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Development of highly regenerable callus lines and biolistic transformation of turf-type common bermudagrass [*Cynodon dactylon* (L.) Pers.]

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Abstract Common bermudagrass, *Cynodon dactylon*, is a widely used warm-season turf and forage species in the temperate and tropical regions of the world. Improvement of bermudagrass via biotechnology depends on improved tissue culture responses, especially in plant regeneration, and a successful scheme to introduce useful transgenes. When the concentration of 6-benzylaminopurine was adjusted in the culture medium, yellowish, compact calluses were observed from young inflorescence tissue culture of var. *J1224*. Nine long-term, highly regenerable callus lines (including a suspension-cultured line) were subsequently established, of which six were used for biolistic transformation. Five independent transgenic events, with four producing green plants, were obtained following hygromycin B selection from one callus line. Three transgenic events displayed resistance to the herbicide glufosinate, and one of these showed β -glucuronidase activity since the co-transformation vector used in the experiments contained both the *gusA* and *bar* genes.

Keywords Bermudagrass · Embryogenic · Plant regeneration · Transgenic · Turfgrass

Abbreviations *ABA*: Abscisic acid · *BAP*: 6-Benzylaminopurine · *2,4-D*: 2,4-Dichlorophenoxyacetic acid · *GA₃*: Gibberellic acid · *GUS*: β -Glucuronidase · *hph*: Hygromycin phosphate transferase · *hyg B*: Hygromycin B · *NAA*: α -Naphthaleneacetic acid · *SEC*: Somatic embryo cluster

Introduction

Bermudagrass is widely used as a warm-season turf and forage grass in the temperate and tropical regions of the world. Turf-type bermudagrasses include a species, *Cynodon dactylon* (common bermudagrass, usually $2n=4x=36$), and an interspecific hybrid, *Cynodon dactylon* \times *C. transvaalensis* (hybrid bermudagrass, $2n=3x=27$). As for most plant species, improvement of bermudagrass via biotechnology depends on improved tissue culture responses, especially in plant regeneration, and a successful scheme to introduce useful transgenes. Although calluses have been readily induced from vegetative tissues such as nodal segments of some turf-type bermudagrass varieties (Chaudhury and Qu 2000), regenerable calluses have mostly been obtained from immature inflorescence culture (Ahn et al. 1985, 1987; Artunduaga et al. 1988, 1989; Chaudhury and Qu 2000; Li and Qu 2002). Albinism has also been encountered in plant regeneration (Artunduaga et al. 1988). Artunduaga et al. (1989) reported an improved tissue culture response of the common bermudagrass cultivar *Zebra* by using a combination of $13.6 \mu\text{M}$ 2,4-D and 200 mg l^{-1} casein hydrolysate in the culture medium. Chaudhury and Qu (2000) substantially improved green plantlet regeneration of turf-type bermudagrass by lowering the level of 2,4-D ($4.5 \mu\text{M}$) and adding BAP ($0.04 \mu\text{M}$) to the MS (Murashige and Skoog 1962) callus induction medium. Scanning electron microscopic studies have revealed that somatic embryogenesis is a major route for plant regeneration in turf-type bermudagrass. It has recently been shown in this laboratory that somatic embryo formation of the hybrid bermudagrass variety *Tifgreen* can be further improved by including ABA in the above callus induction medium and the germination/regeneration of somatic embryos accelerated by supplementing GA_3 in the regeneration medium. Repetitive somatic embryogenesis was observed in tissue culture of common bermudagrass var. *Savannah* (Li and Qu 2002), and genetic transformation of the hybrid bermudagrass variety *TifEagle* has been recently achieved (Zhang et al. 2001, 2003; Gold-

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man et al. 2002). In this communication, we report the establishment of highly regenerable callus lines and, for the first time, the recovery of green transgenic plants from common bermudagrass.

Materials and methods

Plant material and tissue culture conditions

Common bermudagrass var. *J1224* (Jacklin Seeds, Post Falls, Idaho) and nine other varieties (see Results section) were grown at the Turfgrass Field Laboratory of North Carolina State University (Raleigh, N.C.). Young inflorescences wrapped in the sheath were collected throughout the blooming season (May to September) when the flag leaf was first emerged. The sheathed inflorescences were first rinsed with 70% ethanol for 1 min, then sterilized in 50% Clorox (6.0% sodium hypochlorite; Clorox, Oakland, Calif.) with 0.1% Tween 20 for 15–20 min with stirring, and rinsed five times with sterile distilled water. The young inflorescences, approximately 1 cm in length, were sliced into segments of approximately 1 mm and cultured in the callus induction medium for 3–4 weeks at 25°C in the dark. In most of the experiments, the callus induction medium consisted of MS basal medium supplemented with 4.5 μM 2,4-D, 0.04–0.9 μM BAP, 0–1.9 μM ABA, 30 g l⁻¹ sucrose, and 3 g l⁻¹ Phytigel; occasionally NB medium (Li et al. 1993) with the above supplements except for BAP was used. Yellowish, compact sectors of the induced calluses were isolated and subcultured twice at 3-week intervals in the above MS medium supplemented with an elevated BAP level (2.2 μM for long-term maintenance). The embryogenic callus lines were then subcultured every 4 weeks on MS medium supplemented with BAP. The same media without Phytigel was used for suspension cell culture, which was subcultured once a week. The regeneration medium was MS basal medium containing 4.4 μM BAP, 1.05 μM NAA, 1.3 μM GA₃, 30 g l⁻¹ sucrose, and 3.0 g l⁻¹ Phytigel. The rooting medium consisted of half-strength MS medium supplemented with 1.05 μM NAA, 30 g l⁻¹ sucrose, and 3.0 g l⁻¹ Phytigel. The pH of all media was adjusted to 5.8 prior to autoclaving. All chemicals were purchased from Sigma Chemical Co (St. Louis, Mo.). The medium was autoclaved at 121°C for 30 min and cooled down to 50°C before the sterile stock solutions of the growth regulators, ABA, BAP, GA₃, or NAA were added. Cultures for regeneration and rooting were maintained in a lighted (140 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ of cool-white fluorescent light) culture chamber (CU-32L; Percival, Boone, Iowa) at 25°C under a 16/8-h (day/night) photoperiod.

Biolistic transformation of the embryogenic callus lines

Plasmid pACh1 (Wang et al. 1992) carrying an *hph* gene under the control of the rice *Act1* 5' regulatory sequence (promoter and intron 1) was used in the transformation experiments for hyg B resistance. Plasmid pAHC25 (Christensen and Quail 1996) was used for expression of the reporter *gusA* and the herbicide resistance *bar* genes, with both being under the transcriptional regulation of the maize *ubi1* promoter (including its intron 1).

The transformation experiments were performed using a PDS-1000/He Biolistic Particle Delivery system (Bio-Rad, Hercules, Calif.). Based on the preliminary experiments using transient *gusA* gene expression levels as an indicator, we chose the following parameters for the bombardment experiments: 1,550 psi helium pressure, 7 cm distance between the stopping screen and target tissue, two shots per target plate, and 1.0- μm gold particles. Three milligrams of gold particles in 50 μl 50% glycerol were coated with 5 μg plasmid DNA as instructed by the manufacturer (Bio-Rad) and used for six shots. Approximately 50 pieces of callus, each about 0.5 cm in diameter, were sliced into smaller pieces and placed in the middle of a plate containing osmotic medium (MS basal medium, 0.5 M D-mannitol, 30 g l⁻¹ sucrose, 9.0 μM 2, 4-D, and

3 g l⁻¹ Phytigel; Vain et al. 1993) for 4 h prior to bombardment. These were left on the same medium overnight following the bombardment before being transferred to the subculture medium.

The callus selection medium was the same as the subculture medium but contained 200 mg l⁻¹ hyg B, and the regeneration medium contained 100 mg l⁻¹ hyg B. Regenerated shoots were transferred to rooting medium containing 50 mg l⁻¹ or 100 mg l⁻¹ hyg B in Magenta boxes (Magenta, Chicago, Ill.). Rooted plants were transplanted to soil for further growth.

Analysis of transgenic plants

The assay for transient and stable expression of the *gusA* gene was performed as previously described (Li et al. 1993). Leaf painting assays were performed to evaluate the resistance of transgenic plants to the herbicide Finale (11% glufosinate-ammonium, from former AgrEvo USA). A 1% dilution of Finale was generously applied using cotton swabs to both sides of the marked leaf areas.

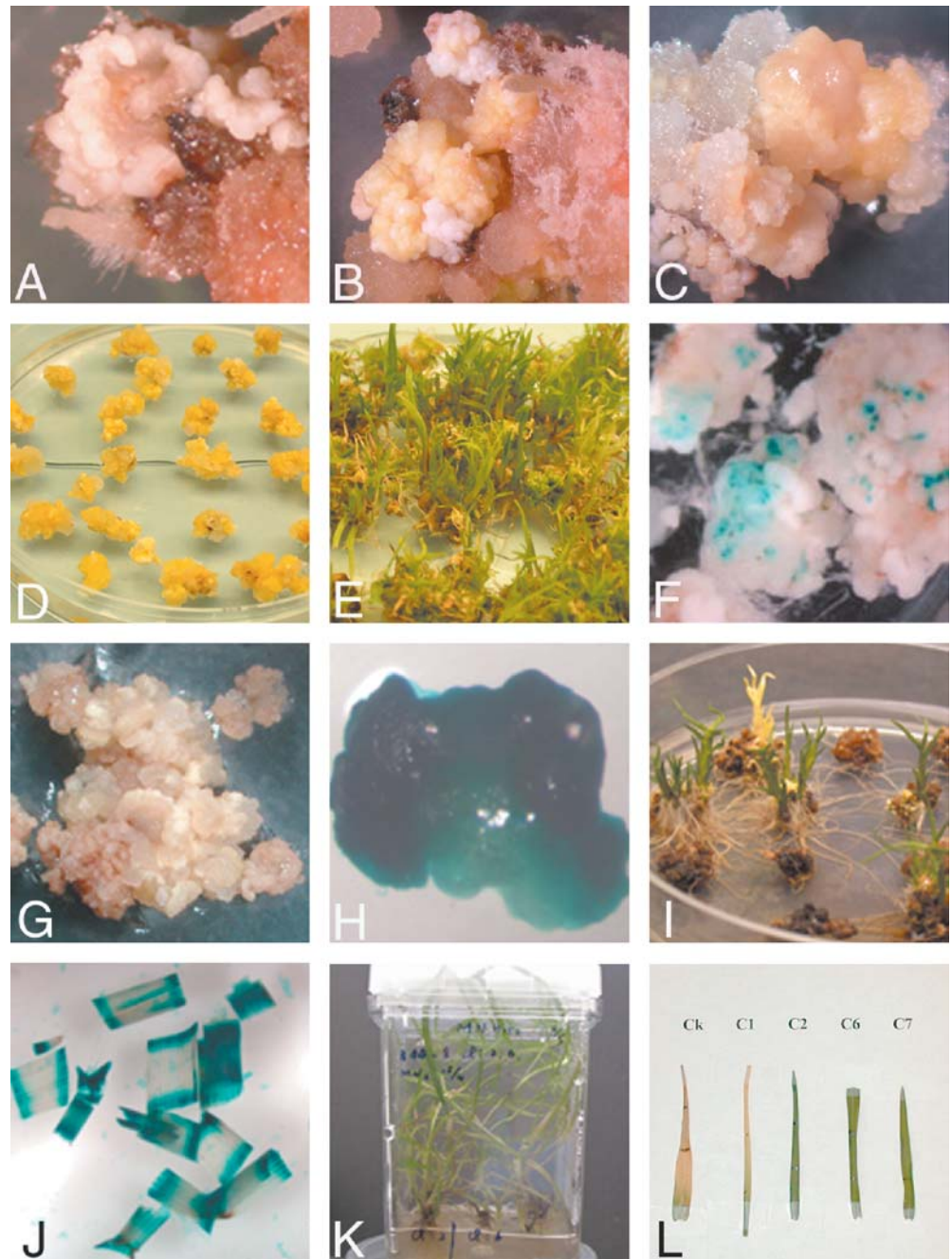
For the Southern hybridization analysis, genomic DNA was extracted from leaf tissues of non-transformed and putative transgenic plants based on the protocol of Dellaporta et al. (1983) with the addition of a DNase-free RNase A treatment (0.5 mg l⁻¹, 37°C, 10 min) before precipitating genomic DNA. A 10- μg aliquot of genomic DNA from each sample was digested with *Kpn*I, which cleaves once at the 3' terminus of the *hph* gene cassette in pACh1 (Fig. 2B) and subsequently subjected to electrophoresis on a 0.8% agarose gel. Plasmid DNA equivalent to one copy of the *hph* gene in a 2C common bermudagrass genome (Arumuganathan et al. 1999)—i.e., 30 pg of pACh1—mixed with 10 μg genomic DNA of a non-transgenic var. *J1224* plant was used as a positive control. Following electrophoresis, the gel was treated with 0.5 M NaOH and 1.5 M NaCl to denature the DNA and then neutralized with a solution containing 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl. The DNA was then blotted to GeneScreen hybridization transfer membrane (NEN Research Products, Boston, Mass.) according to the instructions of the manufacturer. Hybridization was performed with a 1.1-kb *Sma*I fragment of pMON410 (Rogers et al. 1987) containing the full-length coding region of the *hph* gene in the MiracleHyb hybridization solution (Stratagene, Cedar Creek, Tex.). The probes were labeled with α -[³²P]dCTP using the Prime-It II Random Primer Labeling kit (Stratagene) according to the manufacturer's instructions. After overnight hybridization at 68°C, the blot was washed once for 20 min at room temperature with 2 \times SSC buffer and 0.1% SDS solution, followed by two washes, 30 min each, at 65°C with 0.1 \times SSC and 0.1% SDS (Sambrook et al. 1989), and exposed to Kodak BioMax MS film (Eastman Kodak, Rochester, N.Y.).

Results

Establishment of highly regenerable embryogenic callus lines

In a pilot experiment, young inflorescences of ten common bermudagrass varieties (*J1224*, *Yuma*, *Shangri-la*, *Mirage*, *Pyramid*, *Sun Devil*, *Sun Star*, *Sun Dance*, *Cheyenne*, and *Savannah*) were cultured in MS media containing various combinations of auxin and cytokinin to evaluate their tissue culture responses. Approximately 30–40% of var. *J1224* calluses developed somatic embryo clusters (SECs, Fig. 1A) 2 weeks after culture (Li and Qu 2002), which contained white, differentiated somatic embryos, whereas SEC formation on calluses from other varieties on various media was 5–30%. Moreover, yellowish, compact, undifferentiated callus sectors were only occasionally observed from the culture of *J1224*

Fig. 1A–L Highly regenerable callus lines and transformation of bermudagrass. **A** A somatic embryo cluster (*SEC*) on a piece of callus. **B, C** Yellowish, compact callus sectors forming in adjacent to *SEC*s. **D** An established embryogenic callus line. **E** Regeneration of an embryogenic callus line. **F** Transient *GUS* expression 3 days after particle bombardment. **G** A hyg B-resistant callus 10 weeks after bombardment. **H** *GUS* expression in a piece of hyg B-resistant callus. **I** Plantlets regenerated from hyg B-resistant calluses. **J** *GUS* expression in leaves of transgenic plant C2. **K** Two green transgenic plants in rooting medium. **L** Responses of transformation events C1, C2, C6, and C7, and a non-transgenic control plant (*Ck*) to the application of 1% herbicide Finale. Photo was taken 5 days after leaf painting



adjacent to the *SEC*s (Fig. 1B, C). Further tests showed that this distinct type of callus was highly regenerable and could be developed into embryogenic callus lines (Fig. 1D, E). Thus, var. *J1224* was chosen for subsequent experiments. The undifferentiated, embryogenic status of these lines can be maintained for a long period during subculture in media supplemented with an elevated BAP concentration (starting at 0.9 μM for callus line development and increasing to 2.2 μM for culture maintenance). Among the approximately 300 young inflorescences of *J1224* that were cultured, nine embryogenic callus lines, including a suspension callus line, were established. Thus, the frequency at which such embryogenic callus lines

developed from *J1224* young inflorescence culture was approximately 3%. To date, these lines have been subcultured for more than 14 months, and over 80% of the calluses in each line are still able to regenerate into green plantlets.

Genetic transformation and analysis of the transgenic plants

In four transformation experiments, a total of 31 plates of calluses from six highly regenerable callus lines were co-bombarded with vector pACh1 containing the *hph* gene

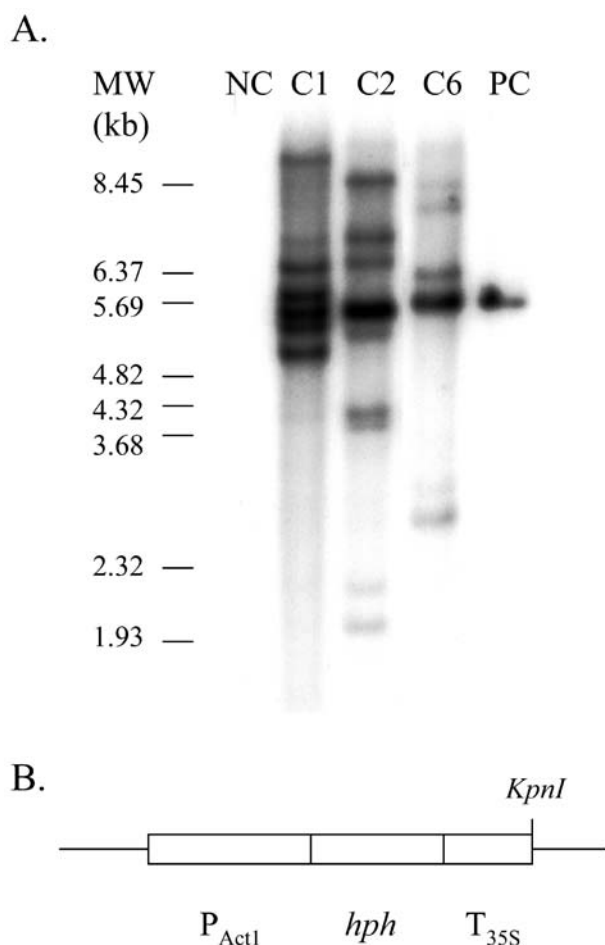


Fig. 2A, B Molecular analysis of the transgenic plants. **A** Southern analysis of three transgenic bermudagrass events, *C1*, *C2*, and *C6*. *NC* Negative control (DNA from a non-transgenic plant of *J1224*), *PC* positive control (*NC* plus one copy/genome equivalent of pACh1). All DNAs were digested with *KpnI*, and the blot was hybridized with *hph* probes. *MW* Molecular-weight markers; lambda DNA digested with *BstEII*. **B** A simplified diagram showing the gene construct and the *KpnI* restriction site in vector pACh1 (5.6 kb). *P_{Act1}* Rice *Act1* promoter including intron 1 in the 5' untranslated region, *hph* hygromycin phosphotransferase gene, *T_{35S}* CaMV 35S terminator

and vector pAHC25, which harbors the *gusA* gene and a *bar* gene. The bombarded calluses were cultured for 3 weeks before being subjected to hyg B selection (200 mg l^{-1}). In one experiment, following 7 weeks of selection in the dark at 25°C , two clusters of hyg B resistant calluses, 0.5–1 cm in diameter, were identified (Fig. 1G). Both were from the embryogenic callus line *J15-1A*, which had been maintained on $2.2 \mu\text{M}$ BAP, and of which two plates had been bombarded. GUS activity was observed throughout whole pieces of callus (Fig. 1H) or in sectors. Six subsets of green plantlets (with occasional albinos) (Fig. 1I, K) and a subset of albino plantlets were regenerated from the hyg B-resistant calluses. The hyg B-resistant albino plants were not further analyzed. After transplanting into soil, four independent transformation events (*C1*, *C2*, *C6*, and *C7*)

were identified among the green transgenic plants based on Southern analysis, GUS assays, morphology, and resistance to the herbicide Finale. Transgenic events *C2*, *C6*, and *C7* showed resistance to herbicide application (Fig. 1L), but only *C2* was GUS-positive (Fig. 1J). *C2* and *C6* had no appreciable morphological changes from the parent variety, while *C1* and *C7* were off-types with shorter and wider leaves (data not shown). Southern analysis of *C1*, *C2* and *C6* is shown in Fig. 2A. The three transformation events have distinct hybridization patterns and multiple *hph* transgene copies. In another transformation experiment, a hyg B-resistant, GUS-positive callus was obtained from line *J15-1A* (data not shown). No transgenics was recovered from the other two experiments. From these four experiments, five independent transformants were obtained; the callus transformation efficiency of var. *J1224* (no. of independent transformant obtained/no. of callus bombarded) was therefore approximately 0.32%. In these experiments, three plates of callus line *J15-1A* was used. Thus, the transformation efficiency for this line was 3.3%

Discussion

Common bermudagrass is a recalcitrant species in tissue culture and is highly recalcitrant with respect to the recovery of transgenic plants. Soft, non-embryogenic calluses have often been induced in culture from bermudagrass tissues that were not regenerable. These calluses were occasionally transformable, but no transgenic plant was recovered (unpublished data). It has been reported that somatic embryo formation can be induced by adding $0.04 \mu\text{M}$ BAP and $7.6\text{--}19 \mu\text{M}$ ABA to the callus induction/subculture media and that embryo germination/regeneration can be improved by supplementing the regeneration medium with $0.5 \mu\text{M}$ GA_3 (Chaudhury and Qu 2000; Li and Qu 2002). However, it remained very difficult to recover transgenic plants from common bermudagrass because the somatic embryos formed too quickly (within 2 weeks) under those conditions. It appears that successful transformation requires a large number of undifferentiated, embryogenic cells which are both regenerable and transformation-competent. Thus, the yellowish, compact, embryogenic calluses, as identified in this report, and the callus lines established from these calluses are a key factor for the recovery of green transgenic plants in common bermudagrass. The embryogenic callus sectors were obtained in most cases after extended culture in media containing a very low level of BAP ($0.04 \mu\text{M}$). The fact that such calluses were only obtained from var. *J1224* suggests an important role of genotype in developing embryogenic callus. Higher concentrations of BAP in the subculture media were necessary to maintain the undifferentiated and embryogenic nature of the cells and to develop callus lines. The addition of ABA to the culture media promoted somatic embryo formation and maturation (Li and Qu 2002), but did not facilitate the formation and maintenance of this

type of callus. Thus, ABA was not included in the media in most of the experiments. Improvement in the induction and maintenance of embryogenic calluses in other grasses by supplementing BAP to callus induction and subculture media has been reported (Altpeter and Posselt 2000; Cho et al. 2000; Bai and Qu 2001; Bradley et al. 2001).

Hyg B selection was first used in rice transformation for obtaining fertile transgenic rice plants (Shimamoto et al. 1989). The selection works efficiently for quite a few non-cereal grass species (Wang et al. 1992; Spangenberg et al. 1995), including common bermudagrass (this report). However, it seems that callus from these grass species has a natural resistance to the antibiotic, and higher concentrations (150–250 mg l⁻¹) are usually necessary for selection of transgenic callus. However, plantlets seem to be more sensitive to hyg B, and 50–100 mg l⁻¹ is sufficient for selection at this stage for tall fescue (Bai and Qu 2001) and bermudagrass (this report).

Among the six embryogenic callus lines used in the transformation experiments, a total of five independent transformation events were recovered in two transformation experiments from only one callus line, J15-1A, suggesting a variation in biolistic transformation competence among the lines although the mechanism is yet to be elucidated.

We have developed a protocol for developing highly regenerable callus lines and, for the first time, for obtaining green transgenic plants from common bermudagrass. This lays the foundation for engineering useful traits, such as nematode resistance and drought tolerance, into the grass species in the near future. The highly regenerable callus lines could also be employed for transformation using other approaches, such as the *Agrobacterium*-mediated transformation.

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