Abstract A transformation system for triploid bermudagrass (*Cynodon dactylon* x *C. transvaalensis* cv. TifEagle) was established with a biolistic bombardment delivery system. Embryogenic callus was induced from stolons and maintained on Murashige and Skoog’s medium supplemented with 30 μM dicamba, 20 μM benzylaminopurine, and 100 mg/l myo-inositol. Using the hygromycin phosphotransferase (*hpt*) gene as the selectable marker gene, we obtained 75 transgenic lines from 18 petri dishes bombarded. Integration of the *hpt* gene into genomic DNA and transcription of *hpt* was confirmed by Southern and Northern blot analyses, respectively. Through suspension culture screening, we obtained homogeneously transformed plants showing stable transcription of the *hpt* gene.

Keywords Triploid bermudagrass · Transformation · Biolistics · Hygromycin phosphotransferase gene

Abbreviations BAP: Benzylaminopurine · *hpt*: Hygromycin phosphotransferase gene · Hyg: Hygromycin B · GUS: β-Glucuronidase

Introduction

Among warm season turfgrasses, triploid bermudagrasses normally provide the highest quality of turf grown in southern regions. They are planted on lawns, golf courses, and other sport fields. Triploid bermudagrass cultivars are sterile F1 hybrids of *Cynodon dactylon* x *C. transvaalensis* and must be propagated vegetatively. Each new cultivar is derived from a selected plant. As sterile triploids, current hybridization breeding procedures cannot be applied for their further improvement. Variability can only result from spontaneous mutation or from γ-ray radiation-induced mutation. They are excellent targets for biotechnological improvement because the sterility of the plant eliminates the gene flow problem occurring in most transgenic plants. Transformed bermudagrass with improved stress tolerance or pest tolerance could yield a new cultivar. To date, successful transformation of other popularly used turfgrasses, such as creeping bentgrass (*Agrostis palustris* L.), perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea* schreb.), Kentucky bluegrass (*Poa pratensis* L.), and zoysiagrass (*Zoysia japonica* Steud.) has been reported (Wang et al. 1992; Hartman et al. 1994; Spangenberg et al. 1995; Inokuma et al. 1997; Dibble et al. 1998). Although Ahn et al. (1985) reported the regeneration of some common bermudagrass and triploid bermudagrass cultivars through somatic embryogenesis, there is no report on the transformation of triploid bermudagrass. We report here the establishment of triploid bermudagrass embryogenic callus culture from nodes of stolons and their successful transformation through biolistic bombardment.

Materials and methods

Plant material

TifEagle, a newly released triploid bermudagrass cultivar, was kindly provided by Dr. Wynne Hanna (USDA, University of Georgia, Tifton). The grass was planted in the greenhouse in which the daytime temperature was maintained at 24–29°C and the nighttime temperature at 19–22°C. Nodes of stolons were used to induce the embryogenic callus.

Induction and maintenance of embryogenic callus and suspensions

Stolons were washed with running tap water, all of the leaves were removed from the nodes, and the stolons were cut into sections with

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each node located in the middle of a section. The sections were submerged in 95% ethanol for 30 s, washed twice with distilled water, and then soaked in 1.2% sodium hypochlorite solution plus 0.1% Tween 20 for 3.5 min. The surface-sterilized sections were then washed four times with sterile distilled water. Nodes (about 0.3 cm long) were cut out of the sections and sliced once along the stem axis. The excised nodes were placed on callus inducing medium consisting of MS medium (Murashige and Skoog 1962) supplemented with 30 μM dicamba, 20 μM BAP, 100 mg/l myo-inositol, 1x B5 vitamins, 30 g/l sucrose, 2.5 g/l Phytogel (Sigma, St. Louis, Mo.), pH 5.80–5.85 and cultured in the dark at 30°C. After 4–6 weeks, compact embryogenic calli were selected and transferred to new dishes containing the same induction medium. The calli were subcultured every 10 days onto the same medium. Suspension culture was also used to proliferate embryogenic callus for bombardment and further selection of putative transformants. The composition of the liquid medium was the same as that of the solid medium but without agar. Suspension cultures were maintained at 28°C with shaking (125 rpm).

Transformation, screening, and regeneration of transformants

The calli were kept on callus inducing medium supplemented with 0.6 M mannitol for 16 h prior to bombardment. For bombardment, embryogenic calli were arranged into an area 5 cm in diameter at the center of a petri plate that contained callus inducing medium supplemented with 0.6 M mannitol. DNA-coated gold particles (1.0 μm in diameter) were prepared as described by Vain et al. (1993). The bombardment was carried out using a Bio-Rad (Hercules, Calif.) PDS-1000 He biolistic delivery system at 1,100 psi, with a target distance of 6 cm. Each petri plate was bombarded twice. The day following bombardment, the calli were transferred to media without mannitol. After 1 week, they were transferred to the selection medium (same as callus inducing medium except supplemented with 200 mg/l Hyg). After 4 weeks of growth on the selection medium, resistant calli were transferred to suspension culture screening medium (the same as the selection medium but without agar) for 4 weeks. Resistant calli were then transferred to regeneration medium (MS medium plus 2.0 mg/l BAP and 200 mg/l Hyg). For regeneration, culture plates were placed in a growth chamber at 28°C (day/night) under a16/8-h (day/night) photoperiod with light supplied at an intensity of 400 μmol/m² per second.

Screening vector

The truncated hpt gene under the control of the rice actin promoter on plasmid pAC1H (kindly provided by Dr. German Spangenberg, Institute for Plant Sciences, Swiss Federal Institute of Technology, Zurich, Switzerland) was used as selectable marker (Bilang et al. 1991; McElroy et al. 1991). The uidA gene contained in plasmid pAHC25 was used for the transient GUS assay to optimize the parameters of transformation (Christensen et al. 1992).

Southern and Northern blot analyses

DNA isolation was based on the method described by Tai and Tanksley (1990) with slight modifications. Instead of dehydrated leaves, 3.0 g fresh leaves were used for DNA extraction, and a chloroform extraction step was added before the final DNA precipitation. Trireagent (Sigma) and its protocol were used for RNA isolation. Southern and Northern analyses were carried out according to methods described by Sambrook et al. (1989). Agarose gel (1.2%) containing formaldehyde and 1x MOPS buffer was used for RNA electrophoresis. The capillary transfer method was used for transferring DNA or RNA to nylon membrane. RadPrime DNA labeling system (GibcoBRL, Gaithersburg, Md.) was used for probe labeling. The final wash condition was 20 min in 0.2× SSC, 0.1% sodium dodecyl sulfate at 65°C with agitation. Nylon membrane (Micron Separations) was used for the DNA and RNA blots.

Results

Embryogenic callus of TifEagle was whitish in color, very compact, and hard to break (Fig. 1A). The calli initially grew rapidly at 28–30°C, but after about 7–9 days

Fig. 1A–F Transformation and regeneration of transformed triploid bermudagrass. A. Embryogenic callus of triploid bermudagrass. B Suspension calli before transformation. C Transient expression of GUS, 2 days after bombardment. D Hyg- resistant calli obtained 4 weeks after bombardment of embryogenic calli. Bombarded calli were subjected to selection on solid culture medium supplemented with 200 mg/l Hyg. E Shoot differentiation on Hyg-resistant callus, observed 3 weeks after the callus was transferred to regeneration medium. F Different Hyg-resistant transgenic lines planted in the greenhouse.
of growth, this fast growth was inhibited by a brown substance that accumulated at the surface between the callus and the medium. When the calli were kept on the same plate, growth gradually ceased. Both frequent transfers to new media and/or the use of suspension culture helped to overcome this problem. Although both methods worked well, suspension culture was more convenient and, consequently, all of the calli used in further transformation experiments came from suspension culture (Fig. 1B).

Using the uidA gene and the transient GUS assay as an indicator (Fig. 1C), we determined that the optimized conditions for transformation were a 16-h incubation (overnight) of the calli on medium supplemented with 0.6 M mannitol, two bombardments per plate at 1,100 psi and a 6-cm target distance (data not shown).

Hyg-resistant calli were observed after 4 weeks of growth on the selection medium (Fig. 1D). After the calli had been transferred to the regeneration medium, green points could be observed within 2 weeks. Within 6 weeks, small plantlets emerged (Fig. 1E). Large plantlets were transferred to regeneration medium without plant hormones for root formation. Normally, roots formed within 1–2 weeks. The regenerated plants with roots were transplanted into the greenhouse (Fig. 1F). From 18 plates of calli bombarded, a total of 163 putative transfectants were selected on solid medium supplemented with 200 mg/l Hyg. From these, 75 regenerated plants were ultimately obtained. The regeneration frequency was 46.0% (75/163).

Successful integration of the hpt gene into transformants' genome was verified by Southern hybridization (Fig. 2A). Approximately 6.0 µg total DNA from independent putative transgenic plants was thoroughly digested with BamHI and used for gel electrophoresis. The SsrI digestion fragment of pAcH1 was used as a probe (Fig. 2B). A typical 1.34-kb band appeared in all samples of putative transgenic plants but not in the untransformed control. Most of the lines checked were multiple-copy transformants, which is a typical result of biolistic bombardment. At the same time, low-copy transformants were also observed. Lines 7 and 69 were probably low-copy transformants. Normal transcription of the hpt gene was confirmed by Northern blot assay (Fig. 3). Aliquots (15 µg) of total RNA from independent transgenic plants and the untransformed control were blotted to a nylon membrane and hybridized with the same probe as that used in the Southern assay. A band of about 1.3 kb was detected in several transformants. This is the size of transcription predicted from the sequence of the hpt gene construct.

A random single-sprig (a single stolon with roots) selection and propagation system was used to check whether the regenerated plants were fully transformed and whether the transcription of the hpt gene was stable in vegetatively propagated progeny (Fig. 4A; Bettany et al. 1998). Transgenic plant lines nos. 12 and 41 were chosen for this investigation. Before carrying out the Southern and Northern analyses, we conducted three generations of random single-sprig propagation. The interval between each generation of propagation was approximately 3 months. Figure 4B shows the results of the Southern blot analysis of different progeny lines derived from transgenic lines 12 and 41, respectively. About 5.0 µg DNA of each sample was thoroughly digested by BamHI. The same hybridization pattern and signal strength were obtained from different sprig lines that came from the same transgenic line. Hybridization with a rice Act gene probe confirmed the similar loading amount of DNA (data not shown). These results confirmed that integration of the hpt gene was homogeneous and stable. Figure 4C shows the results of the Northern hybridization assay with the hpt gene probe and a rice Act gene probe. There was very good correlation between the loading amount of
RNA (shown by Act gene transcription) and the strength of the hpt gene hybridization signals. This correlation further proved that transcription of the hpt gene in the transgenic lines checked was stable and homogeneous.

**Discussion**

Since the cultivar used in our study is a sterile triploid, it is impossible to obtain homogenously transformed progeny through seed. A transformed callus line may be a chimera consisting of both transformed and untransformed cells and, consequently, a regenerated plant from this line would be unstable in further vegetative propagation. In our study, we used suspension culture supplemented with 200 mg/l Hyg to intensify the selection pressure in order to eliminate possible untransformed cells adhering to cells with a high expression of the hpt gene. Two to five pieces of calli from a putative single transformed cell were inoculated into a 200-ml flask with 50 ml suspension selection medium. The part of the callus with transformed cells could divide and grow in the first 2 weeks of culturing, while the other part of the same piece turned brown and eventually died (within 3 weeks). No further browning was observed on those calli successfully growing in the selection medium. Although we were not sure whether this phenomenon was caused by the death of untransformed or unstable expressed cells under stringent selection pressure or whether only some of the cells could adapt to suspension culture, a similar phenomenon was not observed in suspension cultures of non-transformed calli without Hyg. We believed that suspension culture with Hyg provided a more stringent selection pressure to eliminate chimeras and, consequently, the same suspension screening procedure was applied to all 163 Hyg-resistant transformants obtained on solid medium.

Our frequency of transformants per dish after bombardment was about nine (163 in 18 dishes). The regeneration frequency was 46.7% (75/163). The longer it took from transformation to regeneration, the lower the regeneration frequency. In order to obtain pure transformants from possible chimera calli, suspension culture screening was added in addition to the solid medium screen. This step (about 4–6 weeks) lengthened the whole screening time, which could have caused the decline of regeneration frequency. At present, we are trying to shorten the whole screening time by reducing the length of the screening period on solid medium.

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**Fig. 4A–C** Random single-sprig propagation used to verify homogeneous integration and transcription in transgenic lines 12 and 41, respectively. **A** Scheme of random single-sprig propagation from transgenic line 12. **B** DNA blot hybridized with the SstI fragment probe. **C** Northern blots hybridized with the hpt gene probe (the SstI fragment) and a plant Act gene probe, respectively.

| A | Scheme of Random Single Stolon Propagation for Identification of Pure Transformants |
|---|---|
| **Transformant No. 12** | **1ˢᵗ** random single stolon propagation |
| 1 | 2 | 3 | 4 |
| 1’ | 2’ | 3’ | 4’ |
| 1” | 2” | 3” | 4” |

**B** M 12-1 -2 -3 -4 41-1 -2 -3 -4

**C** M 12-1 -2 -3 -4 41-1 -2 -3 -4
During embryogenic callus culture, brown pigments were secreted by calli, and accumulated on the surfaces between calli and the culture media. The pigments are obviously inhibitory to the growth of callus and are deleterious to callus quality. If the pigments were allowed to accumulate during the process of regeneration, the growth of shoots and the initiation of roots were inhibited. A similar phenomenon was reported by Ahn et al. (1987), who suggested frequent subculture to reduce the influence of these pigments. However, their inhibitory effects during the regeneration process were still difficult to overcome. While suspension culture reduced the effect of the pigments, it was not suitable for plant regeneration. In addition, suspension culture is apt to induce somaclonal variation by activating the retrotransposons that commonly exist in plant genomes (Hirochika et al. 1996). Somaclonal variation could be an important reason for extra morphological and physiological changes, which normally are deleterious and should be avoided in biotechnological improvement.

Another problem often encountered during our tissue culture of embryogenic callus was contamination by Clavibacter xyli subsp. cynodontis may be associated with the bermudagrass stunting disease (Liao and Chen 1981). However, the bacteria grew very slowly on callus culture medium, and no direct harmful effects on bermudagrass callus were noticed although their role in the degradation of callus quality in long-term culture, and whether they are connected with the secretion of the brown pigment were not clear.

It is possible to use somaclonal variation in triploid bermudagrass breeding if the regeneration frequency is high. Coughan and Quisenberry (1989) reported their attempts to improve fall armyworm (Spodoptera frugiperda) resistance of common bermudagrass through tissue culture. In our study, obvious somaclonal variation in the regenerated transformed plants was noticed. The relative instability of the allopolloid triploid genomic background, a long period of tissue culture (about 5–6 months since initiation of embryogenic callus), and the absence of suspension culture for further screening may all contribute to the production of these somaclonal variants. If a screening factor, such as pathogen toxin or a high salt concentration, could be applied to bermudagrass tissue culture for preferred mutant selection, somaclonal variation breeding could be promising in “predictive” breeding of triploid bermudagrass.

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