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BRIEF COMMUNICATION

Production of mRNA from the *cry1Ac* transgene differs among Bollgard® lines which correlates to the level of subsequent protein

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Abstract Commercial cultivars of Bollgard® cotton, *Gossypium hirsutum* L., differ in the amount of expressed Cry1Ac protein. However, the plant-mechanism for which this occurs is still unknown. Using quantitative real-time polymerase chain reaction (qPCR), we developed a method to determine if differences in the overall level of Cry1Ac among Bollgard® lines could be correlated to the mRNA transcripts. Our data shows that the *cry1Ac* mRNA transcript differs among Bollgard® lines and are correlated with corresponding Cry1Ac protein levels. In addition, qPCR based methods can efficiently be employed to quantify Cry1Ac protein expression levels in transgenic cotton cultivars. We postulate that qPCR based methods could be successfully employed for quantifying expression levels of transgenes in plants carrying different Bt toxins.

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Introduction

Cotton plants, *Gossypium hirsutum* L., containing the Cry1Ac δ-endotoxin from the soil bacterium, *Bacillus thuringiensis* Berliner (Bt) (Bollgard® or Ingard®, Monsanto Co., St. Louis, MO), have been used as a tool to selectively manage lepidopteran pests for over 10 years. Although highly effective against the tobacco budworm, *Heliothis virescens* (F.) and pink bollworm, *Pectinophora gossypiella* (Saunders) (Williams 2000), supplemental foliar insecticide applications have been used in a number of Bollgard® fields to control fall armyworms, *Spodoptera frugiperda* (J. E. Smith); beet armyworms, *Spodoptera exigua* (Hübner); corn earworms, *Helicoverpa zea* (Boddie); and Old World pests, *Helicoverpa armigera* (Hübner) and *Helicoverpa punctigera* (Wallengren) (Bacheler and Mott 1997; Roof and DuRant 1997; Fitt 1998; Smith 1998; Burd et al. 1999).

The Cry1Ac protein level in Bollgard® cotton appear to decline as the plant matures (Finnegan et al. 1998; Adamczyk et al. 2001). Recently, the overall level of Cry1Ac protein was correlated with increased survival of *H. armigera* on Bollgard® cultivars late in the growing season (Kranthi et al. 2005; Olsen et al. 2005; Wan et al. 2005). Using Northern-hybridization techniques, Olsen et al. (2005) and Xia et al. (2005)

attributed this decline in available Cry1Ac δ-endotoxin to reduced transcription levels. Furthermore, Xia et al. (2005) suggest that the reduction of Cry1Ac late in physiological maturity could be due to overexpression of the transgene earlier in development, which could lead to regulation at the post-translational level, resulting in gene-silencing later in development. Methylation of the promoter may also contribute to this seasonal decline in available protein (Xia et al. 2005; see Dong and Li 2007 for review).

Commercial cultivars of Bollgard® cotton differ in the amount of expressed Cry1Ac protein. Overall Cry1Ac levels among Bollgard® cultivars (expressing the MON 531 event) have been correlated to survival levels in various lepidopteran pests that are intrinsically tolerant to this protein (Adamczyk et al. 2001; Adamczyk and Gore 2004; Wan et al. 2005). These differences in Cry1Ac protein levels among commercial cultivars can vary as much as 5-fold throughout the season (Adamczyk et al. 2001). Furthermore, these cultivar differences are heritable (Adamczyk and Meredith 2004; Rochester 2006) and controlled by a small number (1–2) of effective genetic factors (Adamczyk and Meredith 2004).

Adamczyk and Meredith (2004) and Rochester (2006) showed that the parental background (i.e. the non-Bt cultivar) had a significant influence on the amount of available Cry1Ac protein in Bollgard® cultivars. Furthermore, Adamczyk and Meredith (2006) showed that by using forward breeding (i.e. Bollgard® cultivars crossed with Bollgard® cultivars), cultivars could be selected for the highest overall amount of Cry1Ac in addition to desired agronomic traits. Although increased levels of Cry1Ac protein were obtained in a selected cultivar, the plant-mechanism for which this occurs is still unknown. The purpose of this study was two-fold: (1) to determine if the overall *cry1Ac* mRNA levels differed among Bollgard® lines and (2) to determine if transcription levels correlated with available Cry1Ac levels.

Materials and methods

Forward breeding to obtain different levels of Cry1Ac protein

Details into the development of cotton lines that contained different levels of Cry1Ac protein derived from the Bollgard® trait is described in Adamczyk and Meredith (2004, 2006). Briefly, in 2001 crosses were made from plants derived from distantly related backgrounds that expressed different levels of Cry1Ac. A Stoneville Pedigree Seed Co. cultivar (cv. ST4691B) and a Delta and Pineland Co. cultivar (cv. NuCOTN 33B) were chosen as parental cultivars for the subsequent crosses because NuCOTN 33B expresses significantly more Cry1Ac in all plant structures compared to ST4691B (Adamczyk and Sumerford 2001). Seeds produced from the crosses (F_1) were grown in the greenhouse, allowed to self-pollinate, and the F_2 seed was planted the following spring.

In 2002, the resulting F_2 seeds were planted at multiple locations in single row plots (5.0 m) near Stoneville, MS and arranged in a randomized complete block design. All plots were maintained according to local management practices. Once bolls began to open, five randomly chosen plants/plot were marked with slant-lock tags (A.M. Leonard, Piqua, OH), and the amount of Cry1Ac protein was quantified using the techniques described in Adamczyk and Meredith (2006). After all bolls were mature, F_3 seed was hand-harvested from each tagged plant and planted the following spring.

In 2003, the resulting F_3 seeds obtained from each of the five selected F_2 plants were planted in progeny rows at two locations in Stoneville, MS. Plots consisted of single rows (5.0 m) arranged in a randomized complete block, and each F_3 line was replicated twice within a block (4). After plants began to flower, healthy, uniform plants (5) were selected from each plot for Cry1Ac quantification (Adamczyk and Meredith 2006). Once Cry1Ac protein levels were obtained for all progeny rows, lines were selected for further study. Four lines that consistently had high and low levels of Cry1Ac protein (High: 51-2 and 53-5, Low: 48-5 and 42-1) were identified using Cry1Ac endotoxin quantification procedures described below, and the resulting F_4 seeds were hand-harvested and planted the following spring (April 19, 2004) in two row plots (10.0 m) with four blocks near Stoneville, MS. In addition, cv. NuCOTN 33B also was grown to serve as a positive, high expressing control (Adamczyk and Sumerford 2001). These same lines were grown in the greenhouse (four replicates) in the spring of 2005. One high (51-2) and one low (48-5) Cry1Ac protein expressing line was

selected for further study and planted in two row plots with three blocks on May 17, 2005 near Stoneville, MS.

Cry1Ac endotoxin quantification

Terminal leaves from the plants were used because this tissue accurately reflects overall Cry1Ac differences among cultivars (Adamczyk and Sumerford 2001). For ELISA assays, tissue was excised from the lobed region of a terminal leaf by placing the tissue underneath the attached cap of a 0.5 ml microcentrifuge tube (VWR International). Closing the cap produced a uniform circular sample of ca. 4.8 mg that was self-contained within the microcentrifuge tube. This procedure also minimized desiccation of the leaf samples. The five individual leaf samples per plot were placed into a plastic bag and transported to the laboratory in a cooler with ice. Within 1 h, the five samples/plot were combined into an individual 2.0 ml 96 deep-well microtiter plate (BioSpec Products, Inc., Bartlesville, OK) containing two 6.4 mm stainless steel ball-bearings (BioSpec Products, Inc.). Cry1Ac extraction buffer (1.0 ml) (EnviroLogix, Inc.) was added to each well. The tissue was homogenized for 30 s using a Mini-Beadbeater-96™ (BioSpec Products, Inc.). The microtiter plate was centrifuged at 3,000 rpm for 5 min at 4°C (Avanti™ J-20XP, Beckman Coulter, Inc., Fullerton, CA). For each sample, a 20 µl aliquot was place in an individual 1.1 ml 96 deep-well microtiter plate containing 500 µl of Cry1Ac extraction buffer (EnviroLogix, Inc.) (1:26 dilution). The microtiter plate was covered with a corresponding silicone-based lid (BioSpec Products, Inc.) and placed on an orbital shaker for 1 min at 300 rpm. A commercial quantification plate kit was utilized to quantify the amount of Cry1Ac present for each cultivar/plot (EnviroLogix, Inc.). Samples were plotted against a standard curve with Cry1Ab calibrators supplied in the kit. The known doses for Cry1Ab calibrators were substituted for doses of Cry1Ac as dictated by the kit protocol. The amount of Cry1Ac was measured per gram fresh weight of transgenic cotton leaves after accounting for the proper dilution factors (Dutton et al. 2002). Mean levels of Cry1Ac was analyzed using REML-ANOVA, and means were separated using LSMEANS (Littell et al. 1996; PROC MIXED SAS Institute 2001).

Cry1Ac mRNA quantification

RNA extraction & cDNA synthesis

For all samples, a single terminal leaf was excised from a single plant obtained from all cotton plots and transported on ice to the laboratory. To minimize differences in mRNA expression due to environmental factors (e.g. shading, time of day, temperature), all samples were taken at the same time. In 2005, to correlate mRNA production to corresponding protein expression, a single leaf was equally divided longitudinally for both analyses (as described above). Cry1Ac protein ELISA was conducted using fresh tissue, as described above, utilizing half of the leaf, while the corresponding leaf samples were stored at −80°C until RNA extraction.

RNAEasy Plant reagent kit (Qiagen) was used for isolating total RNA. In-column digestion with DNase I was carried out to remove genomic DNA that may contaminate RNA samples. Complete removal of contaminating DNA for each sample was verified by performing a qPCR using 100 ng of total RNA without reverse transcription using the18S ribosomal RNA (rRNA) endogenous control primer-probe set (Applied Biosystems, Foster City, CA; Assay # Hs99999901_s1). RNA samples that produced detectable 18S products during 40 cycles of qPCR were digested with 10 units of RNase-free DNase (Ambion, San Antonio, TX), extracted with phenol–chloroform–isoamyl alcohol (25:24:1, pH 4.5, Sigma-Aldrich), and precipitated with ethanol. The resulting RNA pellets were re-suspended in water and tested for DNA contamination as outlined above. Total RNA samples were quantified by spectrophotometry (Bio-Rad, Richmond, CA.)

Synthesis of cDNA was carried out by reverse transcription using 500 ng of total RNA from each sample in a reaction volume of 20 µl using random hexamers provided with the first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Samples were heated to 75°C for 15 min. at the end of cDNA synthesis to inactivate the reverse transcriptase enzyme and stored at −20°C until used in qPCR.

Quantitative PCR

All PCR reagents and supplies, including the 2× universal PCR master mix, 96-well PCR plates, and

optical plate sealing film were purchased from Applied Biosystems, Inc., and qPCR was carried out using an ABI 7500 sequence detection system (Applied Biosystems, Inc.). Relative quantification of the *cry1Ac* transcript was conducted using qPCR by the standard curve based method (Livak 1997; reviewed in Wong and Medrano 2005). An endogenous reference and a treatment calibrator were utilized to determine the relative quantity of a target transcript. The detection probe and PCR primers for the target (*cry1Ac*) sequence were purchased through Applied Biosystem's Assays-by-Design service. The *cry1Ac* primer-probe 20× concentrate contained forward primer (5′-CGCGAGGAAATGCGTATTCAAT-3′) and reverse primer (5′-ACAATGGGATAGCTGTGGTCAAG-3′) at a concentration of 18 μM each and the TaqMan MGB probe (5′-FAM-TCAACGACATGAACAGCG-3′) at a concentration of 5 μM. The 18S rRNA universal endogenous control primer-probe set (assay # Hs99999901_s1) was purchased from Applied Biosystem's Assays-on-Demand service. Nucleotide sequences of the PCR primers or the detection probe of this proprietary product were not provided, although provided context sequence (TGGAGGGCAAGTCTGGTGCCAGCAG), around which the detection sequences were designed, matched cotton (*G. hirsutum*) 18S rRNA sequence (Accession # GHU42827) from nucleotide positions 521 to 545. TaqMan MGB detection probes for both *cry1Ac* and 18S rRNA sequences were labeled with a 6-FAM (6-carboxyfluorescein) reporter dye molecule at the 5′ end and with a non-fluorescent quencher DABCYL (4-(4′-dimethylamino-phenylazo)-benzoic acid) at the 3′ end.

Suitability of both target and reference genes (*cry1Ac* and 18S, respectively) for quantifying the respective genes was tested by performing qPCR on cDNA prepared from total RNA of transgenic Bt cotton plants containing the Bollgard® trait. cDNA from reverse transcription reactions were quantified by spectrophotometry and a set of standards containing 50, 5, 0.5, and 0.05 ng of cDNA per microliter was prepared by serially diluting the cDNA in water. Each 25 μl qPCR reaction contained 12.5 μl of 2× universal master mix, 2 μl of cDNA (equivalent to 100, 10, 1, and 0.1 ng cDNA per reaction, respectively), 1.25 μl of primer-detection probe mix, and 9.25 μl of water. Each concentration for *cry1Ac* and 18S quantifications was replicated four times.

After determination of the suitability of the *cry1Ac* and 18S primer/probe sets to quantify cDNA over a wide range of cDNA concentrations, we determined that cDNA equivalent to 50 ng of total RNA was sufficient to provide consistent quantification of both target and reference genes. Quantitative PCR of experimental samples were carried out in a 25 μl reaction volume with 1.25 μl of corresponding probe/primer combination, 12.5 μl of Universal PCR Master Mix, 2 μl of cDNA (equivalent to 50 ng of total RNA), and 9.25 μl of water following the temperature regime: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 10 min, 60°C for 1 min. Detection of fluorescence was carried out during the 60°C annealing/extension step of the PCR profile. In order to quantify *cry1Ac* and 18S quantities in leaf cDNA samples, standard curves were prepared for *cry1Ac* and 18S using triplicate serial dilutions containing 100, 10, 1, and 0.1 ng of transgenic cotton (line 52) cDNA. Relative expression level of *cry1Ac* in each sample was calculated by normalizing the absolute quantities of *cry1Ac* to that of 18S in each sample (Livak 1997). These relative expression values normalized to endogenous controls were divided by the expression value of the leaf sample showing the least expression level (i.e. calibrator) to obtain expression levels relative to the calibrator sample. Samples obtained from greenhouse plants were replicated four times in the laboratory, while field-grown plants obtained from the three blocks were replicated twice in the laboratory. Mean levels of mRNA (Cry1Ac/18S) were analyzed using REML-ANOVA, and means were separated using LSMEANS (Littell et al. 1996; PROC MIXED SAS Institute 2001). A correlation analysis was conducted for Cry1Ac protein versus mRNA (Cry1Ac/18S) obtained from the two lines (48-5 and 51-2) planted in the field in 2005 (PROC CORR SAS Institute 2001).

Results and discussion

After three generations, the four selected lines used for subsequent mRNA studies differed in the amount of expressed Cry1Ac protein. The mean for the two low lines (48-5 and 42-1) was 2.58 μg Cry1Ac g^{-1} fresh weight and for the two high lines (53-5 and 51-2) was 10.32 μg Cry1Ac g^{-1} fresh weight, or a difference of ca. 400% (Table 1.). It is evident that cultivar

improvement in Cry1Ac protein content can be greatly improved by selection at the F2 plant and F2:3 progeny row level. Adamczyk and Meredith (2006) showed that within a segregating F2 population that is homozygous for the *cry1Ac* transgene, large differences in Cry1Ac protein levels among F2 plants can be detected. Further selection of high, and for that matter, low levels of Cry1Ac protein is highly effective, increasing the ability to control target lepidopteran pests (Adamczyk and Gore 2004; Adamczyk and Meredith 2006). Furthermore, similar results in Cry1Ac protein among the lines were detected in the field the following year, indicating that selection for the desired level of Cry1Ac protein can be obtained in only a few generations of selection as first indicated by Adamczyk and Meredith (2006) (Fig. 1a).

It appears that the level of expressed Cry1Ac protein in different Bollgard® lines is correlated with the amount of *cry1Ac* mRNA transcript (Fig. 1). The amount of Cry1Ac protein (measured in 2004) and mRNA (measured in 2005) was significantly different ($P < 0.05$) among the examined cotton lines planted in the field plots (2004) or in the greenhouse (2005) (Protein: $F = 171.64$; df = 4, 11; $P < 0.001$) (mRNA: $F = 22.47$; df = 4, 14; $P < 0.001$). Both lines 48-5 and 42-1 had significantly lower ($P < 0.05$) amounts *cry1Ac* mRNA transcript and corresponding protein compared to all other cotton lines containing the Bollgard® trait (Fig. 1). In addition, both lines 53-5 and 51-2 contained significantly higher ($P < 0.05$) amounts of *cry1Ac* mRNA transcript than all other cotton lines, and at least numerically higher amounts of Cry1Ac protein than the remaining cotton lines. The cv. NuCOTN 33B

Table 1 Mean (±SE) level of Cry1Ac protein found in terminal leaves from lines containing the Bollgard® trait that were utilized for mRNA studies

Lines	μg Cry1Ac g^{-1} Fresh weight
48-5	1.82c ± 0.788
42-1	3.33b ± 0.651
53-5	10.14a ± 0.989
51-2	10.49a ± 0.441
F-value	136.02
df	3, 9
$P > F$	<0.001

Means within a column followed by the same letter are not significantly different according to LSMEANS

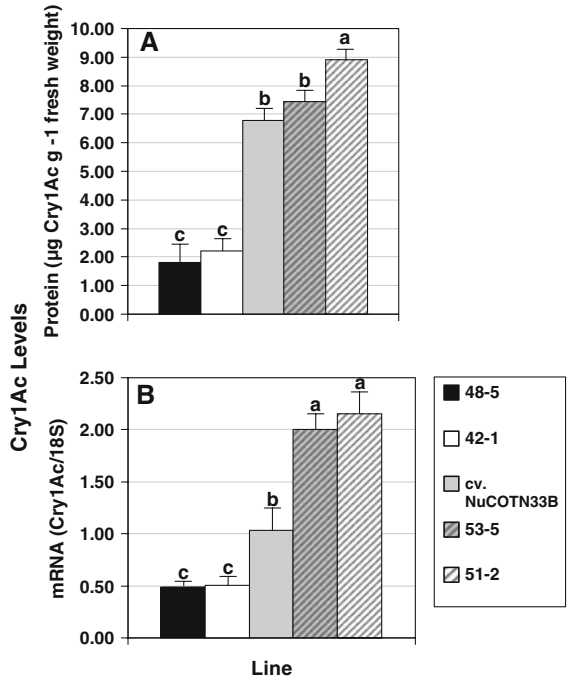

Fig. 1 Mean Cry1Ac protein (**a**) and mRNA transcript levels (**b**) among different lines containing the Bollgard® trait. Bars with a common letter are not significantly different ($\alpha = 0.05$) from one another according to LSMEANS

control had significantly higher levels of *cry1Ac* mRNA transcript and corresponding protein compared to the low lines (42-1 and 48-5). Relative differences between Cry1Ac protein levels among lines 48-5, 42-1, 53-5, and 51-2 were consistent with the F_3 generation shown in Table 1. Furthermore, in the same environment and from the same plant structure, there was a significant correlation ($P < 0.001$) between the amount of *cry1Ac* mRNA transcript and the amount of corresponding Cry1Ac protein (Fig. 2). It is important to note that a full linear relationship was not observed. For example, Cry1Ac protein varied ca. 30-fold when corresponding *cry1Ac* mRNA transcripts were between ca. 2.5 and 3.0 (Cry1Ac/18S). These differences in Cry1Ac protein levels may be partially explained by plant-to-plant variability caused by environmental factors (Dong and Li 2007). However, in general terminal leaves that had higher amounts of *cry1Ac* mRNA transcript had higher amounts of corresponding Cry1Ac protein (Table 2).

Our data shows that the overall level of *cry1Ac* mRNA transcript differs among Bollgard® lines and

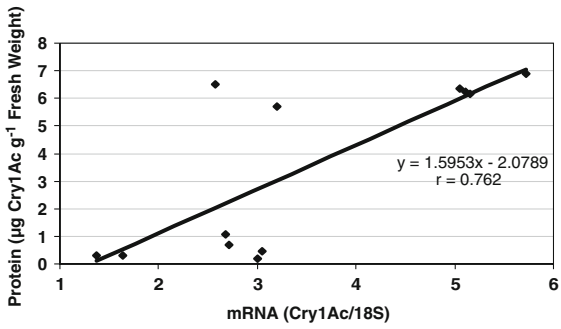

Fig. 2 Correlation between *cry1Ac* mRNA transcript and subsequent Cry1Ac protein levels for samples taken from lines 48-5 and 51-2

Table 2 Mean (±SE) levels of Cry1Ac protein and mRNA found in terminal leaves from two lines containing the Bollgard® trait

Lines	mRNA (Cry1Ac/18S)	µg Cry1Ac g^{-1} Fresh weight
48-5	2.41a ± 0.294	0.50a ± 0.135
51-2	4.47b ± 0.517	6.30b ± 0.161
F-value	21.00	762.76
df	1, 5	1, 10
$P > F$	0.006	<0.001

Means within a column followed by the same letter are not significantly different according to LSMEANS

is correlated with corresponding Cry1Ac protein. Adamczyk and Meredith (2004) showed that only a small number of genetic factors were controlling the amount of Cry1Ac protein in these transgenic cultivars. This current study suggests that these genetic factors are impacting the overall *cry1Ac* mRNA transcript levels among different Bollgard® lines. However, post-translational effects or environmental factors that could further impact available Cry1Ac protein levels cannot be excluded (Xia et al. 2005; Dong and Li 2007).

Commercial seed companies could select for the highest *cry1Ac* expressing Bollgard® cultivar in addition to desired agronomic traits early in plant development (i.e. seeds), virtually excluding environmental factors that could impact Cry1Ac protein expression (Dong and Li 2007). Adamczyk and Sumerford (2001) showed that differences among Cry1Ac protein levels in Bollgard® cultivars could be determined at any stage of plant development using ELISA assays. Our study is the first to show that by using qPCR, the same selections could be obtained without having to use monoclonal antibodies. Furthermore, we postulate that qPCR based methods could be successfully employed for quantifying expression levels of transgenes in plants carrying different Cry genes, and could offer an alternative choice for conducting such studies without the expense of acquiring monoclonal antibodies.

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