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Agrobacterium-mediated transformation of common bermudagrass (*Cynodon dactylon*)

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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. We have been able to transform the species using *Agrobacterium*-mediated approach. In seven experiments reported here, a total of 67 plates of calluses and suspensions were infected with *Agrobacterium tumefaciens* strains, and nine hygromycin B resistant calluses were obtained after selection. Among them two green independent transgenic plants were recovered. The plants growing in pots looked relatively compact at the beginning, but the ploidy level of the plants, as determined by nuclear DNA content, was not altered.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; ABA – abscisic acid; BAP – 6-benzylaminopurine; GA – gibberellic acid; Hph – hygromycin phosphate transferase; hyg B – hygromycin B; NAA – *α-naphthaleneacetic acid;* SEC – somatic embryo cluster

Introduction

Bermudagrass (*Cynodon dactylon* (L.) pers.) is an important warm-season turf and forage grass in the temperate and tropical regions of the world. Bermudagrass actually includes a species, *Cynodon dactylon* (common bermudagrass, usually $2n = 4x = 36$), and an interspecific hybrid, *Cynodon dactylon* × *Cynodon transvaalensis* (hybrid bermudagrass, $2n = 3x = 27$). Overall, bermudagrass is a highly recalcitrant species *in vitro*. However, progress has been made in the last few years in somatic embryogenesis from young inflorescence culture of bermudagrass (Chaudhury and Qu, 2000; Li and Qu, 2002; Li and Qu, 2004). Embryogenic calluses were also obtained by culturing nodal segments of the hybrid bermudagrass elite turf-type cultivar TifEagle (Zhang et al., 2003). Genetic transformation of hybrid and common bermudagrass by microprojectile bombardment was recently reported (Zhang et al., 2003; Goldman et al., 2004; Li and Qu, 2004).

Agrobacterium-mediated transformation of several cereals and other grasses has been reported (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997; Tingay et al., 1997; Yu et al., 2000; Zhao et al., 2000; Bettany et al., 2003). Most of the approaches used the "super-binary" vector, which contains a DNA fragment of the virulence region of pTiBo542, as demonstrated in the report by Hiei et al. (1994). *Agrobacterium*-mediated transformation is often preferred because the approach transfers defined T-DNA fragment, often yields plants with low number of transgene copies, and does not require expensive equipment. In this communication, we report, for the first time, the recovery of green bermudagrass plants transformed with *Agrobacterium tumefaciens*. In most of the experiments, only a "standard" binary vector (Frame et al., 2002) was used, suggesting

that "super-binary" vector may not be necessary for bermudagrass transformation.

Materials and methods

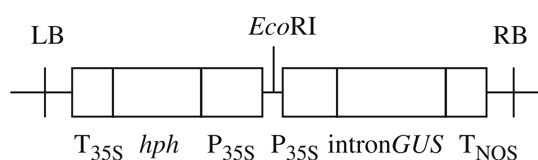
Plant material and tissue culture conditions

Common bermudagrass variety 'J1224' (Jacklin Seeds, Post Falls, ID, USA) was grown at the Turfgrass Field Laboratory of North Carolina State University (Raleigh, NC, USA). Somatic embryo cluster (SEC) induction from young inflorescence and the culture conditions were as previously reported (Li and Qu, 2004). To establish suspension cultures, some SECs were collected and cultured in AA liquid medium (Müller and Grafe, 1978), supplemented with 2,4-D (4.5 μM) and sucrose (30 g l^{-1}), at 25°C in the dark with a shaking speed of 120 rpm. The medium was changed weekly. After two months, the suspension culture was switched to MS liquid medium supplemented with 2,4-D (4.5 μM), 3% sucrose and BAP (gradually increased from 0.8 to 2 μM).

Agrobacterium-mediated transformation

The binary vectors pCAMBIA1301 (Figure 1) and pRQ235, both derivatives of pCAMBIA1300 (CAMBIA, Canberra, Australia), were used in the transformation experiments. The T-DNA of the two vectors includes a selectable marker gene construct for hygromycin B (hyg B) resistance and a construct of a *gus* reporter gene containing the first intron of castor bean CAT−1 gene (Ohta et al., 1990) or a gene of interest (GOI). The freeze-thaw method (An et al. 1988) was used to introduce the binary vector into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993), sometimes together with pTOK47 which carries a 20 kb *Kpn*I fragment of Ti plasmid from pTiBo542 (Jin et al., 1978) containing *virB*, *virC*, and *virG* virulence genes (Le et al., 2001). The resulting strains are EHA105(pCAMBIA1301), EHA105 (pRQ235), and EHA105(pTOK47, pRQ235), designated as E1301, E235 and ET235, respectively. The strains were inoculated in YEP liquid medium (An et al., 1988) in the presence of appropriate antibiotics, and cultured at 26°C for 24 h until the OD_{595} reading reached 1.5–2.0. Seventy-five microliter of the culture was added to 50 ml AB medium (Hiei et al., 1994), supplemented with appropriate antibiotics, and the culture was grown for another 24 h when its OD_{595} reading was 1.5. The bacteria were collected by centrifugation (2000*g*, 10 min) and re-suspended in AAM medium (Hiei et al., 1994), supplemented with 4.5 μM 2,4-D and 100 μM acetosyringone (Aldrich,

(a) pCAMBIA1301



(b) pAcH1

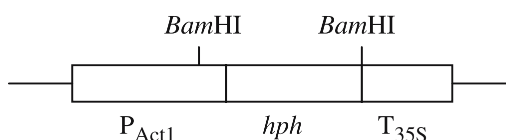


Figure 1. Schematic representation of plasmids *(a)* T-DNA region of plasmid pCAMBIA1301. The position of *Eco*RI restriction site, used in Southern analysis, is marked in pCAMBIA1301. The diagrams are not drawn proportionally. P_{35S}: CaMV 35S promoter; T_{NOS}: *Agrobacterium tumefaciens nos* gene terminator; T_{35S}: CAMV 35S terminator. LB and RB: left border and right border sequences, respectively, of T-DNA. *(b)* A simplified diagram showing the gene construct and the *Bam*HI restriction sites in vector pAcH1 (5.6 kb) used as the positive control in DNA gel blot analysis. *Bam*HI digestion yields a band of 1.35 kb containing the *hph* coding region. P_{Act1}: rice *Act1* promoter including intron 1 inside the 5' untranslated region; *hph*: hygromycin phosphotransferase gene, T_{35S}: CaMV 35S terminator.

Milwaukee, WI, USA), to make the OD_{595} reading 0.5–0.6 for co-cultivation.

Calluses, from callus lines subcultured on solid medium or suspension culture, approximately 1.5 g per plate, were sliced into small pieces (1–2 mm in diameter), and transferred to NB1 medium (Li et al., 1993) supplemented with 4.5 μM 2,4-D and 100 μM acetosyringone. The *Agrobacterium* suspension was dropped to cover the calluses for co-cultivation in the dark at 25°C for 2–3 days. The calluses were then collected and rinsed with sterile water, blot dried, and transferred to MS medium (supplemented with 4.5 μM 2,4-D, 2 μM BAP, 30 g l^{-1} sucrose and 3 g l^{-1} phytagel) containing 150 mg l^{-1} carbenicillin (Apollo Scientific, Stockpork, UK) and 200 mg l^{-1} timentin (GlaxoSmithKline, Research Triangle Park, NC) to inhibit *Agrobacterium* growth (Cheng et al., 1998).

Ten days later, the calluses were transferred to the same medium containing 25 or 50 mg l^{-1} hyg B for selection. Three to 4 weeks later, the calluses were subjected to two more rounds of selection on the same medium containing 100–150 mg l^{-1} hyg B with 3–4 weeks each round. Sometimes, selection in liquid medium using similar scheme was also tested. Calluses growing vigorously under selection were transferred to the regeneration medium with selection (50 mg l^{-1} hyg B). The same concentrations of carbenicillin and timentin were included in the selection medium, and timentin alone in the regeneration medium. Regeneration and rooting culture were carried out in a lighted culture chamber (CU-32L, Percival, Boone, IA, USA) with a 16-h photoperiod (140 μmol m^{-2} s^{-1} of cool white fluorescent light) at 25 °C. Regenerated plants were transferred to rooting medium containing 50 mg l^{-1} hyg B for root development and further selection at plantlet level. Rooted plants were transplanted to soil and grown in the greenhouse.

Analysis of transgenic calluses and plants

Assay for transient and stable expression of the *gus* gene was performed as previously described (Li et al., 1993).

For DNA gel blot 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 ml^{-1}, 37 °C, 10 min) before precipitating genomic DNA. Ten μg of genomic DNA from each sample was digested with *Eco*RI, which cleaves once between the *hph* and *gus* gene cassettes in the pCAMBIA1301 (Figure 1), and was subjected to electrophoresis in a 0.8% agarose gel. Plasmid pAcH1 DNA equivalent to one copy of the *hph* gene in a 2C common bermudagrass genome (Li and Qu, 2004) mixed with 10 μg genomic DNA of a non-transgenic 'J1224' plant, and digested with *Bam*HI to yield an 1.35 kb fragment containing the *hph* coding region (Figure 3a), was used as a positive control. Southern analysis was performed as previously described (Li and Qu, 2004).

Determination of ploidy level of transgenic plants

The plant ploidy level was determined by nuclear DNA content using flow cytometry. Two hundred milligram of fresh stems of the samples were harvested and manually chopped with a razor blade into small segments (< 0.1 mm) to release nuclei with approximately 1 ml of chopping buffer (per liter: 9.14 mg $MgCl_2$, 8.94 g sodium citrate, 4.18 g morpholinopropanesulfonic acid, 1 ml Triton X−100, pH adjusted to 7.0). The liquid was removed, filtered through 50 and 20 (μm nylon mesh screens successively, and centrifuged at 100 g for 8 min at 8 °C. The supernatant was removed and the pellet was re-suspended in 500 (μl propidium iodide at 200 mg l^{-1} stock diluted in the chopping buffer. The samples were then analyzed using a Coulter Epics-XL acquisition flow cytometer (Coulter Corp., Miami, FL, USA) at a wavelength of 488 nm. Samples were run until 5000 nuclei had been analyzed in the largest peak in the histogram (generally the 2C peak).

To quantify DNA content, chicken erythrocyte nuclei (CEN) at 2.33 pg DNA/2C was added to the final sample as an internal standard and reference. Two drops of CEN at 2.0×10^7 cells per (μl were diluted in 100 (μl of chopping buffer, and 5 (μl of this solution was used per sample. Nuclear DNA content was obtained by using the following equation: nuclear DNA content = mean position of sample peak times the standard DNA amount (2.33 pg) divided by mean position of the peak of the standard.

Results and discussion

Establishment of embryogenic callus lines and suspension cultures

The SECs and the yellowish, compact calluses growing adjacent to them were induced, and used to establish embryogenic callus lines as previously described (Li and Qu, 2004). Meanwhile, suspension cultures were initiated by transferring early stage SECs (4 weeks after callus induction) to AA liquid medium. In two months or so, numerous granular, embryogenic structures (0.2–1 cm in diameter) were observed in some cultures and used to establish embryogenic suspension cultures (Figure 2a, b). Gradual increase of BAP in the medium (from 0.4 to 1 μM) helped maintain the regeneration ability of the suspensions (Altpeter and Posselt, 2000; Cho et al., 2000; Li and Qu, 2004). When the regeneration ability declined or albino plants increased after a long-term culture, the embryogenic suspension culture was ‘‘renewed’’ by selecting only the callus pieces that still maintained the ability to regenerate green plantlets to re-establish the suspensions. After such a screening, the regeneration ability of the suspen-

Figure 2. Embryogenic suspension lines and *Agrobacterium*-mediated transformation of bermudagrass. *(a)* A suspension culture in a flask. *(b)* Granular SECs from suspension culture. *(c)* Transient GUS expression after co-cultivation with *Agrobacterium*. *(d)* Selection of hyg B resistant callus. *(e)* GUS expression in a piece of hyg B resistant callus. *(f)* The upper half shows plantlets regeneration from a hyg B resistant callus line. Note the roots are already growing. The lower half demonstrates the selection effects on non-transgenic calluses. *(g)* A green transgenic plant growing in selective rooting medium. *(h)* A green transgenic plant growing in the greenhouse. *(i)* GUS expression in leaves of transgenic plant A125-10.

sion was enhanced from below 50% to more than 80%, and the regeneration ability of the suspension lines could last for more than a year.

Agrobacterium-*mediated transformation and analysis of the transgenics*

In five experiments using *Agrobacterium* strain E1301, a total of 27 plates of calluses from 7 embryogenic callus lines (cultured on solid medium) and 16 plates of calluses from 4 suspension lines were subjected to *Agrobacterium*-mediated transformation. After co-cultivation, transient expression of *gus* gene in bermudagrass cells was observed (Figure 2c). Since the *gus* gene contains an intron (Ohta et al., 1990), the detected GUS enzyme activity indicated T-DNA delivery into the plant cells. After selection, 6 hyg B resistant calluses were obtained (Figure 2d) with 4 showing GUS activity (Figure 2e). Green plantlets from two resistant calluses were recovered (Table 1, Figure 2f, g, h). They were confirmed as two transformation events by Southern analysis (see below). The two events were designated as A125-10 and A125-12. Event A125-10 displayed a strong GUS enzyme activity by histochemical assay (Figure 2i) whereas A125-12 did not show any GUS activity. In some experiments, surfactant Silwet 77 (Clough and Bent, 1998) or amino acid L-cysteine (Olhoft and Somers, 2001; Frame et al., 2002) was added to the medium but no obvious effect was observed (data not shown).

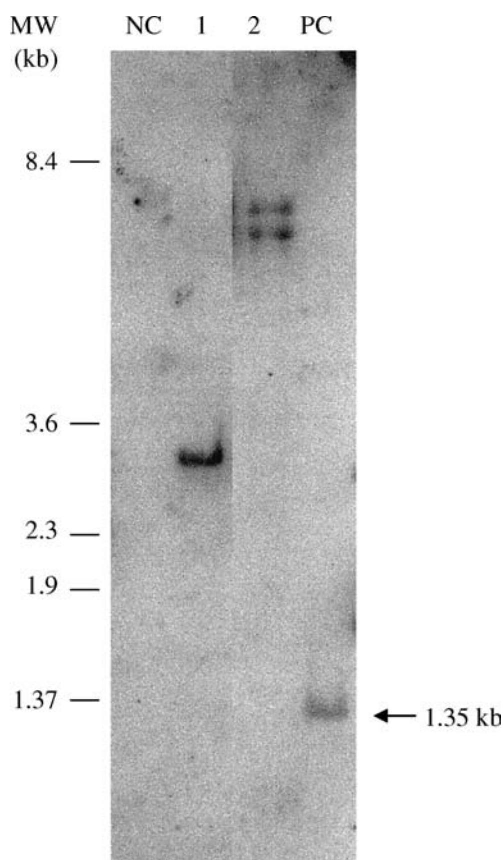


Figure 3. Southern analysis of transgenic plants. Southern analysis of two transgenic bermudagrass plants. Lane 1: A125-10; Lane 2: A125-12; NC: negative control (DNA from a non-transgenic plant of J1224); PC: positive control (NC plus 1 copy/genome equivalent of pAcH1). Plant DNA was digested with *Eco*RI while the positive control DNA was digested with *Bam*HI (Figure 1*b*). The blot was hybridized with *hph* gene probes. MW: molecular weight markers from lambda DNA digested with *Bst*EII.

Two experiments involving 24 plates of calluses from 9 callus lines were performed using strains E235 or ET235 (Table 1). Three hyg B resistant calluses were obtained. Two resistant calluses were from 12 plates of E235 transformation while one from 12 plates transformed with ET235.

Difference in transformation competence was observed among the callus and suspension lines. Of the nine hyg B resistant calluses, seven were from suspension line J21-1 or the callus lines derived from it. Both transgenic plants were recovered from this line.

Southern analysis of the two green, hyg B-resistant plants obtained from the experiments confirmed the transgenic nature of the plants. Since *Eco*RI cleaves only once in the binary vector, the hybridization patterns of genomic DNA of the two transgenic plants digested with *Eco*RI were expected to vary due to the location of the nearest *Eco*RI restriction site in the genome at the T-DNA insertion locus. It is the case here for the two transgenic plants examined, indicating that they were from two transformation events (Figure 3). Event A125-10 is most likely to have one copy of the *hph* transgene, while A125-12 has two. It is not unusual to have more than one transgene copies in *Agrobacterium* transformation. In a large-scale effort in *Agrobacterium* transformation of *Arabidopsis*, the average number of T-DNA insertions per line was found to be ~1.5 (Alonso et al., 2003).

Efforts to recover transgenic plants using the *bar* gene and the bialaphos selection system (De Block et al., 1987) resulted in a couple of stably-transformed, GUS-positive calluses or sectors.

Table 1. Experimental results of *Agrobacterium*-mediated transformation of common bermudagrass

Strain	Plant tissue	No. of plate infected	No. of hyg B resistant callus (No. of GUS^{+})	No. of hyg B resistant plant (No. of GUS^{+})
E1301	Callus lines	27	1 (1)	0
	Suspensions	16	5 (3)	2 (1)
E235	Callus lines	12	2 (N.A.)	0
ET235	Callus lines	12	1 (N.A.)	0

N.A.: not applicable.

Plantlets recovered from calluses survived the selection at 7 or 10 mg l^{-1} bialaphos were escapes as revealed by Southern analysis (data not shown). The results suggest high natural tolerance of common bermudagrass to the herbicide bialaphos.

After the plants were transplanted into soil, the transgenic plants looked relatively compact, in the first few months, with smaller leaves and shorter internodes (data not shown) when compared to their parent cultivar plants. To determine whether the suspension culture or transformation selection somehow altered the ploidy level of the transgenic plants, flow cytometric assays were performed to determine the nuclear DNA content of the transgenic plants and compared to the plants of the parental cultivar, J1224. The results indicated that there was no ploidy level change in the transgenics. The nuclear DNA content in the transgenic plants (both being 1.58 pg) was similar to the one from J1224 plants (1.69 pg).

The transgenic plant event A125-10 flowered in the greenhouse. The inflorescences were curly and abnormal when emerging from the leaf sheath but eventually looked normal when fully extended (data not shown). Microscopic examination with 1% I_2–KI solution revealed that the mature pollen grains had irregular shapes, did not accumulate starch (as indicated by no blue staining), and were deteriorated (data not shown). This plant also failed to set seeds in a crossing attempt, suggesting it be a completely sterile plant. The sterility may be caused by the long-term suspension culture. Plant A125-12 stayed for more than a year in the greenhouse without flowering, similar to the transgenic plants obtained by the biolistic method (Li and Qu, 2004), suggesting a change in the timing of flowering probably due to the *in vitro* conditions and/or the genetic transformation.

To our knowledge, this is the first report of *Agrobacterium*-mediated transformation of bermudagrass. It is only possible to reach this achievement when embryogenic callus and suspension lines are developed. *Agrobacterium*-mediated transformation of bermudagrass seems to yield transgenic plants with less transgene copies than the biolistic approach and thus may reduce gene silencing potential caused by multiple transgene copies (Hiei et al., 1994). Moreover, in general, *Agrobacterium* transformation does not demand special apparatus, is more economical, and could be more efficient (Dong and Qu, 2005) over the biolistic method. The facts that most of the transgenic calluses were obtained with E1301 and E235, and that ET235 did not show advantage in transformation efficiency over E235, may suggest a standard binary vector be sufficient for transforming bermudagrass. Further improvement of the protocol, probably by improving the transformation competence of the culture lines, is still needed to efficiently transform bermudagrass with *Agrobacterium* for its trait improvement.

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