the hexaploids, included an increased leaf width and length. Southern blot hybridization confirmed genomic integration of the *bar* gene in triploid and a subset of hexaploid herbicide-resistant plants. AFLP analysis did not indicate changes in DNA profiles using [^{33}P] and a sample of 32 hexaploid plants recovered from a single bombardment. DNA profiles were very similar to that of the TifEagle control with a semi-automated fluorescence-based AFLP.

Keywords Hybrid bermudagrass · Transformation · Herbicide resistance · Phosphinothricin

Abbreviations *BA::* 6-Benzylaminopurine · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *GFP:* Green fluorescent protein · *GUS::* β-Glucuronidase · *PAT:* Phosphinothricin acetyl transferase · *PPM:* Plant preservative mixture · *PPT:* Phosphinothricin

Introduction

Transgenic turf grass that has been modified for resistance to an environmentally friendly herbicide could serve as a powerful tool for initiating and maintaining high-quality turf grass for sports applications. Homeowners also could benefit from an easy, efficient method to control weed problems on lawns. With the likely ban in the near future of the soil fumigant methyl-bromide, herbicide resistance could be an effective alternative when planting fairways, greens, or athletic fields. Currently, genes conveying resistance to glyphosate (Roundup) and glufosinate (Liberty, Ignite, Basta, Buster, Challenge, and Harvest) have been shown to be extremely effective in agronomic crops such as corn, soybeans, and cotton. For high-performance greens and fairways, creeping bentgrass (*Agrostis stolonifera* L.), north of the transition zone, and hybrid bermudagrass (*Cynodon dactylon* × *Cynodon transvaalensis*), in the south, produce the highest quality turf. Transgenic herbicide-resistant bentgrass plants have been recovered (Hartman et al. 1994). Hybrid bermudagrass is completely male- and female-sterile and therefore re-

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quires vegetative initiation from sod or sprigs, thus assuring containment of a foreign gene. Transformation of elite hybrid bermudagrass cultivars should be an ideal choice as a suitable species for use of this technology. TifEagle hybrid bermudagrass (Hanna and Elsner 1999) was derived from stolons of Tifway 2 which were irradiated with 7,000 rad of gamma irradiation from [^{60}Co]. TifEagle was the best of 48 mutants evaluated and is ideal for putting greens, providing a fast smooth surface with less thatch accumulation than other ultradwarf types.

Since limited reports are available on transformation of hybrid bermudagrass and given that hygromycin resistance has been used to recover transgenic plants derived from TifEagle (Zhang et al. 2003), the objectives of the investigation reported here were: (1) to test the compatibility of our TifEagle tissue culture protocol with biolistic transformation using the *bar* gene, and (2) to determine the extent of somaclonal variation present in herbicide-resistant plants.

Materials and methods

Culture initiation

The use of nodes from the hybrid bermudagrass cultivar, TifEagle, to initiate embryogenic cultures was first described by Zhang et al. (2001; personal communication). We have also been able to produce embryogenic callus from nodes followed by plant regeneration using different culture conditions (Goldman et al., submitted). Approximately 2-mm segments containing individual nodes were cut from stolons and placed in a 100×15-mm petri dish. The nodes were surface sterilized by rinsing in 70% ethanol for 1 min followed by 20 min in 17% Ultra-Clorox (containing 6.15% sodium hypochlorite). They were then rinsed four times in sterile water, sliced in half longitudinally and plated sliced side down on MS medium (Murashige and Skoog 1962) with 1 mg/l 2,4-D, 0.01 mg/l BA (Chaudhury and Qu 2000), 40 g/l sucrose, 7.5 g/l agar (A1296 Sigma, St. Louis, Mo.) and 1 ml/l PPM (Plant Cell Technologies, Washington, D.C.). A plant preservative mixture (PPM) was included as a precautionary measure to prevent potential contamination throughout the in vitro phase of culture. PPM did not appear to interfere with any aspect of the tissue culture pathway through plant regeneration in bermudagrass or previously with pearl millet (Goldman et al. 2003). For all media, the pH was adjusted to 5.8 before autoclaving (20 min, 121°C, 105 kPa). Node-derived embryogenic tissue was used for biolistic transformation as soon as it appeared or after a short maintenance period on MS medium with 0.75 mg/l 2,4-D and 0.01 mg/l BA in the dark.

DNA constructs used for transformation

Plasmid pAHC25 (Christensen and Quail 1996), a gel-purified *bar* cassette, and p524EGFP.1 (Fleming et al. 2000) were used for transformation. Plasmid pAHC25 contains the selectable *bar* gene, which encodes the enzyme phosphinothricin acetyltransferase (PAT), and the reporter gene (*uidA*) encoding β-glucuronidase (GUS), both under control of separate maize ubiquitin promoters (*Ubi1*) and its first intron and the *nos* terminator. The *bar* expression cassette (1,993 bp) is composed of the *bar* gene driven by a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer region and a rice *actin1* 5′ intron and terminated with *nos* poly A. Plasmid p524EGFP.1 contains the double CaMV 35S promoter sequence followed by the alfalfa mosaic virus enhancer sequence (Datla et al. 1993) and terminated with SV40 poly A, which controls the expression of an enhanced green fluorescent protein (*gfp*) gene (Clontech, Palo Alto, Calif.).

DNA delivery

DNA was precipitated onto gold particles according to Goldman et al. (2003). For co-transformation with pAHC25 and *gfp*, a 1:1 molar ratio of the two DNA molecules was used. Tissues were arranged in a 2.5-cm-diameter circle in the center of the petri dish on medium containing 0.75 mg/l 2,4-D, 0.01 mg/l BA, and 1 ml/l PPM 4 h prior to bombardment. The stopping screen was placed 6 cm below the rupture disk and the target tissue 6 cm below the stopping screen. The gun was fired when the vacuum pressure reached 71 cm Hg. Tissues were bombarded twice at 1,100 psi using 0.75-μm or 0.6-μm gold particles.

Selection of transformed plants

After bombardment, the tissue was removed from the target area and re-plated on MS medium with 0.75 mg/l 2,4-D, 0.01 mg/l BA, and 1 ml/l PPM for 2 weeks in the dark. It was then transferred to medium supplemented with 5 mg/l or 10 mg/l phosphinothricin (PPT) [diluted from 60% glufosinate ammonium (a gift from AgrEvo USA, Pikeville, N.C.)] for selection of herbicide-resistant tissue in the dark at 28°C. Following this selection on herbicide-containing medium, tissue with well-developed somatic embryos was transferred to MS medium with 0.13 mg/l 2,4-D and 0.5 mg/l BA (Taylor and Vasil 1996) without PPT and cultured in the light (100 μmol m^{-2} s^{-1}). Germinating somatic embryos were transferred to basal MS medium supplemented with 8 mg/l or 15 mg/l PPT for the further selection of transformed plants and to encourage root and shoot elongation. Surviving plantlets were moved to polypropylene round-bottomed tubes (17×100 mm) containing basal MS with 8 mg/l or 15 mg/l PPT and 50 mg/l chlorophenol red (Kramer et al. 1993). Plants that remained unaffected by the herbicide and turned the medium yellow were transplanted to soil. Liberty (AgrEvo, Wilmington, Del.), a commercial herbicide formulation containing 182 g/l PPT, was diluted to 500 mg/l PPT (lethal to non-transformed TifEagle) with water and sprayed on putative transformed plants to test for herbicide resistance. A standard mist-type spray bottle was used, and plants were sprayed until all leaf surfaces appeared to be wet.

Molecular confirmation of transformed plant recovery

A subset of herbicide-resistant plants was subjected to Southern blot hybridization analysis. Genomic DNA was isolated from 2 g of fresh tissue that was ground in liquid nitrogen and extracted according to Tai and Tanksley (1990) with some modifications (Ozias-Akins et al. 1993). Genomic DNA (10 μg) was digested with *Hin*dIII for linear fragment-derived plants or *Sac*I for plasmid-derived plants. Both enzymes cut within the transforming DNA once and should indicate insert copy number. Digested genomic DNA was subjected to electrophoresis overnight at 25 V on a 0.8% agarose gel in 1× TBE buffer (Sambrook et al. 1989) and then transferred to GeneScreen Plus nylon membrane (NEN Research Products, Boston, Mass.) using 0.4 *N* NaOH (Sambrook et al. 1989). PCR primers specific for the *bar* open reading frame (*bar* forward primer 5′-GCC AGT TCC CGT GCT TGA AGC CGG C-3′; *bar* reverse primer 5′-GGC GGT CTG CAC CAT CGT CAA CCA C-3′) were used to create [^{32}P]-labeled probes. PCR conditions in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) were as follows: initial denaturation at 94°C for 10 min followed by 40 cycles of amplification (50 s at 95°C, 50 s at 60°C and 2 min at 72°C) and a final extension of 10 min at 72°C. Hybridization was carried out in a solution containing 7% sodium dodecyl (SDS) sulfate, 0.25 *M* $NaPO_4$, 1 m*M* EDTA, and 1% bovine serum albumin at 65°C. Blots were washed once in 2× SSPE/1.0% SDS, once in 0.5× SSPE/1.0% SDS, and finally once in

0.1× SSPE/1.0% SDS, each for 20 min at 65°C. Hybridization signals were detected using the Cyclone Imaging System with OptiQuant software (Packard, Meriden, Conn.).

Flow cytometry

Fresh young leaves (approximately 0.5 cm^2) harvested from greenhouse-grown plants were chopped with a double-edged razor blade in 400 μl of commercial Partec nuclei extraction buffer solution to release the nuclei. The suspension was poured through a 50-μm filter to remove debris, and 1.6 ml of commercial Partec DAPI staining buffer solution was added. The suspension was analyzed on a Partec Cell Analyzer PAS III flow cytometer (Partec, Münster, Germany) and at least 10,000 fluorescent particles were counted. Control TifEagle (3*x*), *C. dactylon* (4*x*), and *C. transvaalensis* (2*x*) were routinely run (TifEagle every fifth sample) as external standards. Ploidy level was determined based on the position of the G_1 peaks of transgenic plants relative to the 2*x*, 3*x*, and 4*x* standard G_1 peaks.

Morphological analysis

A randomized complete block design was used to test for differences in morphological characteristics between transformed plants and the TifEagle control. We measured a group of 54 plants that included 47 hexaploid and seven triploid herbicide-resistant plants obtained from five separate bombardments. Measurements included leaf length (in centimeters; three per plant), leaf width (in millimeters; three per plant), canopy height (in centimeters), and the sum of the length (in centimeters) of the three longest stolons. The leaf width, length, and canopy height measurements were recorded as whole numbers and approximated to 0.1 using a ruler and the naked eye. PROC MIXED (SAS Institute 1999) and multiple *t*-tests on differences of least square means were used to detect significant differences (α=0.05) between transgenic plants and TifEagle harvested from the breeder plot in Tifton, Georgia. PROC GLM was also used and means were separated using Duncan's multiple range test.

Amplified fragment length polymorphism

The AFLP protocol followed that of Zabeau and Vos (1993) and Vos et al. (1995). Genomic DNA was isolated from leaf tissue ground in liquid nitrogen according to the hexadecyltrimethyl ammonium bromide (CTAB) method of Rogers and Bendich (1985). Genomic DNA (500 ng) was digested and ligated with *Mse*I and *Eco*RI adapter pairs. After pre-selective amplification, samples were diluted 40× with 0.1× TE. Selective amplification reactions were performed with fluorescently labeled *Eco*RI-AAC or ACG plus *Mse*I-CTA primers or [^{33}P]-labeled *Eco*RI-AGC plus *Mse*I-CAG primers. The [^{33}P]-labeled PCR products were separated by polyacrylamide electrophoresis. The gel was dried and exposed to X-ray film. For fluorescent AFLP, amplification products (1.5–2 μl), 0.25 μl Size Standard-600 marker, and 38 μl sequence loading solution (SLS) were mixed and run on a Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, Calif.). Default fragment analysis parameters were used except that the stage-one and -two ramp durations were changed to 7.5 s and the injection duration was changed to 45 s.

Results

Initiation of embryogenic cultures and plant regeneration

Sliced nodes were suitable explants for generating bombardment-grade embryogenic tissue. Goldman et al. (submitted) observed that 36% of the sliced nodes of TifEagle formed embryogenic tissue. In many cases responsive nodes maintained in the dark produced additional bombardment-grade embryogenic tissue after an initial harvest. Embryogenic tissue proliferated better on medium with a lower concentration of 2,4-D (0.75 mg/l), and lowering the 2,4-D concentration was an effective way to temporally maintain tissue prior to bombardment. Fifty bombardments were tested with TifEagle node-derived callus (Fig. 1a). Tissue that was bombarded twice at 1,100 psi after being transferred to a medium containing a reduced level of 2,4-D showed a high level of transient GUS or GFP expression 24–48 h after bombardment. Herbicide-resistant tissue was selected after approximately 30 days (three transfers) on reduced 2,4-D medium supplemented with 5 mg/l PPT. No GUS expression was observed in herbicide-resistant callus more than 4 weeks after bombardment or in regenerated plants even though the *uidA* gene should be linked to *bar* in most transformants. Stable GFP expression was observed in a herbicide-resistant callus line recovered from a co-transformation. However, somatic embryo germination was not successfully induced with this tissue and no GFP-expressing plants were recovered. For some somatic embryos (bombardment 11), a single pass on germination medium (MS with 0.13 mg/l 2,4-D and 0.5 mg/l BA) was sufficient for plantlet development. For others, multiple transfers to germination medium or basal MS with 10 mg/l PPT were required for germination. Plantlets moved to tubes developed robust root and shoot

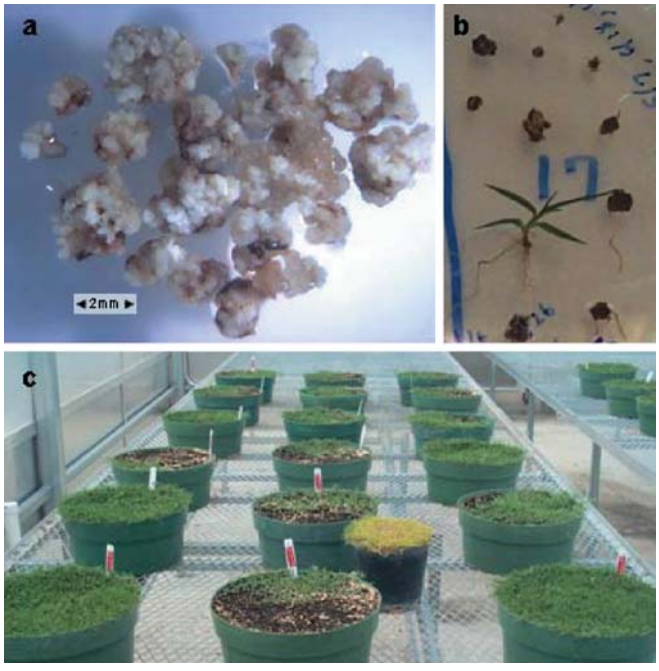

Fig. 1a–c Recovery of herbicide-resistant bermudagrass plants. **a** Embryogenic tissue used to create bombardment 21, **b** a herbicide-resistant plant in the process of rooting on selection medium, **c** TifEagle control (*yellow*) and transgenic plants in the greenhouse 1 week after application of Liberty (500 mg/l)

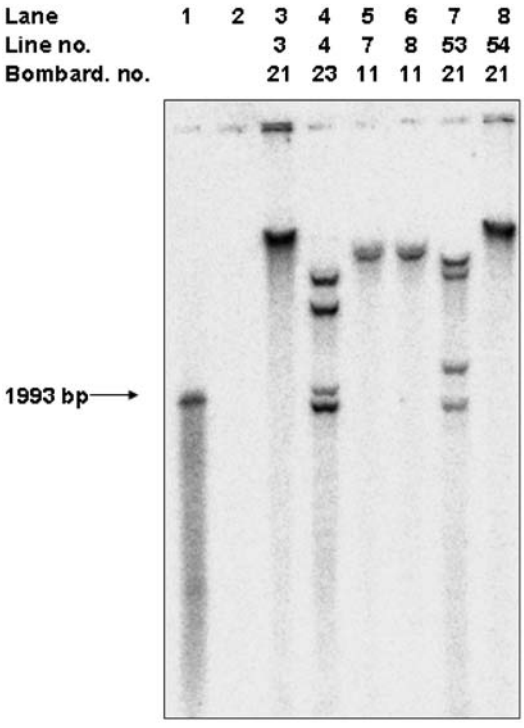

Fig. 2 Southern blot hybridization of *Hin*dIII-digested DNA from six, linear fragment-derived, triploid, herbicide-resistant plants. The blot was probed with an internal 400-bp portion of the *bar* gene. *Hin*dIII cuts within the *bar* expression cassette upstream of the *bar* coding sequence. Line and bombardment numbers are described in Table 1. *Lane 1* Control fragment DNA, *lane 2* TifEagle non-transformed control

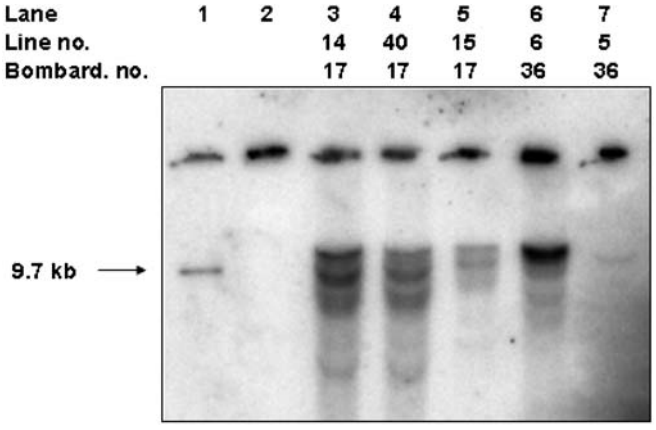

Fig. 3 Southern blot hybridization of *Sac*I-digested DNA from five plasmid-derived transgenic plants. The blot was probed with an internal 400-bp portion of the *bar* gene. *Sac*I cuts upstream of the *bar* gene between the *uidA* coding sequence and *nos* terminator. Line and bombardment numbers are described in Table 1. *Lane 1* Control *Sac*I-digested pAHC25, *lane 2* TifEagle non-transformed control

systems. PPT selection was imposed at the callus, regeneration, and rooting stages (Fig. 1b). The chlorophenol red (CR) assay using 10 mg/l or 15 mg/l PPT was an effective way to further confirm that the plant was herbicide-resistant when the pH indicator turned the medium bright yellow. All plants that passed the CR assay were also resistant to a lethal (500 mg/l) application of Liberty herbicide (Fig. 1c) in the greenhouse. A total of 89 herbicide-resistant plants were recovered from six separate bombardments. The time from embryogenic tissue bombardment to a rooted plant that passed the CR assay ranged from 3 months to 6 months.

Integration of the transgenes into genomic DNA

Southern blot analysis confirmed the genomic integration of *bar* from pAHC25 and the linear *bar* cassette. One to four copies of the insert were detected in the *Hin*dIII digest of DNA from linear-fragment-derived transgenic plants (Fig. 2). Southern blot analysis showed that all regenerated plants from one bombardment were not independent. For example, of three plants analyzed from bombardment 21, two had identical restriction patterns. At least two independent events were confirmed for bombardments 17, 21, and 36 (Figs. 2, 3), and each of the other three bombardments produced at least one event each. Digestion with *Sac*I using plasmid-derived transgenic plants indicated that most of the plants examined contained multiple copies of the insert (Fig. 3). The two plants recovered from bombardment 36 that differed in ploidy appear to be from different transformation events (lanes 6 and 7).

Somaclonal variation

Based on observations of the first group of plants recovered from bombardment 57, subtle differences could be seen in terms of leaf width and length. Morphological data were not collected on this group although they were field tested for one season. Entries in the field lacked vigor compared to the TifEagle control (data not shown). While entering dormancy, the transgenic plants turned a deep purple color, whereas the TifEagle control did not. Flow cytometric analysis later revealed that all of the herbicide-resistant plants recovered from bombardment 57 were hexaploid (Table 1, Fig. 4). Plants recovered from bombardment 17 and a single plant from bombardment 36 were also hexaploid. Bombardment numbers 11, 21, 23, and 36 produced triploid plants. The triploid plants were lighter in color than the hexaploid plants and very similar or slightly lighter in color than the TifEagle control. The plants recovered from all bombardments except bombardment 57 (54 in total) were included in the replicated morphological study. Of the four measurements taken (leaf length, leaf width, canopy height, and stolon length), only leaf length and width showed no significant differences among replicates and were further analyzed. The triploid plants did not differ significantly (α=0.05) for leaf length or width compared with the TifEagle control. Most of the somaclonal variation was observed in the hexaploid

Table 1 Summary of bombardment, flow cytometry, and morphological data from TifEagle transformation experiments (*nr* not recorded)

Bombardment no.	57	11	21	23	36	17	TifEagle control
Gold size (μM)	0.75	0.75	0.6	0.6	0.6	0.6	-
DNA[a]	Fragment	Fragment	Fragment	Fragment	Plasmid + GFP	Plasmid + GFP	-
Total plants[b]	32[c]	2[d]	3[e]	1	2[f]	46[g]	-
Plant ID	57A...	7, 8	3, 53, 54	4	5 (6*x*), 6 (3*x*)	1, 2, 9–52	55
Time to plants[h]	3–4	3	3	3.5	3	4–6	-
Ploidy[i]	6*x*	3*x*	3*x*	3*x*	3*x*,6*x*	6*x*	3*x*
Leaf width[j] (mm)	-	1.0–1.1	1	-	1.0–1.1	1.1–2.3	-
Mean ± SD	-	1.1±0.1	1.0±0	1.3	1.1±0	1.8±0.3	1.1
Leaf length (cm)[j]	-	1.0–1.1	1	-	1.1–1.8	1.3–2.5	-
Mean ± SD	-	1.0±0.1	1.0±0	1.1	1.4±0.5	1.8±0.4	1

[a] Fragment = 1,993-bp linear *bar* expression cassette. Plasmid + GFP = pAHC25 + p524EGFP.1 used for co-bombardment
[b] Total number of herbicide-resistant plants recovered per bombardment, not independent transformation events
[c] Number of independent transformation events not tested by Southern blot hybridization
[d] Same transformation event based on Southern blot data
[e] Numbers 3 and 54 are the same transformation event based on Southern blot data
[f] Likely different events based on Southern blot data
[g] Number of independent events not tested for all plants
[h] Time = Time in months from bombardment to rooted plant in the greenhouse
[i] Ploidy determined by flow cytometry of nuclei from leaves of herbicide-resistant plants
[j] Values based on three measurements per plant averaged over four replications. Data collected as whole numbers and estimated to the nearest 0.1 using a ruler and naked eye. Range is indicated

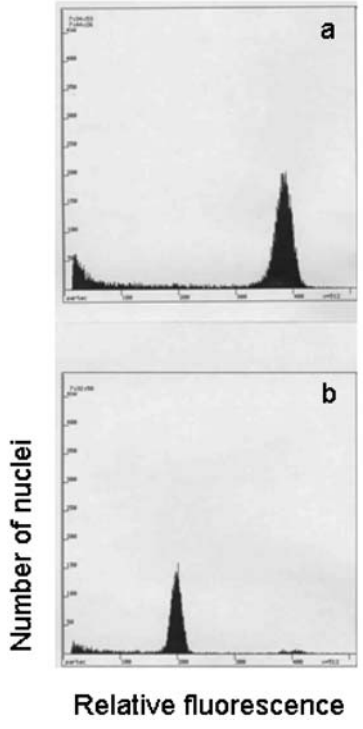

Fig. 4 Flow cytometry results showing G_1 peaks for triploid (3*x*) TifEagle control (**b**) and a hexaploid entry (ID#12) (**a**)

plants from bombardment 17 (Table 1). Further observations revealed obvious differences for leaf width and length (Fig. 5), and quantitative data confirmed that 83% and 91% of the hexaploid plants were significantly different (α=0.05) from the TifEagle control for leaf length and width, respectively. Some hexaploid plants also contained red/purple pigmentation on the leaf edges and other plant parts. It is not clear at the present time if

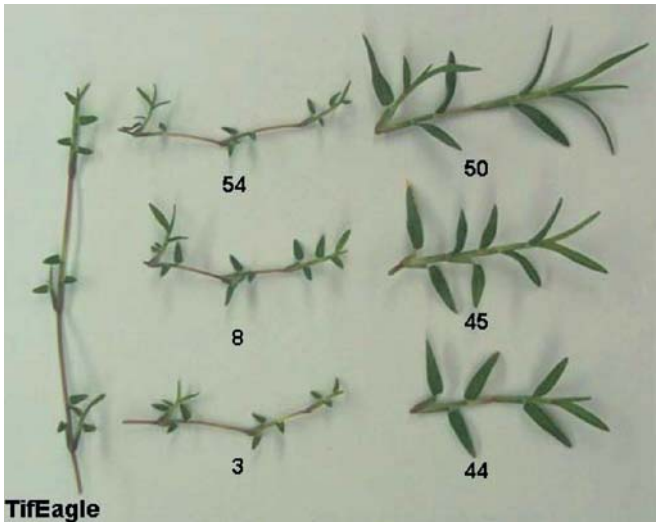

Fig. 5 Somaclonal variation observed in transgenic herbicide-resistant plants. ID numbers are the same as those listed in Table 1

the altered morphological characteristics of the hexaploid plants are stable and will persist in the field.

Amplified fragment length polymorphism

AFLP analysis performed on the 32 hexaploid plants recovered from bombardment 57 did not indicate any differences in DNA patterns compared to the TifEagle control with respect to the major reproducible bands below 400 bp (Fig. 6). The major peaks in DNA profiles were constant between samples using the semi-automated fluorescence-based AFLP analysis with pre-amplified DNA from the [^{33}P] protocol or DNA isolated from bombardments other than bombardment 57 (Fig. 7). There

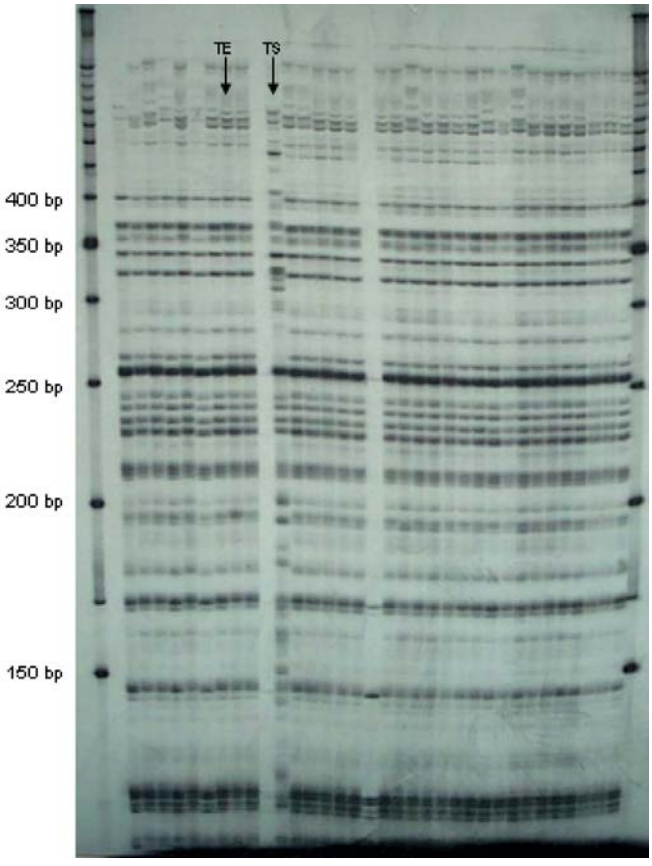

Fig. 6 AFLP (^{33}P method, AGC primer) results using all of the hexaploid plants recovered from bombardment 57. TifSport (*TS*) was also included and is the only lane that contains an altered DNA profile compared to control TifEagle (*TE*)

also were some cases in which smaller peaks were not present in all of the samples tested. Although both AFLP methods were informative, the results could not be directly compared, and in no case was a major difference in DNA profiles observed using either method (^{33}P vs. fluorescent) or selective primer combination.

Discussion

We have developed a transformation system to recover herbicide-resistant hybrid bermudagrass derived from the TifEagle genotype. Zhang et al. (2003) recently also recovered transgenic plants using TifEagle sliced nodes and a hygromycin resistance gene for selection. Aside from using the sliced node to initiate cell cultures, our system is substantially different. One of the goals of our system was to regenerate transgenic plants in the shortest time possible. Although in some cases hygromycin is thought to be a more powerful selectable marker than *bar* (Cho et al. 2000), we found that two to three transfers over a period of 25–30 days on 5 mg/l or 10 mg/l PPT in the dark was sufficient to select herbicide-resistant tissues that were capable of plant regeneration. Zhang et al. (2003) required a lengthy liquid culture phase to increase tissue mass for bombardment and also to select hygromycin-resistant tissue. With our system it was possible to recover rooted transgenic plants 3 months after bombardment.

We have described somaclonal variation present in regenerated plants from TifEagle (Goldman et al. submitted). With the addition of the transformation and selection stages to the protocol, somaclonal variation was again observed, and at a higher frequency. An increase in the frequency of somaclonal variation was also observed with the addition of biolistic transformation and selection using barley (Bregitzer et al. 1998). They used PPT as a selection agent and concluded that it was a significant cause of increased somaclonal variation compared to non-transformed regenerated plants. Zhang et al. (2002, 2003) reported somaclonal variation among regenerated and transformed plants of TifEagle, although the type and extent of variation was not described. Our transformation system resulted in a low frequency (8.5%; 7/82) of transgenic plants that were triploid and very similar to the TifEagle control. This frequency is lower if the number of independent events rather than the number of regenerated plants is considered (6%; 5/82). If the goal is to induce variation for selection purposes, TifEagle is capable of producing a wide range of phenotypic variation in regenerated plants. On the other hand, if the goal is to retain all of the essential characteristics of this ultradwarf cultivar, in addition to a novel agronomic trait such as herbicide resistance, then the efficiency of this transformation system is comparatively low. We used 2,4-D as the auxin source during tissue culture, which may increase the chance of recovering hexaploid plants (Karp 1994, 1995) either by inducing polyploidy or by selecting for polyploid cell growth. Zhang et al. (2003) used dicamba as the auxin source, although differences in ploidy were not determined. The two bombardments (nos. 17 and 57) that resulted in hexaploid plants also produced the majority of the transgenic plants. The hexaploid plant recovered from bombardment 36 retained the fine leaves of TifEagle, was lighter in color, and lacked vigor and canopy density. It is possible that hexaploid tissues may be easier to transform, may grow faster, and may be more likely to regenerate plants than triploid tissues. Goldman et al. (submitted) found that 33% of the regenerated TifEagle plants that had not been subjected to bombardment and selection were hexaploid. In the present investigation, flow cytometry was only performed at the whole plant stage so it is unclear whether the ploidy change occurred in vitro or pre-existed in the explant. None of the hexaploid plants retained the complete TifEagle phenotype, and there was a wide range in degree of somaclonal variation. There are only a few reports of hexaploid bermudagrass (Hanna et al. 1990; Moffett and Hurcombe 1949; Powell et al. 1968). Tifton 10, a vegetatively propagated, hexaploid cultivar, is fertile (Hanna et al. 1990). The hexaploid plants from bombardment 57 that were field tested for one season were not fertile.

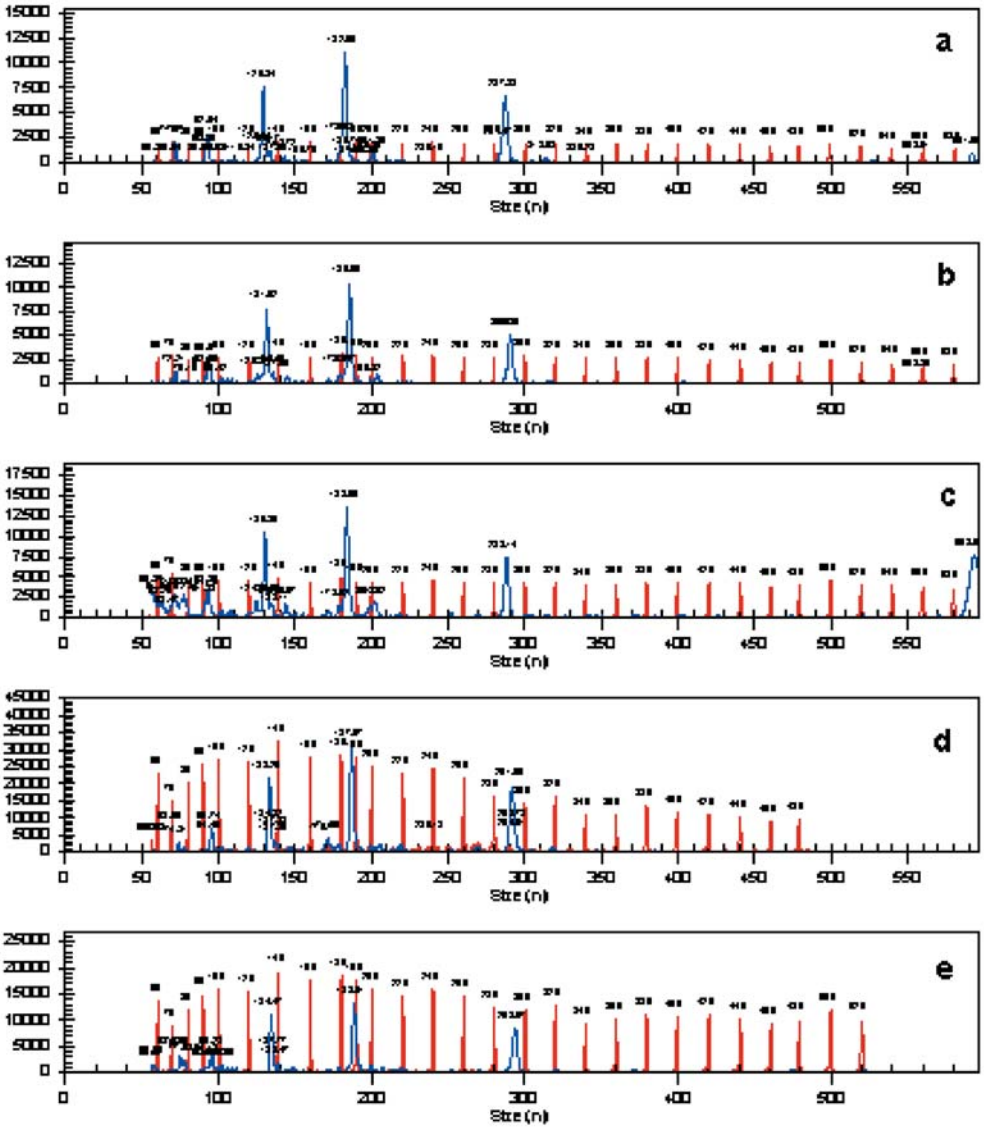

Fig. 7a–e AFLP (fluorescent method, AAC primer) showing similar fragment patterns among transgenic plants and the TifEagle control. **a** ID#46, **b** ID#8, **c** ID#3, **d** TifEagle, **e** TifEagle (^{33}P pre-amplified DNA from bombardment 57 AFLP)

We recovered 89 plants from six petri dish bombardments. On a per bombardment basis, 6/50 (12%) were successful at producing transformed plants, although many of the unsuccessful bombardments did not use improved bombardment or selection conditions. Zhang et al. (2003) recovered 75 transgenic lines from 18 petri dish bombardments. In our case, Southern blot analysis was not performed on all hexaploid plants so it is not clear how many plants are independent transformation events. Recently, Wang et al. (2003) reported that glufosinate may act as a fungicide on some turf pathogens. The herbicide-resistant plants recovered in this study were able to resist glufosinate concentrations that have been reported to reduce fungal growth. A field test is planned to determine if any of the triploid herbicide-resistant plants perform as well or better than TifEagle.

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