

Changes in Cry1Ac Bt Transgenic Cotton in Response to Two Environmental Factors: Temperature and Insect Damage

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ABSTRACT The efficacy of Cry1Ac *Bacillus thuringiensis* (Bt) cotton plants against field populations of *Helicoverpa armigera* (Hübner) has been inconsistent over the growing season. Any reduction in efficacy (where efficacy is the capacity of the plant to affect the survival of the insect) increases the opportunities for *H. armigera* to evolve resistance to Bt toxin. Changes in efficacy could be due to changes at the level of gene expression and/or in the physiological makeup of the plant and may be induced by environmental conditions. Two environmental factors, temperature and insect damage, were investigated. Temperature was found to affect efficacy, whether plants were grown at different temperatures continuously or were exposed to a change in temperature for a short period. Damage caused by chewing insects (*H. armigera* larvae) produced a dramatic increase in the efficacy of presquare Bt cotton. In contrast, damage by sucking insects (aphids) did not induce changes in efficacy. Changes in efficacy seemed to be mediated through modification of the physiological background of the plant rather than changes in the level of *cry1Ac* expression or in the concentration of the Bt toxin. The impact of the non-Bt responses of plants on strains of *H. armigera* should be evaluated. It is possible that by enhancing existing defensive mechanisms of plants, the rate of evolution of resistance to Bt toxins could be retarded by increasing the plants overall toxicity through the additive effects of the toxins and plant defenses.

KEY WORDS *Helicoverpa armigera*, Cry1Ac, mRNA, efficacy, cotton

THE EFFICACY OF FIELD GROWN transgenic cotton plants expressing the *cry1Ac* gene from the *Bacillus thuringiensis* Berliner variety *kurstaki* (Bt cotton), against *Helicoverpa* larvae, has been inconsistent over the growing season, both in the United States against *Helicoverpa zea* (Boddie) and in Australia against *Helicoverpa armigera* (Hübner) (Fitt et al. 1994, Layton et al. 1997). In Australia, late-season reduction in efficacy has been observed, and variation in efficacy also has been recorded early in the season (Fitt et al. 1998). This variable efficacy has consequences for the management of heliothine pests. On occasions, Cry1Ac Bt cotton fields have unexpectedly required applications of insecticide for heliothine control in late spring or early summer, in addition to the late-season applications recommended as part of the resistance management strategy (Pyke and Fitt 1998). Furthermore, reductions in efficacy could impact the rate of evolution of resistance to Bt in *H. armigera* (Daly 1994).

Evidence to date suggests that more than one factor is involved in the seasonal change. Cry1Ac toxin levels and total protein content of the leaves decline as the plant matures, but toxin levels also decline relative to

the total protein (Holt 1998). In addition, we have learned from glasshouse experiments that the growth stage that plants have reached when efficacy is examined is important (Olsen and Daly 2000, Olsen et al. 2005). The variation in efficacy observed could reflect changes at the level of gene expression. The unexpected inactivation of transgenes leading to the loss of a newly introduced trait has been well documented (reviewed in Stam et al. 1997). Transgene silencing in response to environmental factors, such as high light intensity or elevated temperatures, has been reported both for plants growing in the field and in experimental plants growing in controlled environments (Meyer et al. 1992, Walter et al. 1992).

The impact of the environment on Bt efficacy is not totally unexpected, because it is known that secondary products are induced or increase in concentration during periods of physiological stress or physical damage (Dixon and Paiva 1995). Such secondary products have been implicated in affecting the efficacy of Bt against noctuid larvae (Arteel and Lindroth 1992, Sivamani et al. 1992, Navon et al. 1993, Gibson et al. 1995), with orthoquinones possibly acting on Bt toxin itself (Ludlum et al. 1991).

It is difficult, if not impossible, in the field or even a glasshouse, to isolate and test individual environmental parameters, because other factors such as light intensity, water, and nitrogen availability or insect, wind, and hail damage cannot be controlled. Thus,

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experiments to test the interaction between efficacy and the environment must be carried out under controlled conditions such as a phytotron or growth room, where conditions to support normal plant growth can be provided and parameters (light, temperature, and nutrients) altered as required. We have investigated the influence of two environmental factors, temperature and insect damage, on the efficacy of presquare Cry1Ac Bt cotton plants.

The experiments reported here had two principal objectives. The first was to study the effects of environmental factors on the performance of transgenic Bt cotton plants under controlled, insecticide-free conditions. Performance was assessed as the mortality, in bioassays, of neonate *H. armigera* after the ingestion of plant leaf material. The second was to determine whether changes in efficacy of transgenic Bt cotton, in response to environmental factors, are associated with the plants production of toxin by examining the level of *cry1Ac* transcripts. An understanding of the causes and the molecular basis for the early season decline in efficacy of Bt transgenic cotton will provide a basis for making decisions concerning the management of the current Bt cotton lines and the development of new lines with insertions of Bt transgenes.

Materials and Methods

Insects. *H. armigera* and *Aphis gossypii* Glover were used in the experiments. Rearing methods for *H. armigera* were as described in Teakle and Jensen (1985), except that larvae were reared individually in 32-well plastic trays (Oliver Products Company, Grand Rapids, MI). The diet differed from that used by Teakle and Jensen (1985) through substituting chickpea flour for soybean flour and propionic acid (0.08%) for formalin. The laboratory strain was established in the mid-1980s from a series of collections in cotton fields in the Namoi Valley, northern New South Wales (NSW). Since 1990, this strain has been supplemented with collections of larvae from the same region to retain the vigor of the strain. The strain is considered susceptible to Cry1Ac protein toxins. This was monitored regularly using a method in which eggs were surface-treated with a dose of a Cry1Ac that kills $\approx 99\%$ of susceptible neonate larvae. The larvae receive the toxin on hatching through the ingestion of a portion of the contaminated surface of the egg. Controls were set up for all temperature and insect damage experiments to monitor the fitness of the neonate larvae used in bioassays by using leaves of conventional plants not subjected to treatment. *A. gossypii* was reared in a glasshouse at 22°C, on Sicot 189 cotton plants.

Plants. Seed for all cotton, *Gossypium hirsutum* L., plants was obtained from Cotton Seed Distributors Ltd., Wee Waa, NSW, Australia. In all experiments, Bt Siokra V-15i was used as the standard. Siokra V-15i contains the gene for the δ -endotoxin Cry1Ac from *B. thuringiensis* (Monsanto Company, St. Louis, MO) as the transformation event 757. Most experiments also included at least one of the other commercially available Cry1Ac Bt cotton containing the 531 event

(i.e., NuCOTN 37, Siokra V-16i, Sicot 289i, and NuPearl). Varieties with normal and okra leaves were included. The corresponding conventional variety for each Bt variety was used as the non-Bt control.

Most experiments were conducted within plant growth rooms, in conditions appropriate for cotton plants (22–32°C, photoperiod of 16:8 [L:D] h, 14,000–18,000 lx at plant height). In one experiment plants were grown in the glasshouse of a phytotron facility before treatment, followed by a change in temperature regime by transferring them to plant growth cabinets. Plants were grown in 15-cm pots with a 50:50 mixture of sand and potting mix. Four weeks after planting and subsequently at fortnightly intervals, plants received a solution of 1 g/liter of soluble plant fertilizer containing 27% total nitrogen, at a rate of ≈ 500 ml per pot. Up to three plants were grown in a single pot.

All plants sampled were at the presquare growth stage, 4 to 8 wk after planting. Nodes were numbered from the base of the plant, starting at the first true leaves. Cotton plants were sampled up to the first fully expanded leaf, which is usually at the third node down from the apex of the plant. Leaves from Bt plants were collected, divided in half along the main vein, and each half placed in an individually labeled plastic bag. Half of the leaf was used immediately in bioassays, and the remaining half was frozen in liquid nitrogen and stored at -80°C for mRNA and Cry1Ac assays. Leaves from conventional cotton were similarly processed but were bulked.

Bioassays. A leaf disk bioassay method was used in all experiments. The method, described in Olsen and Daly (2000), used 32-well larval rearing trays with a 5-ml mixture of 2% agar and 0.1% sorbic acid per well. A self-adhesive paper dot label placed on the agar limited contact between the leaf disks and the agar. Individual leaves were portioned and distributed among eight wells and one neonate *H. armigera* larva (1–18 h old) was placed in each well before the wells were heat sealed and kept at 25°C and 45–55% RH. Larvae were scored after seven days as either alive (normal movement) or dead (dead, moribund, uncoordinated movement). Each bioassay included a control whereby neonates were exposed to leaf disks of conventional cotton leaves.

Bt Enzyme-Linked Immunosorbent Assay (ELISA). The level of Bt protein toxin was determined in freeze-dried leaf material by an ELISA, by using a kit from Agdia Incorporated (Elkhart, IN). Leaves at node 2, from six plants, were assayed per treatment in the temperature experiments, and 12 leaves per treatment from node 4 (youngest fully expanded leaf) in the insect damage experiment. Leaves at these nodes were chosen for analysis because they showed the greatest difference in efficacy between treatments in bioassays. Leaves were freeze-dried before assay. The protein extraction method used is described in detail by Holt et al. (2002). The extraction buffer was designed to prevent protein degradation and prevent binding by plant secondary compounds.

Three milliliters of buffer was used per 0.1 g of freeze-dried leaf material. Total soluble protein was measured against a bovine gammaglobulin protein standard (Bio-Rad, Hercules, CA) by using a standard protein assay (Bio-Rad). The Bt protein toxin and total plant protein was measured in triplicate at 595 and 405 nm, respectively, by using a Bio-Rad model 3550-UV microplate reader.

RNA Extraction and Northern Analysis. Aliquots of the same leaf material sampled in the experiments and used in the bioassays were analyzed. RNA was isolated from individual leaves according to Jacobsen-Lyon et al. (1995) with the modifications of Townsend and Llewellyn (2002). RNA gel blot assays for *cry1Ac*, *NptII*, and *GUS* expression were done using 10 μ g of total RNA as described in Sheldon et al. (1999). After washing to remove unhybridized probe, filters were exposed for 1–2 h on a PhosphorImager screen and then scanned and visualized using ImageQuant software. Filters were washed again in 2 \times standard saline citrate (SSC) (three by 10 min) and then treated with ribonuclease A (2 μ g/ml) in 2 \times SSC for 15 min at room temperature. The filters were then washed in 0.1 \times SSC, 0.1% SDS at 65°C for 15 min and exposed to a PhosphorImager screen for 24–72 h.

Temperature Experiments. Two responses to temperature were explored. Plants were grown at different temperatures or were exposed to a change of temperature for a limited period. The temperature ranges used in experiments were not considered to constitute a significant stress on the plants. Although the rate of growth was altered, all plants continued to grow.

Plants Grown at Different Temperatures. Two experiments were conducted in growth rooms that provided high (22–32°C) and low (14–24°C) temperature ranges. Two Bt varieties were used: Siokra V-15i in the first experiment and both Sicot 289i and Siokra V-15i in the second experiment. Plants were sampled at the six-node stage (early presquare). Leaves were collected from three nodes (2, 3, and 4) in the first experiment and from two nodes (3 and 4) in the second experiment. Ten plants were used per variety for each temperature.

Plants Exposed to a Change of Temperature for a Limited Period. Plants grown at a medium temperature range were then exposed to the higher or lower temperatures for 7 d. Plants were sampled destructively before and immediately after treatment. Groups of 10 plants were used per variety and treatment at each sampling date. In preliminary experiments, it was observed that the growth-stage of plants could affect the response to short periods of temperature stress. Therefore, separate experiments were performed with early presquare and late presquare plants.

Two experiments were conducted with early presquare plants of the Bt variety Siokra V-15i. Three temperature regimes were used: high (22–36°C), medium (16–28°C), and low (10–20°C) in the first experiment and high (22–32°C), medium (18–28°C), and low (14–24°C) in the second experiment. Ten plants were sampled before treatment, at the six-node

stage and ten plants were sampled immediately after treatment, at the seven- to eight-node stage. Different plants were sampled on each occasion. Leaves were collected from nodes 3 to 6 (if available) from each plant.

Two experiments also were conducted on late presquare plants, in growth rooms providing high (22–32°C), medium (18–28°C), and low (14–24°C) temperature ranges. Siokra V-15i was used in the first experiment and both Siokra V-15i and NuCOTN 37 in the second. Ten plants were sampled before treatment, at the seven- or eight-node stage, and another 10 plants were sampled immediately after treatment at the eight- or nine-node stage. Before treatment, three leaves were collected from each plant. Leaves were collected from different plants after treatment, but at the same node position as well as any new leaves that had expanded at higher nodes during the period of treatment.

Insect Damage Experiments. The influence of chewing insects (*H. armigera* larvae) and sucking insects (*A. gossypii*) on the efficacy of Bt plants were investigated. Six plants were used for each variety and treatment. Conventional varieties were treated and tested along with their respective transformed varieties.

Two experiments were conducted with *H. armigera* larvae, the first with Siokra V-15i alone and the second with Siokra V-15i and three other varieties, Siokra V-16i, Sicot 289i, and NuPearl. Plants were infested by placing 15–30 neonate larvae on the youngest leaves of each plant, the number depending on cotton variety and growth stage. Plants were sampled 7 d after infestation at which time they had six to eight nodes. The two youngest expanded leaves were sampled from each of the damaged and control plants, to test in bioassays, Cry1Ac toxin ELISAs and mRNA assays. All leaves sampled from the damaged plants had minimal damage (<10% tissue eaten).

In the experiments investigating the effect of sucking insects, three varieties of Bt plants (Siokra V-15i, Siokra V-16i, and NuPearl) were infested with 20–30 aphids each for 10 days, with control and infested plants being maintained in separate growth rooms. The experiment was repeated, but on this occasion, the growth rooms for control and infested plants were switched. At the time of sampling, control plants had seven to 10 nodes, and the damaged plants had 6 to 10 nodes. The two youngest expanded leaves were sampled from each plant to test in bioassays and mRNA assays. At the time of sampling, approximately 11% of the damaged leaves used was distorted or curled as a consequence of the aphid infestation. The remaining leaves were normal in appearance but all were infested by aphids.

Data Analysis. F values were calculated for the response of larvae to Bt toxin in the leaves of Bt cotton plants, by using the logit analysis of GLIM version 3.77 (Payne 1985) as in Crawley (1993). GLIM models were examined that contained variety, node, plant, experiment, and treatment as factors. Models were simplified by removing factors if they did not signif-

Table 1. Neonate mortality, Cry1Ac protein toxin and total protein levels of leaves from two varieties of Bt cotton plants grown at high or low temperatures

Leaf/variety	High temp		Low temp		F	df	P
	% mortality ± SE	n	% mortality ± SE	n			
Varieties combined							
Node 3	76.1 ± 12	(238)	49.4 ± 6.9	(239)	15.1	1, 56	0.0003
Node 4	78.5 ± 18.5	(238)	36.1 ± 4.7	(235)	29.1	1, 56	<0.0001
Nodes combined							
Siokra V-15i	76.7 ± 14.3	(318)	46.3 ± 4.8	(317)	32.5	1, 97	<0.0001
Sicot 289i	78.5 ± 15.2	(159)	35.5 ± 8.2	(157)	23	1, 38	<0.0001
Cry1Ac toxin ^a	24.6 ± 6.1	(6)	26.4 ± 4.8	(6)	0.4	1, 10	0.5413
Total protein ^b	93.3 ± 21.2	(6)	113.3 ± 30.9	(6)	1.8	1, 10	0.2094

^a Cry1Ac protein toxin (nanograms per milligram of freeze-dried leaf ± SD) from node 2 leaf of Siokra V-15i.

^b Total protein (micrograms per milligram of freeze-dried leaf ± SD) from node 2 leaf of Siokra V-15i.

icantly contribute to variance. ELISA and protein concentration results were generated by Microplate Manager Software, MPMIII version 1.57. These data were analyzed in GLIM models (with normal error distribution). Factors examined were variety, treatment, and plant.

Results

The controls that monitored the fitness of neonate larvae used in bioassays indicated a consistent level of fitness with low levels of mortality (mortality 5.5 ± 1.09%, mean ± SE, range 0–11.1%, n = 496).

Temperature Effects on Bt Cotton. Plants Grown at Different Temperatures. When assayed for efficacy using the leaf disk method, early presquare Cry1Ac Bt plants grown at the higher temperature regime caused significantly more larval mortality than those grown at the lower regime (F = 54.73; df = 1, 135; P < 0.0001). This was true at all nodes sampled and for both the Siokra V-15i and Sicot 289i varieties (Table 1). Results for node 2 are not included in Table 1 because it was only sampled for one variety and in one experiment, but in that experiment the difference between treatments was also significant (F = 25.68; df = 1, 18; P < 0.0001; n = 168). There was no significant difference between the efficacy at different nodes within a single

temperature regime in these early presquare plants (high: F = 0.92; df = 2, 65; P = 0.404 and low: F = 2.27; df = 2, 67; P = 0.111). Larvae on leaves from conventional (nontransgenic) cotton plants grown at the higher temperatures, were smaller (14.8 ± 2.5 mg, mean ± SE, n = 121) than those fed leaves from plants grown at the lower temperatures (21.7 ± 4.8 mg, n = 111), but the difference was not significant (F = 1.02; df = 1, 8; P = 0.342).

Neither total protein levels nor Cry1Ac concentrations in Siokra V-15i plants tested at node 2 differed significantly between the two treatments (Table 1). The level of *cry1Ac* transcripts in RNA isolated from the equivalent leaves on plants grown at each temperature varied both within the group of plants grown at the same temperature and between plants grown at different temperatures. However, there was no apparent association between the level of *cry1Ac* transcripts and efficacy. For example, in Fig. 1, plant “j” grown at a low temperature, plants “h” and “a” grown at medium temperature, and plant “i” grown at a high temperature all had a similar amount of RNA loaded and a comparable level of *cry1Ac* transcript but bio-efficacy differed up to four-fold between plants. This indicated that the temperature at which plants were grown did not have a predictable effect on the expression of the *cry1Ac* transgene. Similarly, expres-

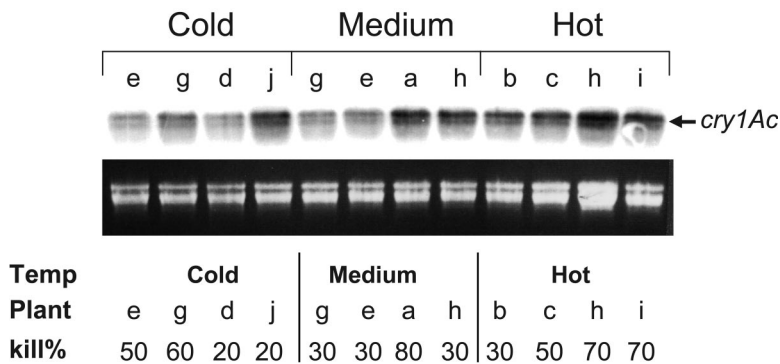


Fig. 1. Northern blot assays of *cry1Ac* transcripts and assays of bioefficacy (% kill) in individual Siokra V-15i plants, show no association. Plants were grown at three different temperatures: high (22–32°C), medium (18–28°C), and low (14–24°C). Assays for leaves sampled at node 3 in the presquare stage are presented. The relative loading for each sample is indicated by the ethidium stained gel shown below the *cry1Ac* transcripts.

Table 2. Neonate mortality, Cry1Ac protein toxin and total protein levels of leaves from Bt cotton plants exposed to high or low temperatures for 7 d

Age/leaf/variety	Before treatment		High temp		Low temp		F-test, high vs. low temp		
	% mortality ± SE	n	% mortality ± SE	n	% mortality ± SE	n	F	df	P
Early presquare									
Node 3	59.3 ± 12.5	(155)	72.1 ± 8.9	(117)	47.8 ± 0.3	(117)	10.5	1, 38	0.0025
Node 4	60 ± 20	(160)	72 ± 9.3	(139)	37.6 ± 0.9	(138)	6.6	1, 38	0.0142
Node 5	n/a ^c		57 ± 6.4	(139)	27 ± 5.6	(113)	10.9	1, 38	0.0021
Node 6	n/a		68 ± 1.7	(130)	n/a			n/a	
Late presquare									
Node 2/3	74.4 ± 10.5	(271)	60.8 ± 10.6	(270)	39.4 ± 7.3	(256)	7.6	1, 62	0.0077
Node 4	59.9 ± 9.4	(267)	42.6 ± 8.5	(271)	26.9 ± 7.9	(254)	5.1	1, 62	0.0275
Node 5/6	41.5 ± 5.5	(215)	20.6 ± 1.9	(268)	14.2 ± 2.8	(240)	1.9	1, 64	0.1725
Node 6/7	n/a		37 ± 10.1	(261)	n/a			n/a	
Variety									
Siokra V-15i	65.6 ± 7.4	(493)	47.2 ± 7	(699)	32.4 ± 5.6	(478)	17.2	1, 153	<0.0001
NuCOTN 37	44.5 ± 8.7	(260)	26.3 ± 4.9	(371)	15.8 ± 5.7	(286)	2.4	1, 81	0.1252
Cry1Ac toxin ^a			30.6 ± 3.7	(6)	26.4 ± 3.9	(6)	4.0	1, 10	0.0735
Total protein ^b			121.2 ± 27.6	(6)	95.5 ± 12.1	(6)	4.4	1, 10	0.0623

One Bt variety, Siokra V-15i, was tested as early presquare plants and two varieties tested at late presquare. n/a, not applicable.

^a Cry1Ac protein toxin (nanograms per milligram of freeze-dried leaf ± SD) from node 2 leaf of Siokra V-15i.

^b Total protein (micrograms per milligram freeze-dried leaf ± SD) from node 2 leaf of Siokra V-15i.

^c Leaf not available at that node.

sion of the 35S::NptII transgene that is linked to the 35S::cry1Ac transgene also was unaffected by the growth temperature (not shown).

Plants Exposed to Change of Temperature for a Limited Period. In early presquare plants, significant changes in efficacy in response to the changed temperature were detected by leaf disk assays ($F = 40.92$; $df = 1, 167$; $P < 0.0001$). Warmer conditions enhanced efficacy (initial versus high: $F = 4.12$; $df = 1, 156$; $P = 0.044$), whereas exposure to cooler conditions decreased levels of mortality (initial versus low: $F = 22.08$; $df = 1, 127$; $P < 0.0001$). This was true at all nodes sampled (Table 2). There was no significant difference in efficacy at different nodes before treatment ($F = 0.57$; $df = 3, 55$; $P = 0.637$) or after treatment within a single temperature regime in these early presquare plants (high: $F = 2.08$; $df = 3, 69$; $P = 0.111$ and low: $F = 2.32$; $df = 5, 84$; $P = 0.051$). Results for node 2 are not included in Table 1 because it was only sampled in one experiment, but the difference between treatments was also significant ($F = 19.95$; $df = 1, 18$; $P = 0.0003$; $n = 160$). Whereas plants (Siokra V-15i) in both experiments showed similar responses to temperature changes, the efficacy in the two experiments were quite different ($F = 8.89$; $df = 1, 200$; $P = 0.0032$), reflecting significant differences in the level of efficacy of plants before treatment ($F = 21.42$; $df = 1, 58$; $P < 0.0001$).

When exposed to brief periods of an increase or decrease in temperature, plants that were close to squaring (late presquare) responded somewhat differently than early presquare plants grown under the same temperature regimes. As for younger plants, limited exposure to a change in temperature regime resulted in highly significant differences in efficacy ($F = 18.36$; $df = 1, 234$; $P < 0.0001$). Again, the change to warmer conditions resulted in significantly enhanced larval mortality (initial versus high: $F = 58.48$; $df = 1,$

194; $P < 0.0001$), and cooler conditions significantly decreased the level of larval mortality (initial versus low: $F = 14.57$; $df = 1, 223$; $P = 0.0002$). Unlike the younger plants, there was a highly significant heterogeneity in efficacy among the leaves at different nodes, both before ($F = 5.66$; $df = 2, 90$; $P = 0.0048$) and after treatment (high: $F = 13.31$; $df = 3, 118$; $P < 0.0001$, low: $F = 6.39$; $df = 3, 100$; $P = 0.0005$) (Table 2). At both sampling times, efficacy was lower in the younger leaves than the more mature leaves (at lower nodes), and this is characteristic of Cry1Ac Bt cotton plants close to or starting to square. Differences observed at the lower nodes (2–4), between treatments, were greater than those for the younger leaves at the higher nodes (5 and 6) where the treatment had no significant effect on efficacy. The two Bt varieties used, Siokra V-15i and NuCOTN 37, showed similar responses to treatment (Table 2), but the resulting differences in efficacy were significant for only Siokra V-15i.

As with the plants grown continually at high, medium or low temperatures, there was no difference in total protein or Cry1Ac levels at node 2 for the early presquare plants tested after temperature treatments. For early presquare plants, a visual assessment was made of the level of cry1Ac transcript in leaves that had completed expansion under the new growth conditions compared with that in leaves from the same plant that had developed before the change in temperature. There was no detectable difference in expression of the cry1Ac transgene in these leaves at different nodes on the same plant (not shown). The level of cry1Ac transcripts in leaves at the same node from different plants that had experienced a change in growth conditions was also compared. Again, there was no apparent association between efficacy and the level of cry1Ac transcripts (Fig. 2).

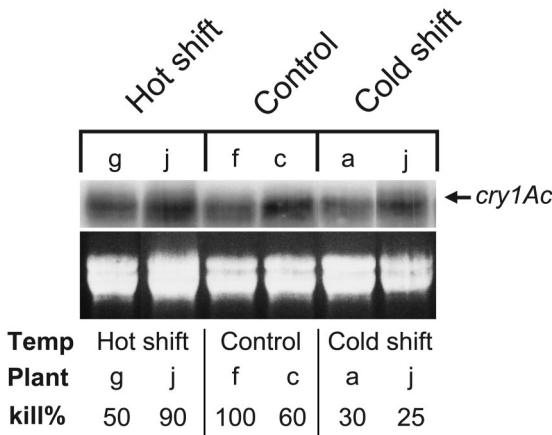


Fig. 2. Northern blot assays of *cry1Ac* transcripts in Siokra V-15i plants, sampled individually in the early presquare stage, show no association with the bioefficacy of the plants after a shift in temperature from a medium control temperature (18–28°C) to hotter (22–32°C) or colder (14–24°C) temperatures. Assays from node 4 leaves shown.

Insect Damage Effects on Bt Cotton. For chewing insects (*H. armigera* larvae), leaf disk assays detected a highly significant increase in the efficacy of damaged compared with undamaged leaves at the nodes tested ($F = 361.24$; $df = 1, 118$; $P < 0.0001$). This was true for all four Cry1Ac Bt varieties tested (Table 3). Prior herbivore damage on conventional varieties also affected the subsequent growth rate of *H. armigera* larvae. Larvae on damaged conventional cotton plants were markedly smaller (1.1 ± 0.3 mg, $n = 93$) than those fed undamaged leaves (13.8 ± 4.03 mg, $n = 147$), and the difference was significant ($F = 9.9$; $df = 1, 6$; $P = 0.02$).

Protein analyses of the leaves detected a significant decrease in protein levels after larval feeding (Table 3). Despite this difference, the actual concentration of Cry1Ac toxin did not change markedly, because of an increase in proportion of Bt toxin in the total protein estimate. It was notable that the initial extract from the damaged leaves seemed considerably more viscous than that from the undamaged leaves. Perhaps related

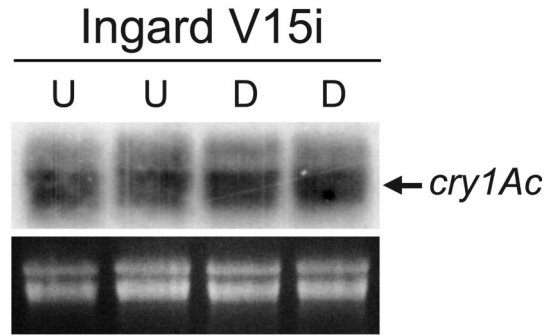


Fig. 3. Northern blot assays of *cry1Ac* transcripts in presquare Siokra V-15i plants, sampled individually, show no response to damage by aphids (D, plant damaged by aphids; U, undamaged plant). Assays from node 5 leaves shown.

to the changes in viscosity, only poor quality RNA could be isolated from these plants. As a result, information at the RNA level on the response of plants to damage by caterpillars is less clear. However, preliminary data suggest that there was no change in *cry1Ac* expression in response to this challenge.

Despite high infestation rates, *A. gossypii* damage failed to induce detectable changes in efficacy in any of the three varieties tested (Table 3) or when all varieties were included in the analyses ($F = 0.12$; $df = 1, 139$; $P = 0.73$). Northern analyses of RNA isolated from insect damaged leaves taken from varieties Siokra V-15i, Siokra V-16i, and NuPearl 37 showed that there was no detectable change in the level of *cry1Ac* transcripts in response to aphid damage (Fig. 3), which is consistent with the findings from the leaf disk assays.

Discussion

This study examined the impact of two environmental factors, temperature and insect damage, on the efficacy of Cry1Ac Bt plants. The survival of *H. armigera* larvae on Bt plants was influenced by exposure to higher or lower temperatures, either for short periods or throughout growth, and by the damage of a

Table 3. Neonate mortality, Cry1Ac protein toxin, and total protein levels of leaves from three varieties of Bt cotton plants after infestation with chewing (*H. armigera* larvae) or sucking insects (*A. gossypii*)

Insect damage	Bt variety	Damaged		Undamaged		F	df	P
		% mortality ± SE	n	% mortality ± SE	n			
<i>H. armigera</i>	Siokra V-15i	95.3 ± 3	(192)	26.8 ± 3.7	(182)	118	1, 46	<0.0001
	Siokra V-16i	100 ± 0	(95)	25.8 ± 3.1	(93)	550.3	1, 22	<0.0001
	Sicot 289i	100 ± 0	(94)	6.8 ± 2.4	(95)	660.5	1, 22	<0.0001
	NuPearl	95.5 ± 2.4	(96)	19.3 ± 10.6	(95)	30.5	1, 22	<0.0001
<i>A. gossypii</i>	Siokra V-15i	58.5 ± 22.5	(186)	67 ± 12	(189)	0.67	1, 45	0.4174
	Siokra V-16i	39 ± 29.0	(198)	38 ± 22	(193)	0.02	1, 45	0.8882
	NuPearl	31.5 ± 14.5	(191)	29.5 ± 15.5	(193)	0.08	1, 45	0.7786
<i>H. armigera</i>	Cry1Ac toxin ^a	11 ± 4.5	(12)	6.4 ± 5.2	(12)	2.9	1, 22	0.1027
	Total protein ^b	91.8 ± 17.9	(12)	160.3 ± 37.3	(12)	33.1	1, 22	<0.0001

^a Cry1Ac protein toxin (nanograms per milligram of freeze-dried leaf ± SD) from node 4 leaf of Siokra V-15i.

^b Total protein (micrograms per milligram of freeze-dried leaf ± SD) from node 4 leaf of Siokra V-15i.

herbivore. Our data predict that cool temperatures, experienced in the early part of the growing season, could markedly reduce the efficacy of Cry1Ac Bt plants, whereas herbivore damage or plant growth at higher temperatures would enhance efficacy. The ELISA and RNA analyses provided evidence that the titer of Cry1Ac toxin and the expression of the *cry1Ac* transgene were not markedly changed in these circumstances. Because Bt production did not seem to be altered in response to environmental factors, we must look for different mechanisms to explain the changes in efficacy.

The results from nontransgenic controls (conventional cotton) subjected to insect damage, indicate that changes in the background physiology of the plants are responsible for the observed altered larval growth. Plants produce an array of chemicals and the production of some of these, particularly phenylpropanoids, seem to be affected by environmental factors such as light, UV, temperature, disease, wounding and nutrient levels (Dixon and Paiva 1995). Insect damage or wounding can elicit a range of responses in cotton plants. For example, infestation of cotton seedlings by *A. gossypii* induces resistance to further colonization by aphids, most likely due to reduced nutrition in the damaged plants, rather than by increased production of metabolites (Wool and Hales 1996). Similarly, insect damage reduced the nutritional quality of cotton plant tissues with a decrease in total protein and ascorbate levels and increased oxidative enzyme activity, producing secondary compounds such as chlorogenic acid (Bi et al. 1997). The growth and development of *Spodoptera exigua* (Hübner) was significantly slower on damaged glanded cotton than undamaged glanded or damaged glandless cotton, suggesting that elevated levels of terpenoid aldehydes were responsible (McAuslane and Alborn 2000). Increased production of phenolic compounds in cotton plants in response to wounding also contributed to induced resistance to *H. zea* (Bi et al. 1997).

The presence of these chemicals or changes in their levels within the plant also have been shown to affect insects' ability to tolerate toxic chemicals. Terpenoids increased the efficacy of Bt toxin against *Heliothis virescens* (F.) (Sachs et al. 1996), and plant phenolics found in cotton, gallic acid and resorcinol, enhanced the efficacy of Bt variety *galleriae* toxin against *H. armigera* (Sivamani et al. 1992). Similarly, chlorogenic acid and polyphenol oxidase, which form orthoquinones in damaged plant tissue, increased the efficacy of Bt against *H. zea* (Ludlum et al. 1991). We observed that even minimal chewing damage to leaves (<10%) induced significant changes in efficacy, consistent with previous reports that plants responded to wounding when little or no physiological damage was evident (Karban 1987, Wool and Hales 1996). Karban (1985) found that abrasion with powdered carborundum was sufficient to induce resistance in cotton seedlings to infestation by *Tetranychus urticae* Koch. Application of a natural chemical inducer, jasmonic acid, induced resistance in cotton plants to *H. zea* (Bi et al.

1997) and to three sucking pests of cotton (Omer et al. 2001).

In a review of plant chemical defenses, Duffy and Stout (1996) emphasize that evaluation of a toxin should always be considered in the context of the plant's chemical background. Although the chemical response of plants to wounding has been well documented, wounding responses may not be responsible for the differences in efficacy found in our temperature experiments. Because the temperature ranges were not extreme, it is more likely that the effects observed are related to the chemistry regulating plant growth. Olsen and Daly (2000) found that leaves from conventional presquare plants (in vegetative growth stage) when spiked with Cry1Ac toxin, were more toxic than similarly spiked leaves from fruiting plants (in reproductive growth stage).

We have concentrated on evaluating the immediate response of cotton plants to certain environmental challenges during a particular growth stage. We have shown that mild temperature differences are capable of inducing a response from cotton that causes marked impacts on insect survival. Plants in the field are exposed to a far greater range of temperatures and environmental assaults (e.g., wind and hail damage; waterlogging, nutritional stresses, insect damage, and root damage), and it might be considered that such plants are in a continual state of stress. Under more extreme environmental conditions of drought and heat, cotton plants have been shown to produce heat shock proteins, at the expense of normal protein synthesis (Burke et al. 1985). In contrast to the mild stresses imposed in our experiments, these more severe stresses may indeed affect the protein toxin production in Bt cotton.

Additional factors that could affect the response of plants to the environment include agronomic factors, growth stage, plant tissues, age, species, and variety. For example, in older plants, Karban and Baldwin (1997) found that the induced response to damage by mites was not as great as in seedlings. Crowding of cotton plants reduced their ability to resist infestations of *T. urticae* (Karban et al. 1989). In studies of the systemic effects of wounding in cotton and soybean, *Glycine max* L., Croxford et al. (1989) found that the reduction in palatability to *Spodoptera littoralis* Boisduval changed with distance from a mechanically produced wound and also over time. Stout et al. (1996), looking at four types of induced responses in tomato plants, found that different mechanisms were active against *H. zea* in different parts of the plant. Differences in the response by cotton plants also may be determined by the feeding method of the insect (Omer et al. 2001). This could partly explain why we saw no induced resistance to *H. armigera* larvae by aphid feeding, but a significant effect caused by larval feeding.

An important outcome of this study relates to the value of ELISA tests on Cry1Ac Bt varieties to predict plant efficacy against *H. armigera*. In general, the test is predictive of efficacy, because it detects the major change in Bt titer associated with plant stage (Olsen

et al. 2005). However, it failed to detect the marked changes in efficacy that were associated with the plant response to environmental stress. Similarly, *cry1Ac* transcript levels did not predict changes in efficacy in response to environmental or physical stresses. Gore et al. (2001) also found that Cry1Ac levels measured in different Bt cotton plant tissues did not correlate with survival of *H. zea*. Plant secondary compounds present at different levels in different tissues, may account for these apparent inconsistencies, by differentially altering the efficacy of the Cry1Ac toxin (Gore et al. 2001). Olsen et al. (2004) noted that the bioassay method used also affects the level of efficacy of Cry1Ac cotton plants. The assay methods chosen for this study were based on that work.

Besides highlighting the fact that cotton plants do respond to environmental factors, this study suggests that the additive effect of the two mechanisms, Cry1Ac toxin production and the plants chemical response to environmental factors, could play a role in reducing the period that insects survive in Cry1Ac Bt crops. This survival effect has two consequences. First, it reduces the duration of the season that synthetic insecticides are required, and second, it delays the period during which individuals of pest species, carrying alleles that confer resistance to Cry1Ac toxin, might be advantaged and thus increase in frequency. Through identifying and understanding the causes of variation in the efficacy of Bt cotton plants, particularly the role of plant background chemistry and changes in the levels of Bt toxin and mRNA, advances can be made in resistance management and in the development of new Bt cotton varieties.

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