Temporal and intra-plant variability of Cry1Ac expression in *Bt*-cotton and its influence on the survival of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera)

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The quantitative levels of Cry1Ac and the seasonal decline in expression differed significantly among the eight commercial Bollgard hybrids tested. The Cry1Ac expression was found to be variable among the hybrids and also between different plant parts. The leaves of *Bt*-cotton plants were found to have the highest levels of Cry1Ac expression followed by squares, bolls and flowers. The toxin expression in the boll-rind, square bud and ovary of flowers was clearly inadequate to confer full protection to the fruiting parts. Increasing levels of *Helicoverpa armigera* survival were correlated with the toxin levels decreasing below 1.8 μg/g in the plant parts. Genotype-independent seasonal decline of the Cry1Ac toxin levels was observed in all the hybrids. Cry1Ac expression decreased consistently as the plant aged. The decline in Cry1Ac was more rapid in some hybrids compared to others. The choice of parental background appeared to be crucial for sustainable expression of the *cry1Ac* transgene. The implications of variability in Cry1Ac expression and the seasonal decline on bollworm management are discussed.

Keywords: *Helicoverpa armigera*, *Bt* cotton, *Bacillus thuringiensis*, Cry1Ac.

THREE *Bt*-cotton transgenic hybrids (Bollgard-MECH-12, Bollgard-MECH-162 and Bollgard-MECH-184) were officially approved and released in 2002 for commercial cultivation in India. The technology was introduced into India by MAHYCO (Maharashtra Hybrid Seeds Company Ltd, Jalna, India) under license from Monsanto, USA. Five more *Bt*-cotton hybrids (Bollgard-RCH-2, Bollgard-RCH-20, Bollgard-RCH-134, Bollgard-RCH-138 and Bollgard-RCH-144 from RASI Seeds Pvt Ltd, Attur, Tamil Nadu) were approved by the Government of India, for large-scale field trials during the 2002 and 2003 cropping seasons, before being released for commercial cultivation. All the *Bt*-cotton hybrids mentioned above were developed using Indian parent varieties into which the *cry1Ac* gene was introgressed from a transgenic *Bt*-cotton variety, Coker 312. All the current Bollgard cotton hybrids have descended from a common parent with a single genetic transformation event[1] 'Monsanto-531', which was transformed with a vector containing a full-length *cry1Ac* coding sequence driven by an enhanced 35S promoter that enables the production of Cry1Ac protein in almost all parts of the plant. When injested by larvae, Cry1Ac binds to specific receptors in the midgut region. Toxin-binding in susceptible insects disrupts midgut epithelium, thereby causing overall toxic effects and ultimately resulting in death of the larvae. The novel transgenic technology was found to be highly beneficial in almost all parts of the world in terms of its capabilities to keep the target pests such as bollworms under check. However, for the *Bt*-transgenic technology to be sustainable, it is important that the toxin expression levels be expressed at adequate quantities in appropriate plant parts at the requisite time of the season to afford protection against major target insect pests, which primarily include the bollworms. Studies in Australia[2–5] and USA[6–11] showed that Cry1Ac toxin expression was variable among *Bt*-cotton plant parts and that the Cry1Ac toxin expression and bollworm mortality levels decreased consistently as the plant aged. Our field experience with *Bt*-cotton hybrids in India showed that the cotton bollworm *Helicoverpa armigera* (Hübner) was able to survive more on some particular fruiting parts, and the pest infestation exceeded economic threshold levels more readily on some specific commercial hybrids compared to others. The main objectives of this study were to determine a critical level of Cry1Ac expression in *Bt*-cotton plants that would be required for effective bollworm control; estimate the variability of Cry1Ac expression among plant parts and elucidate temporal changes in expression of Cry1Ac protein in eight commercial *Bt*-cotton hybrids using ELISA (enzyme linked immunosorbent assay).

Materials and methods

The eight *Bt*-cotton Bollgard hybrids and their corresponding non-*Bt* hybrids (MECH-12, MECH-162, MECH-184,

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RCH-2, RCH-20, RCH-134, RCH-138 and RCH-144) were grown in 150 m^2 plots in three replicates at the Central Institute for Cotton Research (CICR), Nagpur during 2003. Recommended agronomic and crop management practices were followed thereafter. The Cry1Ac content in plant parts was quantified using the commercially available '*Bt*-Quant' ELISA kit (Innovative Biosciences, Nagpur). Cry1Ac was estimated from 4 to 6 replicate samples of leaves and fruiting parts. Generally, 10–15 mg of leaf discs and other plant parts were weighed before being homogenized in Tris-borate buffer (0.1 M Tris; 0.01 M sodium tetraborate; 0.005 M $MgCl_2$; 0.2% L-ascorbic acid and 0.05% Tween-20). ELISA was performed according to the method described by Kranthi *et al.*[12]. Quantification of Cry1Ac was done by plotting absorbance values of the test samples on the standard curve generated with purified Cry1Ac standards on each of the ELISA plates and expressed as µg Cry1Ac per g wet weight of the tissue. Laboratory bioassays with *H. armigera* were conducted regularly on upper canopy leaves at 5–16-day intervals and with fruiting parts using excised parts of 100–107-day-old crop. Plant parts (ten leaves/squares/flowers/bolls per *Bt* and non-*Bt* hybrid) were brought to the insectary and one-day-old (first instar) *H. armigera* larvae were placed on each of the plant parts, individually, in plastic cups. The plant parts were changed each day for seven days until the end of the bioassay. Mortality observations and individual weights of the surviving larvae were recorded on the seventh day after larval release. Analysis of variance was carried out using the methods described by Gomez and Gomez[13].

Results

Upper canopy leaves

Cry1Ac expression (Table 1) was high at 4.42–6.61 µg/g in the upper canopy leaves early in the season at 30 DAS. A gradual decline was observed in expression over time, in all the hybrids. The decline started relatively early in the season in Bollgard-RCH-2, Bollgard-RCH-20 and Bollgard-MECH-162. By 110 DAS, Cry1Ac expression decreased to <0.47 µg/g in all the hybrids. Interestingly, the toxin expression did not decline completely to undetectable levels over the season in all the hybrids. Expression levels of 0.1–0.7 µg/g were observed in Bollgard-RCH-134, Bollgard-RCH-138, Bollgard-RCH-144, Bollgard-MECH-12 and Bollgard-MECH-184 at various times of the season even after 124 DAS. There were significant differences in the expression levels between plant age intervals ($F = 131.2$; df = 8, 24; $P = <0.0001$) and amongst hybrids ($F = 16.8$; df = 7, 189; $P = <0.0001$). Interaction effects between age intervals and hybrids were also significant ($F = 4.1$; df = 56, 189; $P = <0.0001$).

Mid-canopy leaves

The dynamics of Cry1Ac changes in the mid-canopy leaves is presented in Table 2. The Cry1Ac levels were initially high in a range of 2.32–4.26 µg/g during 58–85 DAS in all hybrids. At 95 DAS, Cry1Ac ranged from 0.85 to 1.08 µg/g in Bollgard-RCH-2, Bollgard-RCH-20 and Bollgard-MECH-162, and 1.4 to 2.29 µg/g in rest of the hybrids. Subsequently, after 110 DAS, there was a gradual decline in expression over the season with Cry1Ac content reaching to <1.02 µg/g. There were significant differences between Cry1Ac expressions, between plant age intervals ($F = 3013$; df = 7, 21; $P = <0.0001$) and amongst hybrids ($F = 50.9$; df = 7, 168; $P = <0.0001$). The interaction effect between age intervals and hybrids was significant ($F = 10.9$; df = 49, 168; $P = <0.0001$).

Lower canopy leaves

The Cry1Ac expression in the lower canopy leaves (Table 3), ranged between 2.22 and 6.49 µg/g initially during 58–95 DAS in all the hybrids, except at 95 DAS in Boll-

Table 1. In-season changes in Cry1Ac expression (µg/g fresh weight) in upper canopy leaves of *Bt*-cotton hybrids

	Bt Bollgard hybrids									
DAS	RCH-2	RCH-20	RCH-134	RCH-138	RCH-144	MECH-12	MECH-162	MECH-184	Mean	LSD
30	5.15 ± 0.9	6.61 ± 1.1	4.67 ± 0.7	4.42 ± 0.3	5.56 ± 0.5	6.20 ± 1.0	4.97 ± 0.7	6.47 ± 1.6	5.51	1.37
58	3.43 ± 0.3	3.60 ± 0.3	2.33 ± 0.4	3.91 ± 0.4	2.96 ± 0.5	4.05 ± 0.4	2.68 ± 0.5	3.57 ± 0.6	3.31	0.64
70	2.23 ± 0.9	1.94 ± 0.8	1.26 ± 0.5	2.91 ± 0.5	1.87 ± 0.2	3.32 ± 0.8	1.35 ± 0.6	2.51 ± 0.5	2.17	0.94
85	1.46 ± 0.8	2.40 ± 1.1	1.53 ± 0.9	2.62 ± 1.2	1.63 ± 0.3	2.31 ± 1.2	1.16 ± 0.3	2.60 ± 0.8	1.96	NS
95	0.58 ± 0.9	0.77 ± 0.5	1.66 ± 0.1	0.97 ± 1.2	1.11 ± 1.0	1.07 ± 0.4	0.55 ± 0.4	0.89 ± 0.7	0.95	NS
110	0.21 ± 0.1	0.41 ± 0.2	0.22 ± 0.1	0.14 ± 0.1	0.44 ± 0.2	0.38 ± 0.2	0.16 ± 0.1	0.47 ± 0.2	0.30	0.21
124	0.09 ± 0.1	0.03 ± 0.0	0.07 ± 0.1	0.15 ± 0.1	0.13 ± 0.2	0.32 ± 0.3	0.02 ± 0.0	0.26 ± 0.2	0.13	NS
138	0.02 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.42 ± 0.2	0.49 ± 0.3	0.21 ± 0.1	0.01 ± 0.0	0.71 ± 0.1	0.23	0.20
148	0.01 ± 0.0	0.01 ± 0.0	0.10 ± 0.2	0.03 ± 0.0	0.02 ± 0.0	0.08 ± 0.0	0.01 ± 0.0	0.12 ± 0.1	0.05	NS
Mean	1.46	1.75	1.32	1.73	1.58	1.99	1.21	1.95		
LSD	0.87	0.85	0.64	0.68	0.67	0.94	0.58	1.02		

DAS, Days after sowing; LSD, Least significant difference.

Table 2. In-season changes in Cry1Ac expression (μg/g fresh weight) in mid-canopy leaves of *Bt*-cotton hybrids

	Bt Bollgard hybrids									
DAS	RCH-2	RCH-20	RCH-134	RCH-138	RCH-144	MECH-12	MECH-162	MECH-184	Mean	LSD
58	3.16 ± 0.2	3.22 ± 0.2	3.05 ± 0.2	3.96 ± 0.4	3.17 ± 0.2	4.00 ± 0.2	3.03 ± 0.1	4.26 ± 0.2	3.48	0.32
70	3.41 ± 0.4	3.20 ± 0.4	2.93 ± 0.1	2.93 ± 0.1	3.46 ± 0.2	3.17 ± 0.1	2.56 ± 0.1	3.78 ± 0.2	3.18	0.34
85	3.41 ± 0.4	2.71 ± 0.4	3.61 ± 0.2	2.54 ± 0.4	3.95 ± 0.2	2.61 ± 0.1	2.32 ± 0.2	3.33 ± 0.3	3.06	0.42
95	0.85 ± 0.2	1.08 ± 0.3	2.29 ± 0.3	1.85 ± 0.3	1.94 ± 0.1	1.40 ± 0.1	0.93 ± 0.1	2.03 ± 0.3	1.55	0.34
110	0.37 ± 0.0	0.43 ± 0.1	0.74 ± 0.1	0.32 ± 0.1	0.78 ± 0.1	0.53 ± 0.1	0.18 ± 0.1	1.02 ± 0.2	0.54	0.13
124	0.09 ± 0.1	0.03 ± 0.0	0.07 ± 0.1	0.15 ± 0.1	0.13 ± 0.2	0.32 ± 0.3	0.02 ± 0.0	0.26 ± 0.2	0.13	0.21
138	0.02 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.42 ± 0.2	0.49 ± 0.3	0.21 ± 0.1	0.01 ± 0.0	0.71 ± 0.1	0.23	0.20
148	0.01 ± 0.0	0.01 ± 0.0	0.20 ± 0.2	0.03 ± 0.0	0.02 ± 0.0	0.08 ± 0.0	0.01 ± 0.0	0.12 ± 0.1	0.05	0.11
Mean	1.41	1.33	1.61	1.52	1.74	1.54	1.13	1.94		
LSD	0.34	0.35	0.24	0.36	0.26	0.22	0.15	0.30		

Table 3. In-season changes in Cry1Ac expression (μg/g fresh weight) in bottom canopy leaves of *Bt*-cotton hybrids

	Bt Bollgard hybrids									
DAS	RCH-2	RCH-20	RCH-134	RCH-138	RCH-144	MECH-12	MECH-162	MECH-184	Mean	LSD
58	6.49 ± 0.5	5.63 ± 0.1	5.86 ± 0.2	5.66 ± 0.4	5.58 ± 0.5	5.25 ± 0.0	3.77 ± 0.3	5.66 ± 0.4	5.49	0.51
70	3.53 ± 0.4	4.09 ± 0.3	3.66 ± 0.1	3.29 ± 0.6	3.09 ± 0.1	3.95 ± 0.2	2.80 ± 0.3	4.41 ± 0.3	3.60	0.50
85	2.69 ± 0.7	3.52 ± 0.3	3.04 ± 0.3	2.49 ± 0.4	3.07 ± 0.2	3.54 ± 0.1	2.11 ± 0.2	4.10 ± 0.1	3.07	0.48
95	1.41 ± 0.2	2.22 ± 0.4	2.59 ± 0.2	2.78 ± 0.3	2.42 ± 0.2	2.39 ± 0.1	1.28 ± 0.2	2.48 ± 0.1	2.19	0.35
110	0.85 ± 0.1	0.79 ± 0.0	1.21 ± 0.1	0.91 ± 0.1	1.71 ± 0.2	1.17 ± 0.1	0.42 ± 0.2	1.73 ± 0.3	1.10	0.24
124	0.33 ± 0.0	0.18 ± 0.0	0.24 ± 0.0	0.41 ± 0.1	0.30 ± 0.0	0.65 ± 0.1	0.11 ± 0.0	0.92 ± 0.4	0.39	0.20
138	0.12 ± 0.0	0.06 ± 0.0	0.40 ± 0.2	0.11 ± 0.1	0.31 ± 0.1	0.12 ± 0.1	0.02 ± 0.0	0.26 ± 0.1	0.17	0.09
148	0.03 ± 0.0	0.02 ± 0.0	0.12 ± 0.0	0.10 ± 0.0	0.05 ± 0.0	0.07 ± 0.0	0.01 ± 0.0	0.41 ± 0.2	0.10	0.04
Mean	1.93	1.95	2.14	1.97	2.07	2.14	1.31	2.49		
LSD	0.49	0.25	0.29	0.48	0.32	0.17	0.28	0.38		

gard-MECH-162 and Bollgard-RCH-2, which had 1.28 and 1.41 μg/g respectively. At 110 DAS, the toxin expression was 0.42–1.73 μg/g, but declined steadily thereafter, with especially rapid rate of decrease in Bollgard-MECH-162 and Bollgard-RCH-20. There were significant differences between the Cry1Ac expression, between age intervals ($F = 1876$; df = 7, 21; $P = <0.0001$) and amongst hybrids ($F = 54.1$; df = 7, 168; $P = <0.0001$). Interaction effect between age intervals and hybrids was significant ($F = 12.1$; df = 49, 168; $P = <0.0001$).

Square parts

The square bracts showed variable levels of Cry1Ac that ranged from 0.06 to 0.63 μg/g with significant differences ($F = 2.97$; df = 7, 40; $P = 0.013$) between the hybrids (Figure 1). The expression in square buds was low at 0.05–0.08 μg/g in Bollgard-RCH-20, Bollgard-RCH-138 and Bollgard-MECH-162, and varied from 0.25 to 0.51 μg/g in the rest.

Flower parts

Cry1Ac content in flower petals ranged from 0.25 to 0.80 μg/g with no significant differences between the hybrids (Figure 2). However, the toxin content was highly variable and significantly different between flower bracts ($F = 2.98$; df = 7, 40; $P = 0.013$) and anthers ($F = 6.69$; df = 7, 40; $P = <0.0001$). The ovary contained low levels of Cry1Ac that varied from undetectable to 0.07 μg/g in Bollgard-RCH-144, Bollgard-MECH-12, Bollgard-MECH-162 and Bollgard-MECH-184, and 0.15–0.27 μg/g in rest of the hybrids, with no significant differences among them.

Boll parts

The boll rind contained low levels of Cry1Ac at 0.01–0.05 μg/g in Bollgard-RCH-2, Bollgard-RCH-20 and the three Bollgard-MECH hybrids and ranged from 0.25 to 0.37 μg/g in rest of the three hybrids (Figure 3). Boll bracts, loculi wall and raw seed cotton expressed Cry1Ac within a range of 0.19–1.17, 0.38–1.98 and 0.65–2.02 μg/g respectively. There were no significant differences in Cry1Ac expression in any of the boll parts among all the hybrids.

Bioassays on upper canopy leaves

The larval mortality in bioassays with upper canopy leaves clearly showed that there was a seasonal decline in

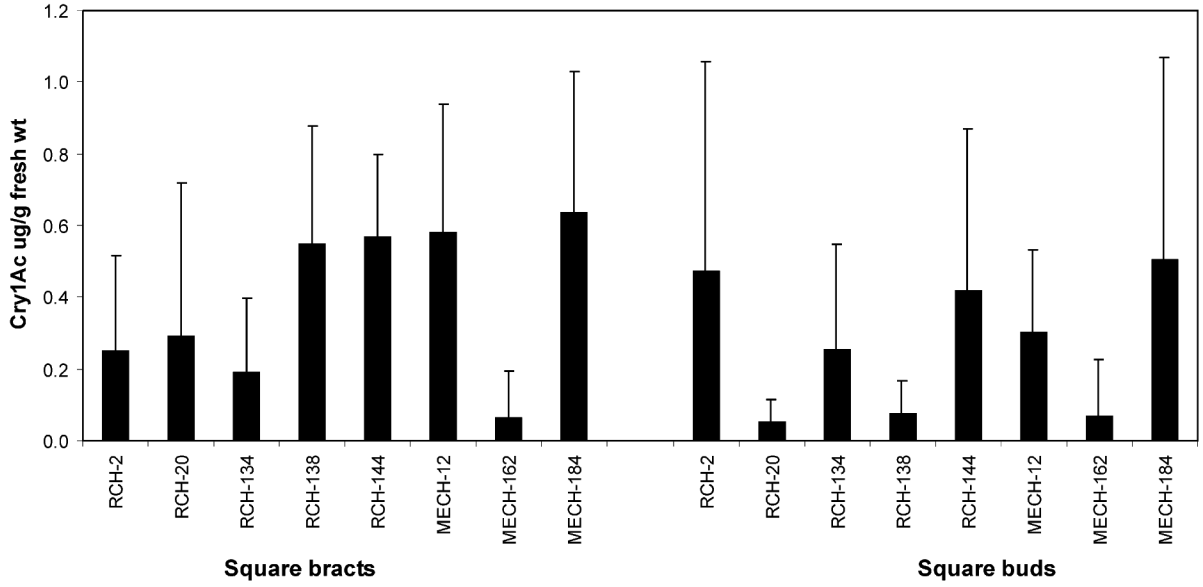

Figure 1. Cry1Ac expression (μg ± SD) in square parts.

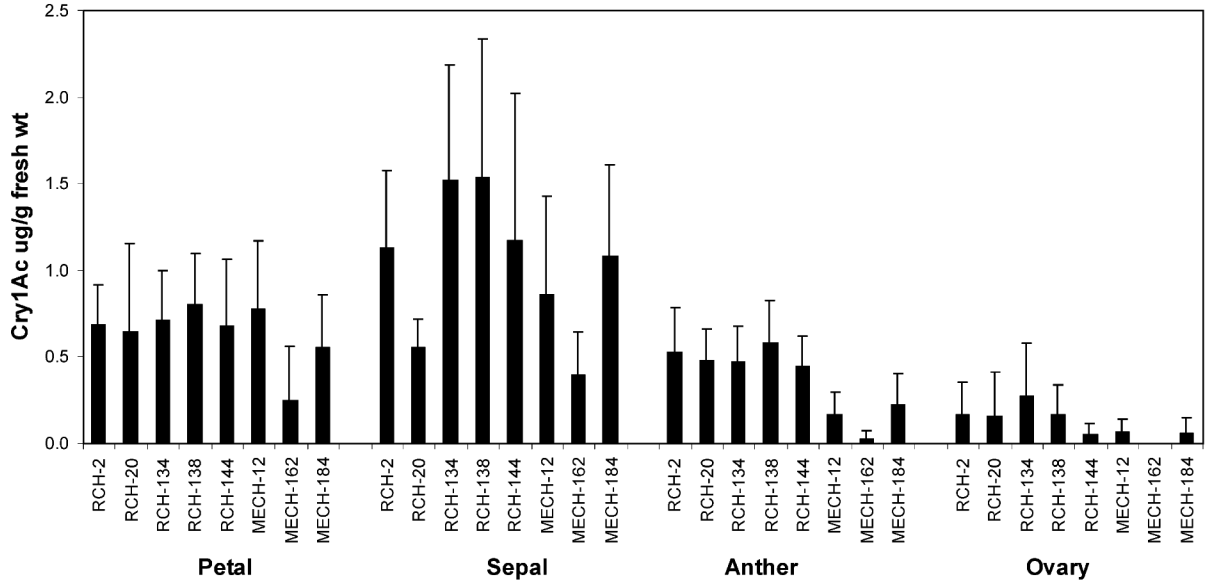

Figure 2. Cry1Ac expression (μg ± SD) in flower parts.

toxicity of the leaves to *H. armigera*. Mortality ranged from 90 to 100% on leaves of 30–96-day-old crops and 15–67% on leaves of 104–131-day-old crops. The control mortality ranged from 3.5 to 13.9% and 13.9 to 21.2% on leaves from 30 to 78- and 87 to 131-day-old corresponding non-*Bt* crops respectively. The temporal change in efficacy of upper canopy leaves of *Bt*-cotton on *H. armigera* larvae is presented as corrected percentage mortality in Figure 4 *a*. The toxicity of *Bt*-cotton leaves to *H. armigera* was correlated with the average Cry1Ac content of the leaves that were used in the bioassays (Figure 4 *b*). Correlation between percentage mortality and the Cry1Ac toxin was significant ($R^2 = 0.902$) at exponential correlation ($y = 0.0155e^{0.0525x}$). Mortality ranged from 10 to 50% at Cry1Ac levels of 0.01–0.2 μg/g, 70 to 100% at 0.3–1.8 μg/g and 100% at >1.9 μg/g.

Bioassays on fruiting parts

Bioassays carried out with fruiting parts from 100 to 107-day-old crops (Table 4) showed that there was significant variability in toxicity of different plant parts to *H. armigera*. The average mortality % ± (SD) was 27.5 ± 18.3 on

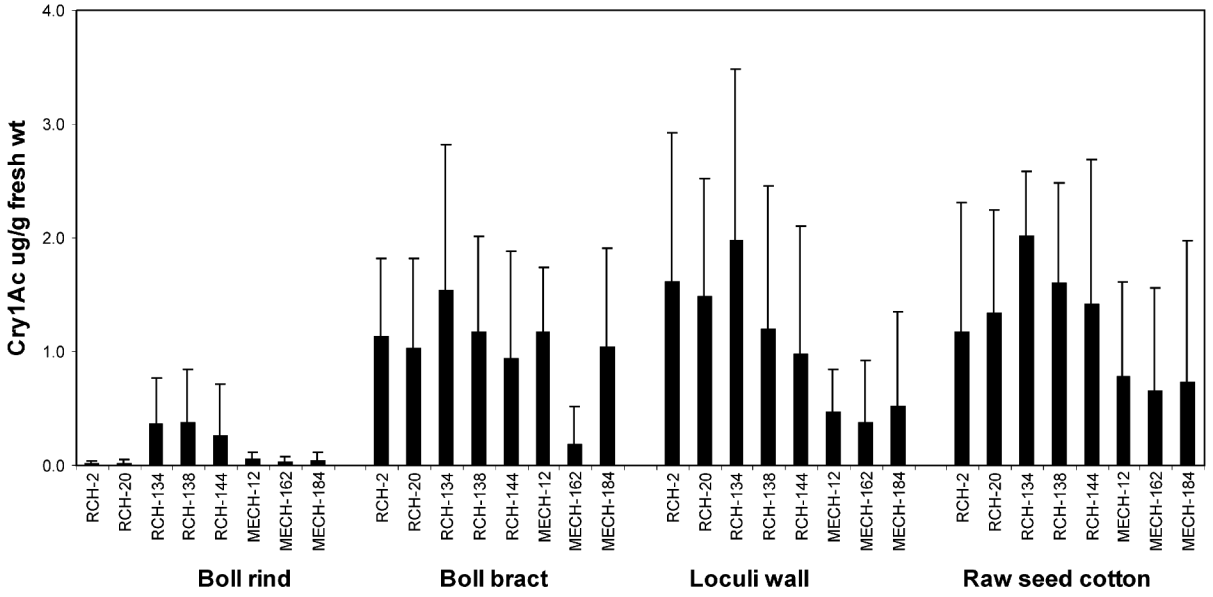

Figure 3. Cry1Ac expression (μg ± SD) in boll parts.

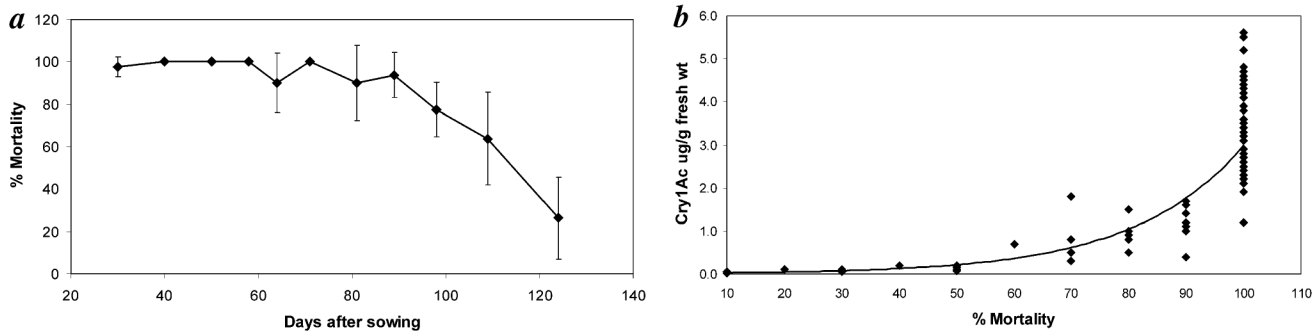

Figure 4. *a*, Mortality of *H. armigera* in bioassays with upper canopy leaves. *b*, Correlation of Cry1Ac expression in upper canopy leaves with *H. armigera* mortality.

squares, 12.5 ± 10.3 on bolls and 6.25 ± 9.2 on flowers. However, the surviving larvae on all the plant parts were stunted with a weight reduction of 48.8–98% compared to growth of the larvae on the counterpart non-*Bt* plant parts.

Discussion

Sustainable expression of Cry1Ac in *Bt*-cotton is crucial for its effectiveness in the control of lepidopteran pests, especially bollworms. This article describes intra-plant and in-seasonal variability in Cry1Ac expression in *Bt*-cotton hybrids in India. More importantly, the results clearly show some hitherto unreported findings that Cry1Ac expression levels were the lowest in the ovary of flowers and boll rind of green bolls, which constitute the most favoured sites of bollworm attack. The study makes a systematic attempt to correlate the Cry1Ac expression in *Bt*-cotton tissues with *H. armigera* mortality, thus identifying a critical expression level for Cry1Ac at 1.9 μg/g in tissues, below which *H. armigera* would be able to survive. From a practical standpoint it helps in understanding farmer complaints related to bollworm survival on *Bt*-cotton plants. For example, data presented here showing that the survival on fruiting parts was >40% on squares, >70% on green bolls and >80% on flowers, clearly help in explaining the differential rate of *H. armigera* survival on fruiting parts in *Bt*-cotton fields in some parts of India, especially under conditions of high pest pressure. The results also help in understanding the late season survival of bollworms on *Bt*-cotton. The results highlight the following: (i) The expression of Cry1Ac in *Bt*-cotton is 2 to 7-fold variable among hybrids. (ii) The Cry1Ac expression declines progressively over the crop growth with toxin levels falling below the critical level of 1.9 μg/g after 110 DAS. Thus despite the variability in toxin expression, the pest control properties are unlikely to be affected significantly at least until the crop becomes 100–115 days old.

Table 4. Survival of *H. armigera* in bioassays on fruiting parts of 100–107-day-old Bollgard cotton hybrids

	Bt-cotton		Non-*Bt*-cotton		Effects of *Bt*-cotton	
	Dead d/10	Wt of surviving larvae mg ± SD	Dead d/10	Wt of surviving larvae mg ± SD	Corrected mortality* %	Larval wt reduction %
Squares						
RCH-2	3	2.6 ± 1.3	0	24.8 ± 12.4	30	89.5
RCH-20	3	3.3 ± 2.9	0	31.1 ± 31.3	30	89.4
RCH-134	3	2.3 ± 1.0	1	16.1 ± 14.5	20	85.5
RCH-138	7	1.0 ± 0.0	1	14.5 ± 8.4	60	93.1
RCH-144	2	3.6 ± 1.4	1	43.7 ± 21.9	10	91.8
MECH-12	3	2.2 ± 1.2	0	22.8 ± 16.8	30	90.4
MECH-162	0	10.4 ± 10.7	0	50.8 ± 30.9	0	79.5
MECH-184	5	1.5 ± 0.7	1	85.1 ± 47.7	40	98.2
Green bolls						
RCH-2	1	35.3 ± 12.6	0	118.5 ± 37.2	10	70.2
RCH-20	2	23.3 ± 22.8	1	64.0 ± 52.2	10	63.6
RCH-134	3	13.3 ± 6.6	1	99.5 ± 41.2	20	86.6
RCH-138	3	21.3 ± 13.9	0	53.0 ± 23.3	30	59.8
RCH-144	3	43.8 ± 26.8	1	100.0 ± 32.9	20	56.2
MECH-12	1	38.8 ± 10.7	0	104.0 ± 28.8	10	62.7
MECH-162	0	71.0 ± 44.2	0	138.7 ± 49.2	0	48.8
MECH-184	0	55.5 ± 38.8	0	153.8 ± 23.4	0	63.9
Flowers						
RCH-2	0	10.5 ± 1.1	0	87.0 ± 2.8	0	87.9
RCH-20	0	16.0 ± 8.5	0	95.5 ± 3.5	0	83.2
RCH-134	2	12.5 ± 2.1	0	101.0 ± 21.2	20	87.6
RCH-138	2	14.5 ± 4.9	0	43.0 ± 19.8	20	66.3
RCH-144	0	8.0 ± 0.0	0	101.0 ± 7.1	0	92.1
MECH-12	1	11.6 ± 2.1	0	98.4 ± 4.8	10	88.2
MECH-162	0	29.0 ± 15.5	0	134.0 ± 14.1	0	78.4
MECH-184	0	32.0 ± 4.2	0	68.5 ± 3.5	0	53.3

*Derived from % mortality on *Bt*-cotton – % mortality on non-*Bt*-cotton.

(iii) Expression of Cry1Ac was found to be highly variable in different plant parts. The leaves of *Bt*-cotton plants, especially from seedlings, were found to have the highest levels of Cry1Ac expression followed by squares, bolls and flowers. Lowest levels of expression were found in the ovary and boll rind.

There were clear differences among the eight hybrids in terms of their quantitative levels of Cry1Ac and the seasonal decline in expression, despite having a common gene insertion event called 'Monsanto-531' from the same transgenic donor parent[1], Coker-312. It is interesting that Adamczyk *et al.*[8] found that there were no significant differences in Cry1Ac expression among the 11 varieties tested by them. We do not know if the 2 to 7-fold variability of Cry1Ac expression among hybrids, as observed in our data, is due to the hemizygous condition of the *cry1Ac* gene in Indian *Bt*-cotton hybrids, a situation that is unique to India thus far. Considering the geographical difference and also the high amount of intra-plant and in-season variability in Cry1Ac expression in *Bt*-cotton plants, it would be difficult to precisely compare the expression estimates among the commercial hybrids (*cry1Ac* gene in a hemizygous form) grown in India, and varieties (*cry1Ac* gene in a homozygous form) cultivated elsewhere in the world. However, a global analysis on the comparative performance of *Bt*-cotton varieties and hybrids against bollworms indicates that *Bt*-cotton varieties appear to be more effective in controlling the *Helicoverpa* species compared to the hybrids being grown in India. Evidence indicates that *Bt* transgenic cotton varieties, including NuCOTN 33B, which express Cry1Ac (event 531) in a homozygous form were found to cause 75–90% mortality in the cotton bollworm, *Helicoverpa zea* in USA[14]; >90% *H. armigera* larval control under field conditions in China[15], and 80–90% mortality of *H. armigera* and *Helicoverpa punctigera* (Wallengren) in Australia[2–5]. In bioassays with NuCOTN 33B plant parts, it was found that squares containing Cry1Ac caused 74% mortality in *H. zea*[8]. In contrast, the *Bt*-cotton hybrids in India do not appear to give such high levels of *H. armigera* control. In a recent study conducted in Central India, Bambawale *et al.*[16] reported a 50% overall reduction in *H. armigera* larval population in Bollgard-MECH-162 compared to the non-*Bt* MECH-162. Their data showed that the total per cent damage to fruiting

bodies, including squares and flowers, green bolls and shed reproductive parts was 65% lower in Bollgard-MECH-162 compared to non-*Bt* MECH-162. Further, the locule damage caused by pink bollworm was found to be 58% lesser in *Bt*-cotton. Udikere *et al.*[17] also showed that the three *Bt*-cotton hybrids, Bollgard-MECH-12, Bollgard-MECH-162 and Bollgard-MECH-184 were able to reduce larval populations of *H. armigera* up to 40%, spotted bollworm (*Earias vittella*) up to 30–40% and pink bollworm (*Pectinophora gossypiella*) up to 60–80% in South India. Their findings are in consonance with our data, which showed that the commercial *Bt*-cotton hybrids in India express less than the critical levels of Cry1Ac required for full protection against bollworms late in the season and also in some plant parts such as boll rind, square bract, bud and flower, which are the main feeding sites of bollworm larvae. Moreover, bolls in *Bt*-cotton F-1 hybrid plants contain segregating seeds, among which only an estimated 75% would express Cry1Ac. Since seeds form the most preferred food source of all the three bollworms, at least 25% of seeds in bolls of a *Bt*-cotton hybrid field, could support susceptible bollworm populations, if infested. Thus, the data available support the presumption that *Bt*-cotton hybrids in India may require more supplemental insecticide sprays than being used on *Bt*-cotton varieties elsewhere in the world. Recent reports[18,19] showed that though yields increased substantially by adopting *Bt*-cotton, farmers in India were able to reduce only up to 2.5 insecticide applications. The most relevant comparison would be with China, Australia, South Africa and Indonesia, which grow *Bt*-cotton varieties in contrast to *Bt*-cotton hybrids in India, but encounter the same target pest, *H. armigera*. Insecticide applications on *Bt*-cotton varieties were reduced up to 14 applications in China[20], 7 in South Africa[21] and 5–6 in Indonesia[21] and Australia[22]. Hence, the Indian farmer would have to be mentally prepared for the possibility of extra supplemental insecticide applications for bollworm control on *Bt*-cotton hybrids. We recommend periodic scouting at weekly intervals during the fruiting phase of the crop, with specific emphasis on locating larvae in fruiting parts.

Out of the eight hybrids tested, Bollgard-MECH-162 is the only one with late maturing and long duration (180–200 days) characteristics. Bollgard-RCH-2 and Bollgard-RCH-20 are of medium duration (160–180 days), while the rest are early maturing short (140–160 days) duration hybrids. The results suggest that the decline in Cry1Ac expression is more rapid in medium-to-long duration hybrids, as was evident with Bollgard-MECH-162, Bollgard-RCH-2 and Bollgard-RCH-20. However, farmers, especially in South and Central India, prefer these hybrids for their big boll size and superior fibre properties. Due to the rapid decline of Cry1Ac expression in these hybrids, it is recommended that 5% NSKE (neem seed kernel extract) followed by HaNPV (*Helicoverpa armigera* nuclear polyhedrosis virus) may be sprayed by 90–100 DAS on Bollgard-MECH-162, Bollgard-RCH-2 and Bollgard-RCH-20 and by 100–110 DAS for rest of the hybrids. Biopesticides are particularly useful to manage younger larvae of the initial bollworm infestation. Subsequently, any of the conventional insecticides such as endosulfan, thiodicarb, quinalphos and chlorpyriphos, or new molecules such as spinosad, emamectin benzoate, novaluron or Indoxacarb can be used at economic threshold levels of one larva per plant. In general, *H. armigera* is still susceptible to these insecticides[23].

It is worth noting that *Helicoverpa* species are at least ten-fold more tolerant to the Cry1Ac protein compared to the tobacco budworm, *Heliothis virescens*, which is the major pest of cotton in USA[22]. *Bt*-cotton varieties in USA cause 99–100% mortality in susceptible *H. virescens*[14]. It is relevant to mention here that *H. virescens* also feeds on leaves apart from the fruiting parts. In contrast, *H. armigera*, which is the major target pest of *Bt*-cotton in India, China and Australia, is primarily a bollworm and prefers feeding on fruiting parts and seldom on foliage. Thus the higher levels of expression in leaves are more advantageous to *Bt*-cotton in USA, where *H. virescens* is a major pest compared to those countries where *H. armigera* is the major pest on cotton. Therefore, biotechnology efforts in these countries, including India should focus on developing transgenic cotton varieties with tissue-specific promoters to enhance the expression of toxin genes in fruiting parts. The current results also point to the fact that the choice of parental background is crucial for sustainable expression of the *cry1Ac* transgene. Therefore, seed companies should evaluate their hybrids critically for highest levels of expression in fruiting parts and also for relatively effective level of toxin expression late in the season. Since the *Bt*-transgenic technology has thus far proven itself to be one of the most environment-friendly methods of bollworm management, it is in the interest of the technology itself that researchers, technology providers and administrators ensure that it must be provided to farmers in a form which gives the best possible returns for the investment.

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