Expression of cry1Fa in Bahiagrass Enhances Resistance to Fall Armyworm

Gabriela Luciani, Fredy Altpeter,* Jessica Yactayo-Chang, Hangning Zhang, Maria Gallo, Robert L. Meagher, and David Wofford

ABSTRACT

Bahiagrass (Paspalum notatum var. saurae) is the predominant forage grass in Florida and in other subtropical regions. To improve pest resistance against fall armyworm [Spodoptera frugiperda (J. E. Smith)], an optimized cry1Fa gene encoding a δ-endotoxin from Bacillus thuringiensis was synthesized, subcloned under the transcriptional control of the constitutive ubi1 promoter, and introduced into the bahiagrass cultivar Tifton 9 by particle bombardment. Three transgenic bahiagrass lines were generated using minimal transgene expression cassettes without vector backbone. Southern blot analyses showed independent cry1Fa transgene integration patterns for the three lines. Transcripts of cry1Fa were detected in all three transgenic lines by reverse transcriptase polymerase chain reaction. Cry1Fa protein was detected in two lines by immuno-chromatography and quantitative Cry1Fa enzyme linked immunosorbent assay (ELISA). The Cry1Fa ELISA also indicated stable cry1Fa transgene expression in vegetative progeny plants of both lines. Cry1Fa expression levels correlated well to resistance levels determined by insect bioassays. An average mortality rate of 83% was observed when neonate larvae of fall armyworm were fed with transgenic leaves of the highest cry1Fa expressing line. These results indicate that minimal expression cassette technology supports stable and high level expression of cry1Fa in bahiagrass which can control fall armyworm, a devastating pest of forage grasses.

doi: 10.2135/cropsci2007.04.0195
© Crop Science Society of America
677 S. Segoe Rd., Madison, WI 53711 USA
All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher.
(Zea mays L.) and sorghum [Sorghum bicolor (L.) Moench], while the rice (Oryza sativa L.) strain is found predominantly in rice, turf grasses, and pasture grasses. The two host strains are morphologically identical, and can only be reliably distinguished by molecular methods (Nagoshi et al., 2004, Nagoshi et al., 2006).

Traditionally, many insect pests are controlled using integrated pest management (IPM) strategies involving the use of pesticides with resistant varieties or biological control agents. However, the indiscriminate use of pesticides produces adverse effects on human health and the environment including the development of insect resistance and the elimination of other beneficial insects (Ranjekar et al., 2003; Ferry et al., 2006). Transgenic crops expressing Bacillus thuringiensis (Bt) δ-endotoxins were a natural choice for controlling insects since Bt crystal protein (Cry) and spore formulation products have been successfully used for many years (Schnepl et al., 1998; Ferre and Van Rie, 2002; Ranjekar et al., 2003; Kaur, 2006).

Insect resistance to Bt toxins in targeted populations arises through different mechanisms and/or at different levels (Ferre and Van Rie, 2002; Griffits and Aroian, 2005). Therefore, to delay insect resistance development, growers must conform to the “High-dose refuge strategy”. The first component of this strategy is to express toxins in plants at a high enough level to kill heterozygotes in the insect population. To increase Bt expression levels in transgenic plants, codon-optimization of cry sequences, the reduction of AT sequences and the truncation of the native cry sequence have been successfully used (Schnepl et al., 1998; Bohorova et al., 2001; Kaur, 2006). Elimination of vector backbone sequences and biolistic transfer of minimal transgene expression constructs (Fu et al., 2000) also supported high transgene expression levels (Agrawal et al., 2005). Stacking of different cry genes (Kaur, 2006), expression of cry fusion constructs (Bohorova et al., 2001), and pyramiding genes including cry genes with genes encoding proteins having alternative insect control mechanisms like vegetative insecticidal proteins or proteinase inhibitors, will reduce the risk of insects developing resistance to Bt toxins (Ferry et al., 2006). The second component of the “High-dose refuge strategy” is to provide structured refuges. Refuges are small areas cultivated with nontransgenic crops which are interspersed with the transgenic crop. Mating of susceptible adults that developed from nontransformed plants with those from transgenic plants allow for the elimination of homozygous resistant individuals and the reduction of resistant alleles (Cannon, 2000; Ranjekar et al., 2003).

Currently, Bt transgenic technology is adopted worldwide and Bt crops are grown on more than 14 million hectares (James, 2005). In the United States, Bt crops are grown on approximately 20% of the crop acreage and their use is directly linked to higher yields and profits and reduced pesticide application (Cannon, 2000). Currently, marketed products include Bt corn containing the cry1Ab, cry1Fa, cry3Bb1, and stacked cry1Ab and cry3Bb1 genes for controlling European corn borer (Ostrinia nubilalis Hübnner), southwestern corn borer (Diatraea grandiosella Dyar) and corn rootworm (Diabrotica barberi Smith and Lawrence), and Bt cotton (Gossypium hirsutum L.) containing cry1Ac, stacked cry1Ac and cry2Ab2, stacked cry1Ac, and cry1Fa for controlling tobacco budworm (Heliothis virescens Fabricius), cotton bollworm (Helicoverpa zea Boddie), and pink bollworm (Pectinophora gossypiella Saunders) (Castle et al., 2006). Cry1Fa has been reported to control fall armyworm in cotton (Adamczyk and Gore, 2004). However, there are no previous reports on Cry proteins expressed in forage and turf grasses and their effects against fall armyworm. Transgenic plants of the noncommercial apomictic genotype ‘Tifton 7’, diploid bahiagrass cultivar Pensacola, and apomictic cultivar Argentine have been recently reported (Smith et al., 2002; Gondo et al., 2005; Alt peter and James, 2005; Sandhu et al., 2007). These genetic transformation protocols allow the introduction of exogenous insect resistance genes into bahiagrass. Hence, the objective of this work was to evaluate the expression of a synthetic cry1Fa gene in transgenic bahiagrass and its effect on resistance to fall armyworm.

**MATERIALS AND METHODS**

**Minimal Transgene Expression Constructs**

Based on the cry1Fa gene sequence available in the NCBI database (M73254), a codon-optimized sequence for the δ-endotoxin was generated. The synthetic cry1Fa gene (1863 bp) was synthesized and subcloned into a pPCR-Script vector by Geneart (Regensburg, Germany). Restriction sites BamHI and HindIII were introduced at the 5‘ and 3‘ ends of the cry1Fa coding sequence, respectively to facilitate subcloning into vector pHZUb1-ox1 (Agharkar et al., 2007). The resulting pHZCRY vector contains the maize ubiquitin 1 promoter and first intron (Christensen et al., 1992), the cry1Fa coding sequence and the nos. 3‘ untranslated region (Fraley et al., 1983) (Fig. 1).

The nptII selectable marker cassette contains the neomycin phosphotransferase II (nptII) coding sequence (Bevan, 1984) under transcriptional control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell et al., 1985) and hsp70 intron (Rochester et al., 1986), and the CaMV 35S polyadenylation signal (Dixon et al., 1986) (Fig. 1). Following the strategy described by Fu et al. (2000), minimal transgene expression constructs (MCs) containing only the expression cassettes without vector backbone were used for biolistic gene transfer. The nptII and cry1Fa gene expression cassettes were excised from their plasmids by restriction digestion with NcoI resulting in a 2.55- or 4.15-kb fragment, respectively (Fig. 1). Transgene expression cassettes were isolated by gel electrophoresis and the corresponding band was excised and purified using the Wizard SV Gel and polymerase chain reaction (PCR) cleanup system (Promega, Madison, WI).
Tissue Culture, Transformation, and Regeneration of Bahiagrass

Embryogenic callus was induced from mature seeds of the diploid bahiagrass cultivar Tifton 9 following a protocol described earlier (Altpeter and Positano, 2005). The callus induction medium (CIM) consisted of 4.3 g L\(^{-1}\) Murashige and Skoog (MS) salts (Murashige and Skoog, 1962), 30 g L\(^{-1}\) sucrose, 1.1 mg L\(^{-1}\) 6-benzylaminopurine (BAP), 3 mg L\(^{-1}\) 3,6-dichloro-2-methoxy benzoic acid (dicamba) and 6 g L\(^{-1}\) agarose (Sigma, St. Louis, MO), supplemented with filter sterilized MS vitamins (Murashige and Skoog, 1962) which were added after the medium was autoclaved for 20 min. Calli were kept in darkness at a temperature of 28°C and subcultured to fresh CIM after 2 wk. Embryogenic calli were placed on CIM medium supplemented with 0.4M sorbitol, for 4 to 6 h before gene transfer and 7 wk after culture initiation. The nptII and cry1Fa gene expression cassettes were used in a 1:2 molar ratio and coprecipitated on 1.0 μm diameter gold particles (Altpeter and James 2005). The BioRad PDS-1000/He device (BioRad Laboratories Inc., Hercules, CA) was used for biolistic gene transfer at 1100 psi (47 atm) with 16 h/8 h light/dark photoperiod, at 28°C, on selection filter sterilized MS vitaminagarose (Sigma, St. Louis, MO) with 16 h/8 h light/dark photoperiod, at 28°C, on selective 0.1 mg L\(^{-1}\) BAP and no dicamba, and transferred to high light (150 μmol m\(^{-2}\) s\(^{-1}\)) intensity with 16 h/8 h light/dark photoperiod at 28°C. After 2 wk, calli were transferred to hormone-free CIM to induce root formation, and kept in the dark for 10 dw before being transferred to low intensity light conditions (30 μmol m\(^{-2}\) s\(^{-1}\)), with 16 h/8 h light/dark photoperiod, at 28°C, on selection CIM containing 50 μg L\(^{-1}\) of paromomycin. After 4 wk, calli were subcultured on shoot regeneration medium, similar to CIM but containing 0.1 mg L\(^{-1}\) BAP and no dicamba, and transferred to high light (150 μmol m\(^{-2}\) s\(^{-1}\)) intensity with a 16 h/8 h light/dark photoperiod at 28°C. After 2 wk, calli were transferred to hormone-free CIM to induce root formation. After 4 to 6 wk, regenerated plantlets were transplanted into Fafard 2 mix (Fafard Inc., Apopka, FL) and acclimatized in growth chambers at 400 μmol m\(^{-2}\) s\(^{-1}\) light intensity with a 16 h/8 h light/dark photoperiod at 28°C/20°C day/night. Two weeks later plants were transferred to an air-conditioned greenhouse at 30°C/20°C day/night and natural photoperiod. Plants were fertilized biweekly with Miracle-Gro Lawn Food (Scotts Miracle-Gro, Marysville, OH) at the recommended rate.

Polymerase Chain Reaction, Reverse Transcriptase Polymerase Chain Reaction, and Southern Blot Analysis

Genomic DNA was extracted from the transgenic lines and wild-type as described by Dellaporta et al. (1983). The forward primer 5’ATGGTTTCAACAGGGCTGAG3’ and the reverse primer 5’CCCTCACACAGGGAAATCTGTA3’ were designed for amplifying a 570-bp fragment internal to the coding sequence of the cry1Fa gene. Approximately 100 ng genomic DNA was used as template for PCR in a BioRad iCycler (Bio-Rad Laboratories Inc., Hercules, CA). The PCR was performed using the HotStart PCR kit (Qiagen, Valencia, CA). The cycling conditions were 95°C for 15 min initial denaturation, 35 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 15 min final extension. The PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

For Southern blot analysis, genomic DNA from wild-type and transgenic lines was isolated using the CTAB method as described by Doyle and Doyle (1987). Genomic DNA (15 μg) was digested with BamHI and fractionated on a 1% agarose gel, transferred onto a nitrocellulose membrane (Hybond, Amersham BioSciences, Piscataway, NJ) and hybridized using the complete cry1Fa coding sequence (1.8 kb) as a probe, labeled with 32P using the Prime-a-Gene kit (Promega). Hybridization and detection were performed according to the instructions of the manufacturer.

Immunological Assays

Quantitative expression of the cry1Fa endotoxin in leaf tissue from the transgenic lines was evaluated using the Quickstix kit for cry1Fa (EnviroLogix) originally developed for Herculex I corn and following the recommendations of the manufacturer. Relative levels of expression of the cry1Fa endotoxin in leaf tissue were estimated by using the enzyme linked immunosorbent assay (ELISA) QualiPlate kit for cry1Fa (EnviroLogix) originally developed for Herculex I corn. Protein extracts from cry1Fa expressing corn grain were quantified and used as a positive control in a dilution series. Following 8 mo of vegetative propagation of the primary transformants, protein extracts were obtained from wild-type and the three transgenic lines including three different vegetative clones per line and three different replicates per clone. Protein concentration of the extracts was determined using the

Figure 1. Expression cassettes used for the generation of transgenic bahiagrass by biolistic gene transfer. Schematic representation of the minimal expression cassettes for nptII (2554 bp) excised from vector pHZ3SSNPTII (top) and the synthetic cry1Fa gene (4155 bp) excised from vector pHZCRY (bottom).
Bradford assay (Bradford 1976) and absorbance was measured at 595 nm. Bovine serum albumin was used to prepare a standard curve (R² value of 96%). Ten micrograms of total protein were loaded per well. The immunoassay was performed according to the instructions of the manufacturer. Reaction kinetics was recorded at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories Inc., Model 680). Optical densitometry values for each line were compared within the linear range of the reaction kinetics after addition of the ELISA substrate.

**Insect Bioassays**

Insecticidal activity of the transgenic lines was evaluated by following a modified version of the protocol described by Adamczyk and Gore (2004). Fall armyworm neonates (rice host strain) were obtained from egg masses hatched the same day, placed in Petri dishes and fed on four leaf pieces of 2 cm length of the third fully emerged leaf. A completely randomized experimental design was used. There were 10 replications per transgenic line represented by individual Petri dishes with leaves and larvae, and the experiment was repeated four times. For estimating fall armyworm resistance, the survival rate from transgenic lines was compared to the wild-type after 5 d of feeding.

**Statistical Analysis**

Optical densitometry data resulting from Cry1F ELISA of wild-type and transgenic lines were analyzed by Proc Anova and means were separated according to Tukey’s test (P < 0.05) (Littell et al., 1996, SAS Institute, 2002).

Survival rates of neonate larvae of fall armyworm, expressed as a percentage, were analyzed by Proc Mixed and means were separated according to Fisher’s protected LSD (Littell et al., 1996, SAS Institute, 2002). Standard errors are shown in figures as vertical bars.

**RESULTS**

**Generation of Transgenic Bahiagrass**

Cobombardment of 300 ‘Tifton 9’ calli with MCs of the pHZCRY and the pHZ3SSNPITII vectors (Fig. 1) and selection on paromomycin-containing culture medium resulted in the regeneration of three independent transgenic lines. These lines were transferred to soil under controlled environment conditions and did not differ phenotypically from the wild type.

**Molecular Characterization of Transgenic Bahiagrass**

Initial screening for the presence of the cry1Fa gene by PCR analysis showed that all three regenerated bahiagrass lines contained the expected 570-bp cry1Fa fragment. This fragment also was amplified from the plasmid control and was absent in the wild-type bahiagrass (Fig. 2A). For expression analysis, reverse transcriptase polymerase chain reaction (RT–PCR), using the same cry1Fa specific primers, showed that the three lines amplified a 570-bp cry1Fa fragment, confirming the presence of cry1Fa transcripts in all transgenic plants (Fig. 2B).

Southern blot analysis showed an independent integration pattern for each transgenic line. Line 1 showed seven hybridization bands while lines 2 and 3 displayed two and four hybridization bands respectively (Fig. 2C).

A qualitative immuno-chromatographic assay showed that transgenic lines 2 and 3 contained Cry1Fa protein above the detection level of the kit. However, the Cry1Fa protein was not detected in protein extracts of line 1 (Fig. 3A). Therefore, a quantitative Cry1Fa ELISA was employed. This method revealed that line 1 produced relatively low levels of Cry1Fa. Lines 2 and 3 displayed cry1Fa expression levels that were 4- and 12-fold higher than those levels observed in line
1, respectively ($P < 0.05$, Fig. 3B). Also, all three vegetative propagated clones from these lines expressed the transgene. No significant differences in $cry1Fa$ expression between clones of the same line were found ($P < 0.05$; data not shown). $Cry1Fa$ protein levels in bahiagrass leaves were estimated by comparison with a crude $Cry1Fa$ protein standard supplied by the manufacturer and were approximately 1.4 and 4.5 μg protein g$^{-1}$ fresh weight for lines 2 and 3, respectively.

**Insect Bioassays**

Differences in feeding patterns were observed between larvae fed leaves from wild type and line 1 vs. lines 2 or 3 (Fig. 4A). Larvae fed consistently on leaves of line 1 or wild-type plants resulting in larger larvae and almost complete consumption of the leaves after 5 d. In contrast, most neonates foraged little on the leaves from lines 2 and 3, and died within the first three days of feeding (Fig. 4A).

Larvae fed leaves from lines 2 and 3 showed a significantly lower survival rate (35% and 17.5%, respectively), than wild type which had an 80% neonate survival rate. Larvae fed leaves of line 1, with low $Cry1Fa$ expression, did not differ significantly from those fed wild-type leaves ($P < 0.05$,Fig. 4B).

**DISCUSSION**

This is the first report of stable, transgene expression of a Bt crystal protein gene that confers insect resistance in a forage and turfgrass. Bahiagrass is an important subtropical forage grass that is also used as low input turf. Constitutive overexpression of the $cry1Fa$ gene in bahiagrass resulted in increased resistance to fall armyworm.

Analysis of the complexity of MC integration patterns had resulted in controversial results in the past. Fu et al. (2000) described that biolistic transfer of MCs resulted in simpler integration patterns and lower copy numbers than plasmids. In contrast, no differences between the two DNA forms were reported by Breitler et al. (2002) and Romano et al. (2003). Southern blot analysis of the three transgenic bahiagrass lines transformed with MCs of the $cry1Fa$ gene showed multiple transgene copies in all lines with line 1 displaying the most complex transgene integration pattern. This complex transgene integration pattern following biolistic transfer of MCs into bahiagrass is in agreement with findings of Breitler et al. (2002) and Romano et al. (2003). It suggests that the complexity of transgene integration is more likely dependent on factors intrinsic to the plant than on the form of DNA as proposed by Agrawal et al. (2005). Nevertheless, clean DNA technology by employing the use of MCs for biolistic transformation is capable of producing similar or higher transformation and expression efficiencies than whole plasmids (Agrawal et al., 2005). In the present study, two of the three lines expressed the $cry1Fa$ transgene in vegetative progeny at a high enough level to control fall armyworm.

Transgenic corn expressing the Bt $cry1Ab$ gene was first commercially released in 1996 (Castle et al., 2006). Constitutive $cry1Ab$ expression of 3.3 or 10.3 μg g$^{-1}$ fresh weight of leaves resulted in 50 to 75% or 98% control of the European corn borer ($O. nubilalis$) in corn field trials (Mendelsohn et al., 2003). This pest is considered the most important corn insect pest in the midwestern and northeastern regions of the United States (Wiatrak et al., 2004); while fall armyworm is the most important pest on grasses and other crops in the southeastern United States. Recently, corn expressing the $cry1Fa$ gene (Herculex I) was commercially released by Pioneer Hi-Bred International and Dow Agrosciences (Events TC1507 and DAS-06275-8). Field trials indicated that these transgenic corn lines effectively controlled multiple insect pests like $O. nubilalis$, $D. grandiosella$, $H. zea$, $S. frugiperda$, $A. ipsilon$, and $R. albicosta$ (USEPA, 2001). In cotton, fall armyworm bioassays indicated that neonate mortality was significantly higher when larvae were fed on leaves expressing $cry1Fa$ (80%) compared with nontransgenic leaves (48%) or leaves expressing $cry1Ac$ (45%) (Adamczyk and Gore, 2004). $Cry1Fa$ concentrations were estimated at 1.4 and 4.5 μg g$^{-1}$ fresh weight in bahiagrass transgenic lines 2 and 3, respectively. These expression levels were associated with 65 and 83% neonate mortality, respectively, while wild-type and transgenic bahiagrass with barely detectable $cry1Fa$ expression showed a significantly lower fall armyworm mortality rate. These results indicate the potential...
of *cry1Fa* to control fall armyworm in accordance with the results reported for *cry1Fa* expressing cotton (Adamczyk and Gore, 2004) and corn (USEPA, 2001).

In conclusion, stable expression of minimal synthetic *cry1Fa* expression constructs in bahiagrass conferred resistance to the difficult to control, and important insect pest, fall armyworm.

**Acknowledgments**

We would like to thank USDA/CSREES T-STAR and the University of Florida-IFAS for financial support. We also thank Jeff Seib for training in safe handling of radioisotopes.

**References**


Sandhu, S., F. Altpeter, and A.R. Blount. 2007. Apomictic bahiagrass expressing the bar gene is highly resistant to glufosinate under field conditions. Crop Sci. 47:1691–1697.


