Expression of the cry1Ac in ‘Arizona Common’ Common Bermudagrass via Agrobacterium-mediated Transformation and Control of Black Cutworm

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Additional Index Words. Agrotis ipsilon, Bacillus thuringiensis Berliner, Cynodon dactylon, gene transfer, insect resistance, tissue culture, turfgrass

Abstract. Bermudagrass (Cynodon L.C. Rich.) is grown on more than 4 million ha in the southern United States. The black cutworm (Agrotis ipsilon Hufnagel) is the most commonly encountered pest of bermudagrass, especially on golf course greens. Developing insect-resistant cultivars is a very desirable substitute, both environmentally and economically, to using current synthetic pesticides. Here we report, for the first time, Agrobacterium-mediated transformation of ‘Arizona Common’ common bermudagrass [Cynodon dactylon (L.) Pers.] with the Bacillus thuringiensis Berliner cry1Ac gene encoding an endotoxin active against black cutworm. Mature seeds were used for producing embryogenic callus, and calli were transformed with a plasmid containing a synthetic cry1Ac and the kanamycin resistance (nptII) genes. Putative transgenic calli and plantlets were selected on media containing 100 and 50 mg·L⁻¹ G418, respectively. RNA-blot analysis of PCR-positive lines revealed the expression of the cry1Ac transgene in three out of five putative transgenic lines. The larvae fed on transgenic plant leaves experienced highly significant (over 80%) mortality.

Common bermudagrass is a warm-season perennial species widely used for turf, forage, and soil stabilization (Anderson et al., 1998). The benefits of turf bermudagrass include a high level of wearability, salt and drought tolerance, and many pleasing aesthetic qualities (Gatschet, 1993). Bermudagrass is grown on more than 4 million ha in the southern United States (Burton, 1975) out of 12.2 million ha of turfgrass coverage nationwide (Potter, 1998).

In general, turf-infesting caterpillars (Noctuidae, Crambinae, and Pyralinae) are the major insect pests that feed on turf foliage (Shetlar, 2003). These insects cause severe discoloration, thinning, and other aesthetic problems. The black cutworm is the most commonly encountered pest, especially on golf course greens, whether bentgrass (Agrostis L.) or bermudagrass (Shetlar, 2003). There is no report on the impact of black cutworm feeding on the yield of bermudagrass. However, it has been reported that fall armyworm (Spodoptera frugiperda J.E. Smith), another lepidopteran insect, caused 0.5 to 1.1 t·ha⁻¹ yield loss of ‘Coastal’ common bermudagrass when artificially infested with population densities of 1.1 to 9.9 larvae per 0.1 m² (Jamjanya and Quisenberry, 1988).

Traditionally, turfgrass improvement has relied on conventional breeding methods (Chai and Sticklen, 1998). Recent advanced molecular genetic techniques can accomplish the transfer of insect resistance within a much shorter time period, and transgenic insect-resistant cultivars are very desirable substitutes, both environmentally and economically, to using synthetic pesticides (Yamamoto and Engelke, 1998). There is only one report on gene transfer into common bermudagrass using Biolistic (Bio-Rad Lab, Hercules, Calif.) bombardment (Li and Qu, 2004) and two other reports on using Biolistic bombardment for genetic transformation of hybrid bermudagrass (C. dactylon × C. transvaalensis Burtt-Davy) (Goldman et al., 2004; Zhang et al., 2003). In all of the above three reports, only marker genes have been transferred to the plants.

Common bermudagrass is a recalcitrant species in tissue culture and is highly recalcitrant with respect to the recovery of transgenic plants (Li and Qu, 2004). To perform efficient genetic transformation of turfgrass species, it is necessary to identify the ideal explant type and optimize the in vitro conditions, mainly using different concentrations of various growth regulators for each genotype (Altpeter et al., 2000; Bai, 2001; Foster and Spanenberg, 1999). Monocotyledons such as grasses may not produce phenolic compounds like acetylsyringone (Cheng et al., 2004). Using acetylsyringone or other phenolic compounds in the culture medium might be useful for more efficient transformation.

Bacillus thuringiensis (Bt) formulations have been used for more than 50 years as biological insecticides to control agricultural pests (Ahmad et al., 2002). The Bt insecticidal genes encode δ-endotoxin crystal proteins, which are active against lepidopteran insects. These genes are widely accepted as important components of integrated pest management programs (Entwistle et al., 1993; Sticklen, 1991).

The objectives of the present study included development of systems for producing embryogenic calli and Agrobacterium-mediated transformation of common bermudagrass using cry1Ac, and testing the transgenic plants for resistance against black cutworm.

Materials and Methods

Plant Materials. Mature hulled seeds of ‘Arizona Common’ common bermudagrass were used as explants. Seeds were
prewashed in water with 0.2% Tween-20 for 15 min and rinsed three times with distilled water.

**Tissue Culture Conditions.** Tissue culture conditions followed Salehi and Khosh-Khui (2005) with minor alterations. The seeds were surface sterilized in 70% (v/v) ethanol for 1 min, followed by 100% laundry bleach (5.25% sodium hypochlorite) for 20 min, and then rinsed six times with sterilized distilled water. Preliminary callus induction experiments were performed using Murashige and Skoog (1962) (MS) basal medium (Sigma-Aldrich, St. Louis) containing 30 g·L⁻¹ maltose, 2.5 g·L⁻¹ gelrite (Sigma-Aldrich), 4.5 to 40 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0 to 0.9 μM N⁰-benzylamino purine (BAP). The medium containing the combination of 4.5 μM 2,4-D and 0.45 μM BAP was selected for callus induction. Four weeks later, the calli were cut to 3 × 3-mm pieces and subcultured every 4 weeks to the same fresh medium for 8 weeks more. Preliminary plant regeneration experiments were performed using MS medium containing 30 g·L⁻¹ maltose and 2.5 g·L⁻¹ gelrite, and different combinations of BAP (0 to 7.5 μM), α-naphthaleneacetic acid (NAA) (0 to 1 μM), 2,4-D (0 to 0.6 μM) and gibberellic acid (GA₃) (0 to 2 μM). The combination of 2 μM GA₃ and 0.6 μM 2,4-D or only 0.6 μM 2,4-D were selected for use in plant regeneration medium. The calli were kept in the same medium for 8 to 12 weeks, with one or two subcultures at 4-week intervals. Plantlets were transferred into the rooting medium, which included half-strength MS supplemented with 30 g·L⁻¹ maltose and 2.5 g·L⁻¹ gelrite. Media were autoclaved for 20 min at 121 °C and 147 kPa. All cultures were kept at 25 °C. Cultures were kept in the dark for callus induction and were transferred to a 16/8 h light/dark photoperiod under 70 mol·m⁻²·s⁻¹ light intensity for plant regeneration and rooting.

Two-week-old well-rooted plantlets were transferred to 10-cm pots containing Bacco potting medium (Michigan Peat Co., Houston), acclimatized, and transferred to the greenhouse. Greenhouse conditions were set to 28 °C, 90% to 95% relative humidity and continuous 190 μmol·m⁻²·s⁻¹ light intensity.

**Plasmid Construct.** Transformation experiments were conducted using A. tumefaciens E. F. Sm. & Towns., Conn. strain LBA 4404 (Hoekema et al., 1983) containing the binary vector KUC (Fig. 1). Plasmid KUC (Cheng et al., 1998) contains a synthetic cryIAc from B. thuringiensis, under the control of ubiquitin promoter and nopaline synthase (N) terminator. This plasmid also contains a hygromycin-B resistance gene (hpt), and a β-glucoronidase gene (gus), each under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter and N terminator, and a kanamycin resistance gene (nptII) under the control of N promoter and terminator (Fig. 1).

**Preparation of bacterial suspension.** A single colony of A. tumefaciens containing pKUC was grown in 10 mL YEP medium [containing 10 g·L⁻¹ Bacto peptone (Difco, Detroit), 10 g·L⁻¹ Bacto yeast extract (Difco), 5 g·L⁻¹ NaCl, pH 7.2] supplemented with 50 mg·L⁻¹ of both hygromycin-B and kanamycin (Ahmad et al., 2002), incubated at 28 °C and 250 rpm for 72 h. The cultures with optical density (OD) of ≈1.0 at A₆₀₀ were used for transformation.

**Sensitivity of Cynodon calli and stem pieces to G418.** The untransformed callus and stem pieces were tested in MS basal medium containing 0, 25, 50, 75, 100, 125, and 150 mg·L⁻¹ of G418 (Stratagene Co., La Jolla, Calif.) for 2 weeks. The selected concentrations (100 and 50 mg·L⁻¹) were used for further transgenic callus and plantlet selection, respectively.

**Tobacco leaf extract preparation.** Eight grams of tobacco (Nicotiana tabacum L. ‘Samsun’) leaves were ground using liquid nitrogen, and the powder was dissolved in distilled water in a 15 mL polypropylene tube (Corning, Corning, N.Y.) up to 10 mL. After centrifugation of the solution at 850 g for 20 min, the supernatant was carefully passed through Whatman #1 filter paper (Sigma-Aldrich) and transferred to a new tube. Then, distilled water was added to the solution to make 10 mL volume. This stock was maintained at −20 °C until used.

**Agrobacterium-mediated transformation.** Four-week-old embryogenic calli, 3 × 3-mm pieces, were infected by immersing in the Agrobacterium culture under 400 mm Hg pressure for 30 min. After inoculation, the calli were blotted on sterilized filter papers, and cultured on co-cultivation medium (callus induction medium supplemented with 100 μM acetosyringone (Sigma-Aldrich), 0.2% (v/v) tobacco leaf extract, or a combination of both). After three days of co-cultivation in the dark, the calli were rinsed three times in washing medium containing liquid MS basal medium and 400 mg·L⁻¹ cefotaxime (Sigma-Aldrich) to prevent Agrobacterium overgrowth. Samples were gently hand-agitated for a few minutes at each rinsing time to help exposure of explants to cefotaxime and the removal of the Agrobacterium. Samples were then blotted onto sterilized filter papers and cultured on callus induction medium supplemented with 250 mg·L⁻¹ cefotaxime, and 100 mg·L⁻¹ G418 for selection of the putative transformants. The washing was performed every 4 weeks for a total of 12 to 16 weeks. G418-resistant calli were then transferred to plant regeneration medium, supplemented with 250 mg·L⁻¹ cefotaxime and 50 mg·L⁻¹ G418 under light conditions.

Plantlets were transferred to rooting medium supplemented with 250 mg·L⁻¹ cefotaxime and 50 mg·L⁻¹ G418. In all of the above media, acetosyringone, tobacco leaf extract, cefotaxime and G418 were filter-sterilized and added in the appropriate media after autoclaving and cooling to 50 °C. Two weeks later, the well-rooted plantlets were transferred to pots, acclimatized and then transferred to the greenhouse.

**GUS assay.** Histochemical assay for GUS activity in putative transgenic calli was done as described by Jefferson et al. (1987). The callus pieces were placed in 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc) (Amersham Biosciences, Piscataway, N.J.) solution, incubated at 37 °C overnight, then the color changes observed under a dissecting microscope.

**Polymerase chain reaction (PCR) analysis.** PCR analysis of plants was used to screen the putatively transgenic plants for cryIAc transgene incorporation. Total genomic DNA of control and transgenic plants was extracted from shoots as
described by Edwards et al. (1991). The following set of primers was used: *cry1Ac* F, 5′-ACA GAA GAC CCT TCA ATA TC-3′ (forward primer) and *cry1Ac* R, 5′-GTT ACC GAG TGA AGA TGT AA-3′ (reverse primer). The predicted size of the amplified DNA fragments was 606 bp. DNA amplifications were performed in a thermocycler (Perkin Elmer/Applied Biosystem, Foster City, Calif.) using REDTaq ReadyMix PCR Reaction Mix with MgCl₂ (Sigma-Aldrich). The PCR profile had an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of 1 min at 94 °C (denaturation), 2 min at 60 °C (annealing), and 3 min at 72 °C (extension). The reaction mixture was loaded directly onto a 1.0% (w/v) agarose gel, stained with ethidium bromide and visualized with ultraviolet light.

**RNA-BLOT ANALYSIS.** Total RNA of control and five putative transgenic lines was isolated from shoots using the TRI Reagent (Sigma-Aldrich) according to the manufacturer’s instructions. Aliquots of RNA (20 μg) were fractionated in a 1.2% agarose formaldehyde denaturing gel and blotted to a Hybond-N+ nylon membrane (Amersham Pharmatica Biotech., Piscataway, N.J.) as specified by the manufacturer. The probe was generated by digesting plasmid DNA with BamHI and EcoRI, and releasing the 1.87-kb fragment containing the *cry1Ac*-coding region. The restriction fragment was gel-purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, Calif.). The probe labeling and transcript detection were obtained using the DIG-High Prime DNA Labeling and Detection Starter Kit II (kit for chemiluminescent detection with CSPD; Roche Diagnostics Co., Indianapolis) following the manufacturer’s protocol.

**INSECT BIOASSAYS.** Insect toxicity of the transgenic Cynodon plants expressing *cry1Ac* (lines 1, 4, and 5) was tested by feeding assays with black cutworm larvae. Five leaf pieces (60 mg in total) were cut from greenhouse-grown control and transgenic plants and placed on moist filter papers in petri dishes (60 × 15 mm). Five first neonate larvae, each 100 μg, were placed on leaf segments with a soft camel-hair brush, and allowed to feed on the leaf pieces for 5 d. The experiment was repeated twice. Each experiment included four replications. Data on insect mortality, increase in larval mass and decrease in leaf fresh mass were recorded 5 d after starting the feeding assay. Analysis of variance was conducted using PROC GLM (SAS institute, 2002). The percentage data were transformed to arcsine before analysis. The means were compared using *t* test when ANOVA results were statistically significant (*P* ≤ 0.01).

**Results and Discussion**

**TISSUE CULTURE STUDIES.** Using 40 μM 2,4-D alone in callus induction medium we were not able to produce any embryogenic callus in ‘Arizona Common’ common bermudagrass. Salehi and Khosh-Khui (2005) used the same amount of 2,4-D and produced 100% embryogenic callus in common bermudagrass (Californian origin). Li and Qu (2004) included 0.9 μM BAP along with 4.5 μM 2,4-D in their callus induction medium for ‘J1224’ common bermudagrass. Our results show that production of embryogenic callus of ‘Arizona Common’ requires a combination of both 2,4-D and BAP with concentrations of 4.5 and 0.45 μM, respectively (Fig. 2A).

Our observations with plantlet regeneration of common bermudagrass showed that ‘Arizona Common’ is a recalcitrant cultivar in tissue culture and confirmed the results of Li and Qu (2004) who worked on other cultivars of common bermudagrass. None of the combinations of BAP, NAA, and GA₃ resulted in plantlet regeneration. However, a combination of 2 μM GA₃ and 0.6 μM 2,4-D or only 0.6 μM 2,4-D resulted in shootlet regeneration and elongation. Therefore, we conclude that regeneration of common bermudagrass is highly genotype specific.

**GUS EXPRESSION.** Histochemical analysis showed GUS expression in transformed calli, after three days co-cultivation (Fig. 2C). None of the control calli showed GUS expression.

**AGROBACTERIUM-MEDIATED TRANSFORMATION.** Our experiment showed that using a combination of both acetosyringone and tobacco leaf extract could result in 100% transient GUS expression as compared to ≥60% when acetosyringone or tobacco leaf extract were used alone (data not shown). The same amount of acetosyringone was used by Luo et al. (2004) for Agrostis stolonifera L., by Chai et al. (2004) for Agrostis tenuis Sibth., and by Bettany et al. (2003) for Festuca arundinacea Schreb. and Lolium multiflorum Lam. Luo et al. (2004) observed transient GUS expression in ≥60% of calli tested, the same as our experiment when using either acetosyringone or tobacco leaf extract. Acetosyringone is a phenolic compound that can induce vir genes that are responsible for the transfer of T-DNA from *A. tumefaciens* to the wounded

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Fig. 2. Tissue culture and transformation of common bermudagrass. Seed-derived callus after 4 weeks of culture on callus induction medium (A). G418-resistant calli 8 weeks after transformation (B). GUS expression in callus 3 d after co-cultivation (C). Regenerated plantlets from G418-resistant calli (D). Transgenic plants in the greenhouse (E). Transgenic (f) and control (e) leaves 5 d after insect feeding (F). Insects fed 5 d on transgenic (f) vs. control (e) leaves (G).
host cell (Cheng et al., 2004; Toyama et al., 2002; Stachel et al., 1985). Monocotyledons, particularly the grasses, may not produce phenolic compounds like acetosyringone, or if they do, not at a level sufficient to serve as signal molecules (Cheng et al., 2004). Tobacco contains phenolic compounds including acetosyringone and α-hydroxy acetosyringone. More studies are needed to see which of the tobacco phenolic compounds may have contributed toward the improved transformation efficiency.

The G418-resistant calli (Fig. 2B) regenerated into somatic embryos which were rooted (Fig. 2D) and grown in the greenhouse (Fig. 2E). All the transgenic plants resembled untransformed plants in morphology. A transformation efficiency of 5% was obtained (data not shown).

**Polymerase Chain Reaction.** As shown in Fig. 3, in addition to the band seen with the positive control, a band of the expected size of 606 bp was amplified from the genomic DNA from five regenerated plantlets, originating from separate calli. No band was detected in the untransformed control.

**Expression of cry1Ac.** RNA-blot analysis of PCR-positive transformed plants showed the presence of cry1Ac mRNA transcripts in shoot tissues of three lines (1, 4, and 5) at detectable levels (Fig. 4). There was no detectable transcript for the untransformed control plant. Therefore, based on RNA-blot analysis, three out of five (60%) PCR-positive lines showed cry1Ac expression.

**Insect Feeding Assays.** We chose the black cutworm because it has been reported to be the most commonly encountered pest of bermudagrass (Shetlar, 2003). Larvae mortality of 80% to 100% with the mean of 82.5% was observed in transgenic plants, whereas the control showed 20% to 40% with the mean of 30% (Table 1). The insect mortality in the control plants is related to the damages made to the tiny first star larvae during their transfer using a camel brush. It is assumed that some of the mortality of insects feeding on transgenic plants might be due to the same reason.

There is no report of expression of the cry1Ac gene in any of the turfgrasses. In transgenic rice, varying degrees of mortality (0% to 100%) have been reported against rice yellow stem borer (Scirpophaga internutula Wlk.), striped stem borer (Chilo suppressalis Walker), rice leaf folder (Cnaphalocrocis medinalis Guenee), and European corn borer (Ostrinia nubilalis Hubner) feeding on cry1Ab and cry1Ac transgenic plants (Ahmad et al., 2002; Cheng et al., 1998; Fujimoto et al., 1993; Maqbool et al., 2001; Nayak et al., 1997).

Significant leaf damage was seen in control plants as a result of black cutworm feeding, whereas in transgenic leaves, there was little detectable damage (Fig. 2F). Decrease in leaf fresh mass in the control was more than twice that of the transgenic plants (Table 1).

There was a significant increase in fresh weight of the larvae that were fed on untransformed control leaves compared to those fed on transgenic leaves (Table 1, Fig. 2G). The feeding assay confirmed the accuracy of RNA-blot analysis results in common bermudagrass plants. The transgenic cry1Ac common bermudagrass should be a very desirable alternative to synthetic pesticides, for both environmental and economical concerns.

In this study, we report an Agrobacterium-mediated transformation system using an agronomically important gene in common bermudagrass. Agrobacterium-mediated transformation of higher plants has been favored due to the generally low copy number of the integrated transgene and the fact that transgenes are usually transmitted to progeny in an expected Mendelian fashion (Luo et al., 2004). The Agrobacterium-mediated transformation system might be useful for other cultivars of common bermudagrass or hybrid bermudagrass. However, we recommend investigating efficient in vitro regeneration for each cultivar before transformation.

**Literature Cited**


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**Table 1. Insecticidal activity and performance of transgenic common bermudagrass plants to black cutworm after 5 d feeding.**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Larvae mortality (± SD)</th>
<th>Increase in each larval mass (μg ± SD)</th>
<th>Decrease in leaf mass (mg ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.0 ± 15.12</td>
<td>267.5 ± 118.80</td>
<td>4.0 ± 0.35</td>
</tr>
<tr>
<td>Transformed</td>
<td>82.5 ± 19.82</td>
<td>56.2 ± 102.50</td>
<td>1.9 ± 0.32</td>
</tr>
</tbody>
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*Means of control and transformed plant in each column are significantly different (P ≤ 0.01) using t test.*

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**Fig. 3. Polymerase chain reaction (PCR) analysis of putatively transgenic common bermudagrass plants showing 606 bp fragment corresponding to cry1Ac. Lanes: M = 1 kb Plus marker; P = plasmid (KUC) as positive control; C = untransformed control; L1 to L5 = putative transgenic lines.**

**Fig. 4. The RNA-blot showing expression of cry1Ac in putative transgenic common bermudagrass plants. Lanes: C = untransformed control; L1 to L5 = putative transgenic lines.**
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