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

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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 *cryIAc* 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 *cryIAc* 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 *cryIAc* 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, stunting, 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* Burt-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 Spangenberg, 1999). Monocotyledons such as grasses may not produce phenolic compounds like acetosyringone (Cheng et al., 2004). Using acetosyringone 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 *cryIAc*, 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

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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 N⁶-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 µmol·m⁻²·s⁻¹ light intensity for plant regeneration and rooting.

Two-week-old well-rooted plantlets were transferred to 10-cm pots containing Baccopotting medium (Michigan Peat Co., Houston), acclimatized, and transferred to the greenhouse. Greenhouse conditions were 25 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-glucuronidase gene (*gus*), each under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter and N terminator, and a kanamycin resistance gene (*kan*) 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) supple-

mented 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_n 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, acclimated 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-β-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

Fig. 1. Restriction map of the T-DNA of pKUC. BR, T-DNA right border; BL, T-DNA left border; *cryIAC* synthetic insecticidal gene from *B. thuringiensis*; *hpt*, hygromycin phosphotransferase gene; *gus* = β-glucuronidase gene; *nptII* = neomycin phosphotransferase gene; 35S = CaMV 35S promoter; Ubi = maize ubiquitin promoter; N = nopaline synthase terminator; n = nopaline synthase promoter.

described by Edwards et al. (1991). The following set of primers was used: *cryIac* F, 5'-ACA GAA GAC CCT TCA ATA TC-3' (forward primer) and *cryIac* 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 Pharmacia Biotech., Piscataway, N.J.) as specified by the manufacturer. The probe was generated by digesting plasmid DNA with *Bam*HI and *Eco*RI, and releasing the 1.87-kb fragment containing the *cryIac*-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 *cryIac* (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 larvae 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 ($P \leq 0.01$).

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 (California 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

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 (t) and control (c) leaves 5 d after insect feeding (F). Insects fed 5 d on transgenic (t) vs. control (c) 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 *CRYIAC*. RNA-blot analysis of PCR-positive transformed plants showed the presence of *cryIac* 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 *cryIac* 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 *cryIac* 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 insertulas* Wlk.), striped stem borer (*Chilo suppressalis* Walker), rice leaf folder (*Cnaphalocrocis medinalis* Guenee), and european corn borer (*Ostrinia nubilalis* Hubner) feeding on *cryIAb* and *cryIac* 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 *cryIac* 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

Fig. 3. Polymerase chain reaction (PCR) analysis of putatively transgenic common bermudagrass plants showing 606 bp fragment corresponding to *cryIac*. 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 *cryIac* in putative transgenic common bermudagrass plants. Lanes: C = untransformed control; L1 to L5 = putative transgenic lines.

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

Plant	Larvae mortality (% $\pm$ SD)	Increase in each larvae mass (μ g $\pm$ SD)	Decrease in leaf mass (mg $\pm$ SD) ²
Control	30.0 $\pm$ 15.12 ³	267.5 $\pm$ 118.80	4.0 $\pm$ 0.35
Transformed	82.5 $\pm$ 19.82	56.2 $\pm$ 102.50	1.9 $\pm$ 0.32

²Decrease in total mass (five leaf pieces).

³Means of control and transformed plant in each column are significantly different ($P \leq 0.01$) using *t* test.

hybrid bermudagrass. However, we recommend investigating efficient in vitro regeneration for each cultivar before transformation.

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