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# ARTHROPOD MANAGEMENT

## Effect of Genotype, Edaphic, Environmental Conditions, and Agronomic Practices on Cry1Ac Protein Expression in Transgenic Cotton

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### ABSTRACT

**Poor Cry1Ac protein expression was common in the first years of transgenic (Bt) cotton in Australia, when single-gene Cry1Ac cotton (called Ingard in Australia and Bollgard in USA) was grown. Two-gene cultivars (called Bollgard II in both countries) show enhanced Cry1Ac protein expression compared with Ingard cultivars. Environment, soil properties, and agronomic management are believed to affect Cry1Ac protein expression. This research evaluated the impact of crop nutrition, plant population density, light intensity, water management, herbicide application, soil fertility, plant growth regulator application, and cotton cultivars on Cry1Ac protein expression in field and glasshouse experiments, as measured in cotton leaves using commercial quantitative ELISA assays. Cultivars provided the major source of variation in leaf Cry1Ac protein expression. Cry1Ac protein concentration ranged from 0.27 to 6.01 mg kg$^{-1}$ in 15 experiments conducted over 4 yr. There was considerable variation among individual plants of a cultivar. Cry1Ac protein expression was highly heritable ($h^2 = 0.94$), as parent plants produced progeny with a similar level of Cry1Ac protein expression. Cry1Ac protein expression was higher in older (lower) leaves. Treatment effects were often more evident in older than younger leaves. Short episodes of waterlogging, shading, herbicide application, or plant growth regulator application did not significantly affect leaf Cry1Ac protein expression, while severely wilted plants exhibited reduced Cry1Ac expression. Cry1Ac protein expression was reduced under conditions that affected cotton growth and development or plant survival, such as drought or sodic/saline soil that severely impaired crop nutrition. Cry1Ac protein synthesis may be limited or the protein metabolized in plants subjected to environmental or edaphic stresses.**

The Australian cotton industry regards transgenic (Bt) cotton containing genes for the Cry1Ac protein from *Bacillus thuringensis* Berliner as a major component of its integrated insect management strategy for reducing the economic burden of Heliothine control and the environmental consequences of insecticide use. Initially, single Bt gene cultivars (called Bollgard in USA, but called Ingard in Austraila and denoted with 'i' in the cultivar name) carrying the Cry1Ac gene provided protection until flowering at best. More recently, cultivars with two Bt genes (called Bollgard II in Australia and USA) have shown enhanced levels of Cry1Ac protein expression compared with earlier cultivars containing the single gene. Bollgard II cultivars (denoted with 'B' in the cultivar name in Australia) carry both the Cry1Ac and Cry2Ab genes and can provide protection from Heliothine pests throughout the cotton growing season. Greenplate (1999) indicated that late-season pest control was from the Cry2Ab component.

The Heliothine pests in Australian crops pose a more serious threat than those in many other countries. Compared with *Helicoverpa zea* (Boddie) and *Heliothis virescens* (F.), *Helicoverpa amigera* (Hübner) and *Helicoverpa punctigera* (Wallengren), the dominant Australian Heliothine pests, are more difficult to control with Bt proteins, because *H. zea* and *H. armigera* are more tolerant of Cry toxins (Fitt and Wilson, 2000). *H. armigera* has developed widespread insecticide resistence internationally (Plapp et al., 1994). The concentration of Cry1Ac protein required to control Heliothine larvae is variable because of numerous factors, particularly the size and age of the larvae, so there is no definitive Cry1Ac concentration that correlates with the ability to control larvae.

In South Carolina, Bt cotton has reduced and almost eliminated insecticide applications (Chitkowski et al., 2003). As Bt technology was introduced to the Australian cotton industry, the effectiveness

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of Bt was variable. This variation was attributed to soil, climatic, and agronomic factors, as well as variation between individual plants. Greenplate (1999) observed significant differences in Cry1Ac protein expression between six field sites, indicating environmental influences on production or stability of the Cry1Ac protein. Cry1Ac concentrations in plants also showed no specific trend over time despite variation among sampling timings and tissue types, which indicated that environmental conditions may influence Cry1Ac protein expression (Greenplate et al., 2000). Often, Bt protein levels decline with time, but Wan et al. (2005) observed that levels rebounded late in the season. Different plant tissues, growth stages, and cultivars provide significant sources of variation in protein levels, but some cultivars were more variable than others.

Maximizing the expression of the Cry1Ac protein would help realize the full potential of Bt cotton, which would assist growers economically and help avoid the development of resistance in insect pests to these proteins and the need for insecticide use. Development of insect resistance would limit the gains in cotton sustainability brought about by the Bt technology.

Enzyme-linked immunosorbent assays (ELISA) can be used to detect presence of Cry proteins and to follow the activity or expression of Cry proteins in the field throughout the growing season (Kranthi and Kranthi, 2000). Quantitative ELISA assays for Bt, which have only recently become available as a research tool, not only assist cotton breeders in identifying lines that express more Bt protein, but they can indicate agronomic and/or environmental factors that impact Bt expression in the cotton plant. Prior to ELISA assays, bioassays that were labor-intensive and slow were used to assess Cry1Ac protein expression (Deeba et al., 2003). Also, the possibility of insecticide spray drift from nearby cotton fields poses a serious limitation to bioassays on leaves taken from field experiments.

The objective of this research was to identified agronomic, edaphic, and environmental factors that can have a significant impact on Bt expression in commercial transgenic cotton crops.

## MATERIALS AND METHODS

All experiments were conducted at the Australian Cotton Research Institute, Narrabri, New South Wales, Australia. For field experiments, cotton was grown under commercial conditions with up to six flood irrigations per growing season. All cotton cultivars were developed by CSIRO Australia (Wee Waa, New South Wales) and distributed by Cotton Seed Distributors. Weeds were controlled with pre-emergent herbicides and with mechanical cultivation and manual chopping. Insect pests were controlled with chemical insecticides when twice-weekly scouting indicated pest populations exceeded commercial thresholds. In field experiments other than those where N responses were investigated, N fertilizer (as anhydrous ammonia) was applied preplant at 150 and 200 kg $ha^{-1}$ N, which was sufficient to maximize lint yield. The soil was a fertile alkaline dark greyish brown cracking Gommel medium clay (fine, thermic, montmorillonitic Typic Haplustert). Crop nutrient uptake was assessed at cut-out (mid- to late boll fill), about 130 d after planting. To determine crop dry matter, the above-ground plant tissue was removed from 1 m of crop row. A subsample of 2 to 3 plants was selected to determine nutrient concentration following drying at 70 °C for 72 h in a forced-draught dehydrator. The plant material was milled and analyzed for N by Kjeldahl digestion. All other nutrient concentrations were determined by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES) after acid digestion. Nutrient uptake was determined as the product of nutrient concentration and the mass of crop dry matter. For experiments dealing with genotype comparisons, N and K fertilizer rates, plant density, shading, and growth regulator and herbicide applications, the treatments were applied to the plots. Each plot consisted of 8 rows each 1 m wide and 20 m long. Two of the eight rows were used for destructive sampling, including leaf sampling. Following chemical defoliation, the two center rows of each 8-row plot were mechanically picked and weighed, and a subsample (~300 g) of seed cotton was ginned to determine lint yield.

For the experiments reported herein, quantitative concentrations of Cry1Ac protein expression was measured on fresh leaf tissue using the Envirologix sandwich Cry1Ab/Ac ELISA kit (EnviroLogix Inc.; Portland, ME). Duplicate assays were performed on each sample. Since variation in leaf moisture content was not significantly different among treatments, results were expressed on a fresh weight basis, except for the water stress experiment where Cry1Ac was expressed on a dry weight basis. Node positions of main stem leaves were counted up from the cotyle-

dons with the cotyledonary node being node zero. Only main stem node leaves were assayed.

The methodology provided in the Envirologix kit was followed closely. Seven to eight discs avoiding large leaf veins were cut from the leaf using a 1.5 mL snap-top microcentrifuge tube. The discs were 9 mm in diameter and the total fresh weight (80 to 100 mg) of leaf material was recorded. For the experiments conducted in the final year of the study (Experiments A, F, G and L – see below), 20 leaf discs each 5 mm diameter were cut using a polymerase chain reaction (PCR) tube. The smaller discs were cut from a larger number of leaves, but a similar amount of fresh leaf material was analyzed compared with the previous years' experiments. One mL of the extractant solution was added to the tube, and two, 4-mm chrome-plated ball bearings were included in each tube. These tubes were then mounted in a mini-bead beater (Biospec Products; Bartlesville, OK), and the samples were homogenized for 60 s. The tubes were then centrifuged at 11,000 rpm for 4 min. An aliquot of the supernatant was pipetted from each vial and diluted with the extraction buffer. This solution was added to the wells of the ELISA test strip, and the three reagents supplied were added and mixed using an orbital shaker per the kit instructions. Color development was measured using a BioRad 'Benchmark' microplate reader (BioRad Laboratories; Tokyo, Japan) at a wavelength of 450 nm. Concentration of Cry1Ac protein was determined from the standard curve prepared using the standard solutions included with the kit.

For the glasshouse experiments, fertile topsoil (0 to 30-cm depth) was collected from a field planted to cotton for several consecutive seasons. Six-liter plastic pots were filled with 4 kg of soil, and sufficient N (equivalent to 80 kg $ha^{-1}$ N, as urea) and Zn (equivalent to 200 g $ha^{-1}$ Zn, as zinc sulfate heptahydrate) was applied to ensure healthy crop growth. Both fertilizers are required for field-grown cotton in these alkaline soils. Glasshouse temperature was maintained between 34 °C and 14 °C without supplementary lighting.

The Genstat program (8th edition; VSN International Ltd.; Hemel Hempstead, UK) was used for statistical analyses (Payne, 1987). Experimental designs were either randomized complete block or split plot designs for field and glasshouse experiments. Data were analyzed using ANOVA, and LSD ($P = 0.05$) values were reported where significant differences between treatment means were detected.

A wide range of agronomic practices and edaphic conditions were investigated with respect to their impact on Cry1Ac expression. Variation between and within cotton cultivars was also assessed. Generic agronomic and management details have been provided. Procedures specific to individual experiments will be provided below.

**Variation among and within cultivars**. ***Experiment A***. Six Bollgard II cultivars, CSX415, CSX55, CSX56, CSX5, CSX2, and CSX48, grown in the field during the 2003-2004 season were assessed for leaf Cry1Ac protein expression. Five replicates were sampled and assayed 14 d prior to defoliation on 9 Mar. 2004.

***Experiment B***. Two Ingard cultivars, Sicot 289i and Sicala 40i, were grown in the field during the 2000-2001 season. Leaf tissue located at nodes 9, 12, 15, 18, and 21 were assayed for the Cry1Ac protein expression on 15 Mar. 2001. Three replicates were sampled.

***Experiment C***. Sixteen individual plants of the cultivar Sicot 289i were grown under glasshouse conditions in 2001-2002, and Cry1Ac expression was assessed at first flower for each plant. At maturity, seed was collected from each of the 16 plants and planted into fresh soil. At the 8-leaf stage, Cry1Ac protein expression in the leaf was determined in each plant to indicate heritability of the trait. Heritability was estimated using the regression method of Frey and Horner (1957).

**Nitrogen (N) nutrition**. ***Experiments D***. Leaves were collected from cotton during the 2001-2002, 2002-2003, and 2003-2004 cropping seasons in a long-established crop rotation by N fertilizer experiment. Lint yield was determined for each experiment. In Experiment D, leaves were collected from plots with a deficient, adequate, and excessive N fertility. The plots deficient in N were continuous cotton with no additional fertilizer, the plots with adequate N were from a cereal rotation supplemented with 100 kg $ha^{-1}$ N, and the plots with excessive N were from a legume rotation supplemented with 200 kg $ha^{-1}$ N. Leaves from Sicot 289i were sampled from nodes 8, 13, and 18 and assayed on 29 Jan. 2002. There were four replicates.

***Experiment E.*** In the 2002-2003 season, leaves were collected from a low N-fertility system (continuous cotton) or from a high N-fertility system (cotton/wheat/legume rotation) in which N fertilizer had been applied at 0, 100, or 200 kg $ha^{-1}$ N. The second leaf from the terminal of Sicot 289RRi plants was sampled and assayed at mid-flowering (30 Jan. 2003). Treatments were replicated four times.

***Experiment F.*** In the 2003-2004 season, N fertilizer was applied at 0, 70, 140, 210, 280 kg $ha^{-1}$ N to plots in a cotton/wheat rotation. The fifth uppermost leaf from the terminal of Sicot 289BR plants was sampled at flowering (22 January), mid-boll fill (12 February), and 20% open boll (22 March). A substantial rainfall that produced minor waterlogging occurred prior to sampling on 22 Jan. 2004. Each N treatment was replicated four times.

**Potassium (K) nutrition**. ***Experiment G***. Seven days prior to planting Sicot 289BR cotton, potassium fertilizers were applied to the soil at 200 kg $ha^{-1}$ K, as either KCl or $K_2SO_4$, or as foliar applications of dissolved $KNO_3$ in three doses of 10 kg $ha^{-1}$ K during the growing season. Cry1Ac protein was assayed 4 d prior to crop defoliation. The treatments were replicated six times and included a control with no supplemental K.

**Other nutrients.** Cotton leaves were sampled from a commercial field where severe nutrient stress was apparent. Healthy and deficient leaves were sampled from adjacent plants and analyzed for nutrient content using ICP-AES.

**Soil salinity and sodicity**. ***Experiment H***. A glasshouse experiment was set up to simulate the extremes of salinity and sodicity observed on commercial cotton farms. This was achieved by mixing sodium sulfate into pots of a fertile control soil prior to planting cotton. Soil exchangeable sodium percentage (ESP) was increased from 2.1 to 21.8%, while soil salinity, measured as electrical conductivity (EC), increased from 2.1 to 12.9 dS $m^{-1}$ (saturated extract method). The seven sodicity treatments (ESP at 2.1, 4.8, 6.1, 11.7, 16.7, 18.6, and 21.8%) were replicated five times. The seed planted in the pots were collected from a single plant of Sicot 289i grown the previous season to reduce variability in Cry1Ac protein expression between plants. The leaves were assayed for Cy1Ac on 28 September and 3 October. Leaf area was measured using a Li-Cor leaf area meter (LI-3100C; Li-Cor Biosciences; Lincoln, NE) on 30 September.

**Plant population**. ***Experiment I***. In 2001, Sicot 289i and Sicala V-3RRi were planted in a field experiment at 2, 8, and 20 plants $m^{-1}$. Leaf Cry1Ac protein expression was measured from node 5, 10, and 15 at mid-flowering. The treatments were replicated four times.

**Shading**. ***Experiment J***. To simulate the effects of prolonged cloudy weather, a shading experiment was conducted in 2001-2002 on field-grown Sicot 289i and Sicala V-3RRi. The two shading treatments were replicated twice for each cultivar. Shade was applied on 21 Dec. 2001 (early flowering) by covering 2 m of crop row with shade cloth that reduced incident light by 70%. Shade was removed after 7 d. Leaf Cry1Ac protein concentration was assayed on the day of shade removal and 21 d after removal of shade.

**Water stress - waterlogging and drought**. ***Experiment K***. The initial waterlogging experiment used two cotton cultivars (Sicot 289i and Siokra V-16i). The waterlogging treatment was imposed 20 Sept. 2001 by flooding the pots and ensuring that free water remained on the soil surface for 23 d until 12 October, when the plants were sampled. The waterlogged treatment pots were allowed to begin drying, but soil in the pots remained waterlogged for an additional 7 d. The waterlogged and control treatments were replicated four times.

This glasshouse experiment was repeated in January 2002 with Sicot 289i. Soil in the waterlogged treatment remained saturated for 8 d, while the control soil remained moist. The treatments were replicated eight times.

***Experiment L***. Severe waterlogging often occurs in field-grown cotton, particularly when rain follows flood irrigation and soil fails to drain, and plants exhibit yellowing from restricted nutrient and water uptake because of anaerobic soil conditions. Soil was severely waterlogged in some treatments of an irrigation timing experiment in 2003-2004. One irrigation treatment, which was to receive 8 ML $ha^{-1}$ for the growing season, was flood irrigated prior to 100 mm rainfall on 14 Jan. 2004, so soil was severely waterlogged for more than 7 d. The other irrigation treatments (2 and 4 ML $ha^{-1}$) had not been irrigated prior to the rainfall and were not waterlogged. The irrigation treatments were replicated three times. Leaves of Sicot 289BR cotton plants were sampled and assayed on 28 Jan. 2004.

***Experiment M***. Cry1Ac protein expression in upper leaves of glasshouse-grown Sicot 289RRi and Sicala V-3RRi cotton was measured after a period of drought stress. Two treatments (control or drought stress) were applied to each cultivar and replicated six times. Drought stress was initiated when the plants began flowering. Plants in the stressed treatment received no water for 4 d (until 16 Sept. 2002). Plants in the control pots were watered daily. The plants were severely wilted for most of this time, and then leaves were sampled for Cry1Ac assays. Starting 16 Sept. 2002, all pots were watered

daily until 23 Sept. 2002, when leaves were again sampled (7 d after removal of drought stress) and assayed. Results (from this experiment only) were expressed on a dry weight basis to correct for the difference in moisture content between wilted and non-wilted leaves.

**Plant growth regulator**. ***Experiment N***. The plant growth regulator, mepiquat chloride (Pix; BASF Corp.; Florham Park, NJ), which is commonly used in commercial cotton production to arrest excessive vegetative growth, was evaluated for its effect on Cry1Ac protein expression. Two growth regulator treatments and a control were replicated four times in a field experiment. The commercial growth regulator mepiquat chloride was applied to two cotton cultivars (Sicala 40i and Sicot 289i) on 12 Jan. 2001 (flowering) and 27 Feb. 2001 (cut-out). The control treatment with no mepiquat chloride was compared with applications of 600 mL $ha^{-1}$ mepiquat chloride at flowering and 600 mL $ha^{-1}$ mepiquat chloride at flowering + 1 L $ha^{-1}$ mepiquat chloride at cut-out. Leaf Cry1Ac protein expression was assessed on 15 Mar. 2001 in all treatments. Four replicates of each treatment were included.

**Herbicides**. ***Experiment O***. Seven herbicides; diuron (Diuron; E. I. duPont de Nemours and Co.; Wilmington, DE) and pyrithiobac (Staple; E. I. duPont de Nemours and Co.), s-metolachlor (Dual; Syngenta Crop Protection, Inc.; Greensboro, NC), trifloxysulfuron sodium (Envoke; Syngenta Crop Protection Inc.) and prometryne (Gesagard; Syngenta Crop Protection Inc.), pendimethalin (Stomp; BASF Corp.; Florham Park, NJ) and trifluralin (Treflan; Dow AgroSciences, LLC; Abington Oxfordshire, UK) were applied to field-grown cotton, either preplant or post-emergent as used in commercial situations. Each treatment and control was replicated five times. Cotton (Sicot 289RRi) was planted either before or after herbicide application as specified by the label. Leaves were sampled and assayed for Cry1Ac protein expression on 29 November and 4 December prior to flowering.

## RESULTS

**Variation between and within cultivars.** The five cotton genotypes evaluated in Experiment A varied significantly in leaf Cry1Ac protein expression at the end of the growing season prior to crop defoliation (Table 1). In Experiment B, Sicot 289i plants displayed a more uniform and higher Cry1Ac protein expression throughout the leaf profile than Sicala 40i (Fig. 1). Leaf Cry1Ac protein expression was higher in older leaves near the base of the plant. Differences between cultivars were greater in younger leaves at higher nodes of the plant.

**Table 1. Variation in Cry1Ac protein concentrations among Bollgard II breeding lines 2 weeks prior to application of chemical defoliant (Experiment A)**

| Bollgard II genotype | Leaf Cry1Ac (mg $kg^{-1}$)[z] |
|---|---|
| CSX415 | 5.02 a |
| CSX55 | 3.61 b |
| CSX56 | 2.59 c |
| CSX5 | 2.30 c |
| CSX2 | 2.14 c |
| CSX48 | 2.03 c |

[z] **Means followed by the same letter are not significantly different according to LSD (*P* = 0.05).**

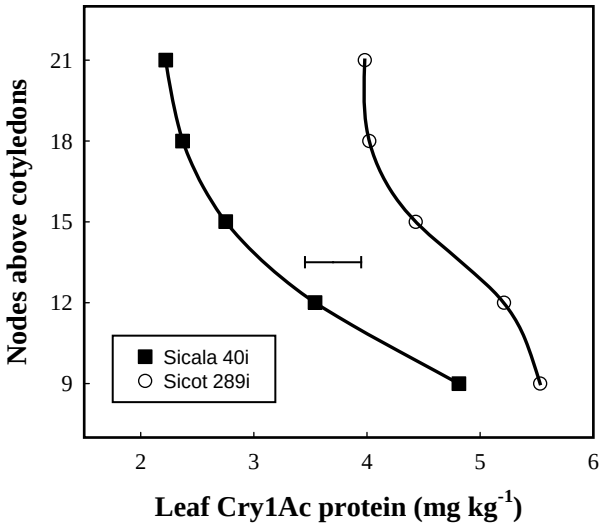


**Figure 1. Leaf Cry1Ac protein expression at five node positions in two Ingard cultivars (Experiment B). The error bar indicates the LSD (*P* = 0.05) of the node by cultivar interaction.**

In Experiment C, there was considerable variation in leaf Cry1Ac protein expression between individual plants of the cultivar Sicot 289i grown under glasshouse conditions. Of the 16 plants assayed in the first generation, four had leaf Cry1Ac protein concentrations about 70% lower than the other 12 plants (Fig. 2). In the second generation, leaf Cry1Ac protein expression was significantly lower in the plants derived from parents expressing low Cry1Ac protein (Fig. 2). Plants that expressed either high or low levels of Cry1Ac protein expression produced progeny with similar high or low Cry1Ac protein

expression. Expression was slightly higher in the second generation, since those plants were younger when sampled. Heritability ($h^2$) of Cry1Ac expression was estimated at 0.94.

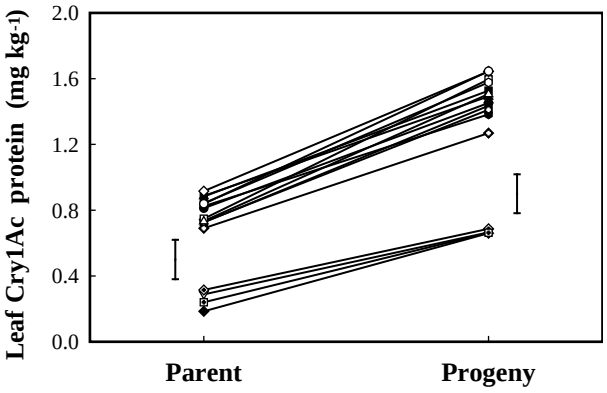


**Figure 2. Leaf Cry1Ac protein expression in the parent and progeny of Sicot 289i plants (Experiment C). Error bars indicate LSD (*P* = 0.05).**

**Nitrogen nutrition.** In Experiment D, during the 2001-2002 season, leaf Cry1Ac protein expression significantly increased with increasing N fertilizer application (Table 2). As the season progressed, symptoms of N deficiency (shorter and light green plants) or excess N (dark green vegetative plants) were observed in the deficient and excessive N rate treatments, respectively. Cry1Ac protein expression was significantly higher in the older leaves (node 8) than the younger leaves (node 18). The N-deficient plants had 2 to 3 fewer nodes than those in the over-fertilized N treatment, which may have influenced the relative differences between the N treatments.

In Experiment E, N application significantly increased leaf Cry1Ac protein expression in both the low and high N systems (Table 3), but the crop rotation sequence and its interaction with N fertilizer application were not statistically significant.

Leaf Cry1Ac protein expression of Experiment F (Table 4) remained relatively high compared with the 2001-2002 season (Table 2) until the last sampling on 22 Mar. 2004. No significant effect of N rate was evident at any sampling date, although a consistent increasing trend was evident at the last sampling on 22 March. Lint yield was not improved above 70 kg $ha^{-1}$ N, indicating the relatively high soil N fertility at this site. Greater responses in lint yield were observed in the previous two experiments.

**Potassium nutrition.** The trend in leaf Cry1Ac protein expression at the end of the growing season in Experiment G was not significant for K applied to the soil or the leaves (Table 5). There was no evidence of K deficiency in the plant. Insect pest observations indicated that Cry1Ac protein expression was sufficient to control Heliothine larvae in this experiment. Uptake of potassium by the cotton plants was not

**Table 2. Concentrations (mg $kg^{-1}$) of Cry1Ac protein in the leaves at mid-flowering and lint yields of cotton grown under three N fertility conditions (Experiment D – Sicot 289i)**

| N applied (kg $ha^{-1}$) | N status [y] | Cry1Ac (mg $kg^{-1}$) [z] | | | Lint yield (kg $ha^{-1}$) [z] |
|---|---|---|---|---|---|
| | | Node 8 | Node 13 | Node 18 | |
| 0 | Deficient | 0.48 cd | 0.53 b | 0.44 de | 1793 b |
| 100 | Adequate | 0.71 a | 0.43 de | 0.42 e | 2238 a |
| 200 | Excessive | 0.76 a | 0.51 bc | 0.46 de | 1899 b |

[y] Deficient plots were from a continuous cotton rotation; adequate plots were from a cotton/wheat rotation; excessive plots were from a cotton/legume rotation.

[z] Means followed by the same letter are not significantly different according to LSD (*P* = 0.05).

**Table 3. Leaf Cry1Ac protein concentrations and lint yield under high and low soil N fertility with supplemental N fertilizer application (Experiment E – Sicot 289RRi)**

| Fertilizer N (kg $ha^{-1}$) | Cry1Ac protein (mg $kg^{-1}$) [z] | | Lint yield (kg $ha^{-1}$) [z] | |
|---|---|---|---|---|
| | Low N | High N | Low N | High N |
| 0 | 2.23 c | 2.39 bc | 987 b | 1557 a |
| 100 | 2.87 a | 2.52 b | 1116 b | 1493 a |
| 200 | 2.66 ab | 2.61 b | 1152 b | 1429 a |

[z] Low N were plots from a continuous cotton rotation; high N were plots from a cotton/wheat/legume rotation. Means followed by the same letter are not significantly different according to LSD (*P* = 0.05).

increased by K application in any form, as K fertility was adequate in the unamended soil. Lint yields were not significantly affected by K application by any method (Table 5).

**Other nutrients.** The leaves collected from field-grown cotton that showed nutrient deficiency symptoms were significantly lower in phosphorus (P) and lower in K (1400 cf 8100 mg $kg^{-1}$) but higher in Na (3900 cf 2800 mg $kg^{-1}$) compared with well-nourished leaves (1800 cf 2900 mg $kg^{-1}$). Cry1Ac protein expression was 35% lower in the nutrient deficient leaves compared with well-nourished leaves (758 cf 1022 mg $kg^{-1}$ Cry1Ac protein).

**Soil salinity and sodicity.** In Experiment H, plant dry matter and leaf area were significantly reduced as soil ESP increased above 17% (Table 6), but leaf Cry1Ac protein concentration remained at around 2.1 mg $kg^{-1}$ even at these higher levels of soil sodicity and salinity. Mineral nutrient uptake (data not shown) was significantly depressed at the higher levels of sodicity. Yield decline in cotton is expected where soil salinity reaches about 8 dS $m^{-1}$ (Kumari et al., 2004; Jiang et al., 2006).

**Plant population.** In Experiment I, high plant population increased leaf Cry1Ac protein expression especially at lower node positions (Table 7).

**Table 4. Cry1Ac protein in cotton leaves at flowering, mid-boll fill, and 20% open bolls and lint yield under five rates of N fertilizer applied as anhydrous ammonia prior to planting (Experiment F – Sicot 289BR)**

| Fertilizer N (kg $ha^{-1}$) [y] | Cry1Ac (mg $kg^{-1}$) [z] | | | Lint yield (kg $ha^{-1}$) [z] |
|---|---|---|---|---|
| | 22 Jan. | 12 Feb. | 22 Mar. | |
| 0 | 2.39 a | 4.87 a | 2.96 a | 1839 b |
| 70 | 2.35 a | 5.05 a | 2.76 a | 2202 a |
| 140 | 2.63 a | 4.07 a | 3.45 a | 2166 a |
| 210 | 2.37 a | 4.71 a | 3.58 a | 2193 a |
| 280 | 2.72 a | 4.82 a | 3.73 a | 2143 a |

[y] Plots were from a cotton/wheat rotation.

[z] Means within a column followed by the same letter are not significantly different according to LSD ($P = 0.05$).

**Table 5. Effect of potassium fertilizer applications on leaf Cry1Ac protein concentration measured 4 d prior to crop defoliation (Experiment G – Sicot 289BR)**

| Treatment | Leaf Cry1Ac protein (mg $kg^{-1}$) [z] | Shoot K uptake (kg $ha^{-1}$) [z] | Lint yield (kg $ha^{-1}$) [z] |
|---|---|---|---|
| Control | 3.53 a | 197 a | 2001 a |
| Foliar $KNO_3$ (30 kg K $ha^{-1}$) | 3.63 a | 209 a | 2034 a |
| Soil-applied KCl (200 kg K $ha^{-1}$) | 3.21 a | 212 a | 2047 a |
| Soil-applied $K_2SO_4$ (200 kg K $ha^{-1}$) | 3.24 a | 182 a | 1982 a |

[z] Means within a column followed by the same letter are not significantly different according to LSD ($P = 0.05$).

**Table 6. Cry1Ac protein concentrations (mg $kg^{-1}$) in cotton leaves grown in soil with increasing soil exchangeable sodium percentage (ESP) and salinity (1:5 soil: water suspension) (Experiment H – Sicot 289i)**

| Sodicity (ESP) | Salinity (dS $m^{-1}$) | Plant DM (g $pot^{-1}$)[z] | Leaf area 30 Sept. ($cm^2$)[z] | Cry1Ac 28 Sept. (mg $kg^{-1}$)[z] | Cry1Ac 3 Oct. (mg $kg^{-1}$)[z] |
|---|---|---|---|---|---|
| 2.1 | 2.1 | 28.0 a | 923 a | 2.14 a | 1.87 a |
| 4.8 | 2.8 | 26.2 a | 814 ab | 2.06 a | 2.29 a |
| 6.1 | 3.4 | 26.2 a | 663 ab | 2.07 a | 2.23 a |
| 11.7 | 6.4 | 28.8 a | 686 ab | 1.97 a | 1.91 a |
| 16.7 | 9.2 | 24.6 a | 619 ab | 1.99 a | 2.06 a |
| 18.6 | 11.0 | 19.5 b | 542 bc | 2.11 a | 2.17 a |
| 21.8 | 12.9 | 11.8 c | 238 c | 2.13 a | 2.22 a |

[z] Means within a column followed by the same letter are not significantly different according to LSD ($P = 0.05$).

Significant differences between the two cultivars were evident at the three node positions examined. Cultivar by plant population interaction was evident at node 15, where leaf Cry1Ac protein expression in Sicot 289i increased with higher plant populations, but remained constant in Sicala V-3RRi.

**Shading.** Shading had no effect on leaf Cry1Ac expression in Experiment J at either sampling time. There was a significant difference between the two cultivars tested. Sicot 289i averaged 1.19 mg $kg^{-1}$ Cry1Ac protein, and Sicala V-3RRi averaged 0.70 mg $kg^{-1}$ Cry1Ac protein. While the shade cloth reduced incident light by 60%, temperature under the shade cloth was also reduced, which would occur during prolonged periods of cloudy weather.

**Water stress - waterlogging and drought. Glasshouse experiments.** Differences in leaf Cry1Ac protein expression were not statistically significant between treatments in Experiment K, despite means for the waterlogged treatment being 20% lower than the control (Table 8). Cry1Ac protein concentrations were low in this experiment. No obvious symptoms of nutritional stress appeared in the waterlogged plants until they had been waterlogged for 20 d, because it was difficult to effectively waterlog soil in the glasshouse environment. The plants were last sampled on 29 Oct. 2001, 10 d after soil moisture conditions had returned to normal. There was a significant difference in leaf Cry1Ac protein expression between the two cultivars, but the effect of waterlogging treatment was not statistically significant, although consistent between cultivars (Table 8). This glasshouse experiment was repeated with more severely waterlogged soil, but no significant differences in leaf Cry1Ac protein expression between the treatments were apparent.

**Waterlogging - field experiment.** The additive effects of irrigation and following 100 mm rainfall produced severe waterlogging for more than 7 d in Experiment L. Leaf Cry1Ac protein concentrations were 2.5, 2.6, and 2.2 mg $kg^{-1}$, and Kjeldahl N concentrations were 4.37, 3.24, and 3.16% N for the 2, 4, and 8 ML $ha^{-1}$ irrigation treatments, respectively. These differences were not statistically significant, despite the severe waterlogging and yellow appearance of the foliage.

**Water stress – drought.** In Experiment M, significantly less Cry1Ac protein was expressed in the leaves of the stressed plants compared with watered plants (Table 9). Sicot 289RRi recovered more quickly from the drought stress than Sicala V-3RRi. The lower expression of Cry1Ac protein in the control treatment for Sicot 289RRi after 4 d drought cannot be explained.

**Plant growth regulators.** In Experiment N, the mepiquat chloride applications did not significantly influence Cry1Ac protein expression. The values were 3.60, 3.51, and 3.47 mg $kg^{-1}$ Cry1Ac protein for the control, 600 mL $ha^{-1}$ mepiquat chloride at flowering, and 600 mL $ha^{-1}$ mepiquat chloride at flowering plus 1 L $ha^{-1}$ mepiquat chloride treatments, respectively.

**Table 7. Effect of plant density on Cry1Ac protein concentrations in leaves of two Ingard cultivars at mid-flowering (Experiment I - Sicot 289i and Sicala V-3RRi)**

| Plant population (plants $m^{-1}$) | Cry1Ac (mg $kg^{-1}$) [z] | | | | | |
|---|---|---|---|---|---|---|
| | Node 5 | | Node 10 | | Node 15 | |
| | 289i | V-3RRi | 289i | V-3RRi | 289i | V-3RRi |
| 2 | 1.06 b | 0.49 ef | 0.78 cd | 0.24 g | 0.77 cd | 0.32 fg |
| 8 | 1.17 b | 0.65 de | 0.96 b | 0.34 fg | 0.96 bc | 0.35 fg |
| 20 | 1.67 a | 1.06 b | 1.12 b | 0.33 fg | 1.05 b | 0.29 fg |

[z]Nodes were counted from the base of the plant and data were meaned over cultivars. Means followed by the same letter are not significantly different according to LSD ($P = 0.05$).

**Table 8. Leaf Cry1Ac protein concentration in two cotton cultivars following 23 days of waterlogging (Experiment K)**

| Treatment | Cry1Ac (mg $kg^{-1}$) [z] | |
|---|---|---|
| | Sicot 289i | Siokra V-16i |
| Control | 0.653 a | 0.335 a |
| Waterlogged | 0.520 a | 0.272 a |

[z]Means within a column followed by the same letter are not significantly different according to LSD ($P = 0.05$).

**Herbicides.** Leaf Cry1Ac protein expression was not significantly affected by the application of any herbicide in Experiment O (Table 10), although symptoms of herbicide damage were observed following some applications (e.g., pyrithiobac).

## DISCUSSION

A wide range of Cry1Ac protein expression was evident among cotton cultivars, so it is possible for cotton producers to reduce the risk of damage from Heliothine larvae by selecting cultivars that express high levels of Cry1Ac protein. Differences in Cry1Ac protein expression between cultivars were due largely to the genetic background of parents; therefore, selection of high expressing parents was crucial to improve Bt efficacy (Adamczyk and Meredith, 2004). Newer Bollgard II cultivars had higher Cry1Ac protein expression than the older Ingard cultivars, indicating the importance of selecting higher Cry1Ac-expressing genotypes for lepidopterous pest control. The addition of other Cry proteins may not significantly alter the expression of Cry1Ac protein (Adamczyk et al., 2001), although dual or stacked Cry proteins may control lepidopterous pests more effectively than plants expressing a single Cry protein (Stewart et al., 2000). Olsen et al. (2005) indicated that Cry1Ac expression declined post-squaring in their glasshouse experiments with Ingard cotton, a phenomenon not observed in Bollgard II cotton in these field experiments (Tables 4 and 5). Some genotypes may not have expressed sufficient Cry1Ac protein to control Heliothine larvae at the end of the growing season, although this was not proven.

The observation of lower Cry1Ac expression in leaves of the youngest nodes (Fig. 1) was contrary to the results of Greenplate (1999), who observed greater Cry1Ac protein expression in the terminal foliage.

Substantial variation was noted among experiments that used the same cultivar. Importantly, this was more closely related to growing conditions than analytical procedures, since lower Cry1Ac expression was observed in glasshouse-grown cotton than field-grown cotton.

Nitrogen nutrition has the potential to affect protein synthesis and Cry1Ac protein levels in Bt plants. Applying N fertilizer at rates beyond that required to optimize lint yield did not afford greater expression of Cry1Ac protein (Tables 2, 3, and 4). Cry1Ac expression tended to be lower with low N

**Table 9. Cry1Ac protein concentrations in the leaves of drought-stressed plants of two cotton cultivars as drought-stress was removed (Experiment M)**

| Treatment | Cry1Ac (mg kg$^{-1}$) | | | |
|---|---|---|---|---|
| | After 4 d drought[z] | | 7 d after stress removed[z] | |
| | Sicot 289RRi | Sicala V-3RRi | Sicot 289RRi | Sicala V-3RRi |
| Control | 6.01 a | 2.76 c | 4.70 a | 3.24 b |
| Drought-stress | 4.79 b | 1.72 d | 5.53 a | 2.19 b |

[z]Means followed by the same letter are not significantly different according to LSD ($P$ = 0.05).

**Table 10. Effect of herbicide applications on leaf Cry1Ac protein concentration in Sicot 289RRi plants (Experiment O)**

| Herbicide | Trade name | Cry1Ac (mg kg$^{-1}$) [z] | |
|---|---|---|---|
| | | 29 Nov. 2002 | 4 Dec. 2002 |
| Control | | 2.83 | 2.76 |
| Diuron | Diuron | 2.45 | 2.75 |
| Metolachlor | Dual | 2.53 | 2.79 |
| Trifloxysulfuron sodium | Envoke | 2.38 | 3.06 |
| Prometryne | Gesagard | 2.40 | 2.97 |
| Pyrithiobac | Staple | 2.29 | 2.73 |
| Pendimethalin | Stomp | 2.44 | 3.24 |
| Trifluralin | Treflan | 2.52 | 3.17 |

[z]Means within each column are not significantly different according to analysis of variance.

fertilizer inputs and in low N fertility sites (Tables 2, 3, and 4). The high fertility sites required about 50 kg ha$^{-1}$ N, while the lower fertility sites required 100-150 kg ha$^{-1}$ N. Deficiencies of other nutrients may also reduce Cry1Ac protein expression.

One 4-d episode of severe drought was sufficient to significantly reduce Cry1Ac protein expression, but Sicot 289RRi recovered its Cry1Ac protein expression more quickly than Sicala V-3RRi after irrigation. Under laboratory conditions, excised leaves showed increased Cry1Ac protein expression as water transpired, but Cry1Ac protein expression remained stable when leaf desiccation was prevented (Adamczyk and Gore, 2003).

Waterlogging poses a substantial threat to the productivity of cotton through poor nutrient uptake and loss of nutrients (e.g., N through denitrification), even though it did not affect leaf Cry1Ac protein expression.

Plant population density significantly influenced Cry1Ac protein expression, although the extremes of the treatments used in this experiment are rarely observed in commercial plantings. Low plant populations may be encountered at the edge of the field. In commercial fields, plant density may average eight to ten plants per linear m row, but wider plant spacing at the edge of the field may improve the survival of Heliothine larvae, possibly due to less shading among plants.

In strongly saline and sodic soils, plant production and nutrient uptake were severely impaired. This problem is currently the major nutritional constraint to cotton production in Australia, as P and K uptake are impaired (Rochester, 2004). Cry1Ac protein expression was unaffected in Experiment H in this study. Jiang et al. (2006) reported significantly reduced Cry protein levels in seedling cotton exposed to increasing salinity. Where severe P and K deficiency symptoms appeared in field-grown cotton, Cry1Ac protein expression was reduced. It appeared that deficiencies of P and K and other nutrients were less influential than the deficiency of N, possibly because protein metabolism was more strongly affected.

Shading had no significant effect on Cry1Ac protein expression. Reduced light can often be associated with waterlogging events when several inclement days follow flood irrigation, so additive effects in this type of scenario may accentuate reduction in Cry1Ac expression where low light, cool temperatures, waterlogging, and reduced nutrient uptake are combined.

Nutrient concentrations (particularly N and hence protein) may have increased in leaves where growth was slowed by herbicides and growth regulators, but the changes in leaf color associated with herbicide or growth regulator applications were not associated with Cry1Ac protein expression.

Only in the most severe instances of enviromental or agronomic variation was the expression of Cry1Ac protein significantly reduced. Extreme stresses that endangered the long term health or survival of cotton, such as drought or growing in sodic/saline soil, impaired crop nutrition and thus reduced Cry1Ac protein expression. Although no specific proteases have been found for Cry proteins in cotton, Chen et al. (2004) suggested that high ambient temperatures during boll filling resulted in the degradation of soluble protein in cotton plants that expressed reduced Cry1A protein levels.

Cry1Ac protein expression in transgenic cotton was relatively robust and was not significantly compromised by adverse growing conditions. Current cotton management practices do not appear to interfere with the efficacy of the Bt technology. Continued assessment of new cotton cultivars is essential to identify cotton cultivars that express more Cry1Ac protein.

## CONCLUSIONS

Cry1Ac protein expression in cotton has improved over time with the breeding of new cultivars. Leaf Cry1Ac protein expression was significantly lower when plants were subjected to severe drought, highly sodic/saline soil conditions, or extreme environmental conditions.

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