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Effect of Pyramiding Bt and CpTI Genes on Resistance of Cotton to *Helicoverpa armigera* (Lepidoptera: Noctuidae) Under Laboratory and Field Conditions

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ABSTRACT Transgenic cotton (*Gossypium hirsutum* L.) varieties, adapted to China, have been bred that express two genes for resistance to insects. the Cry1Ac gene from *Bacillus thuringiensis* (Berliner) (Bt), and a trypsin inhibitor gene from cowpea (CpTI). Effectiveness of the double gene modification in conferring resistance to cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), was studied in laboratory and field experiments. In each experiment, performance of Bt+CpTI cotton was compared with Bt cotton and to a conventional nontransgenic variety. Larval survival was lower on both types of transgenic variety, compared with the conventional cotton. Survival of first-, second-, and third-stage larvae was lower on Bt+CpTI cotton than on Bt cotton. Plant structures differed in level of resistance, and these differences were similar on Bt and Bt+CpTI cotton. Likewise, seasonal trends in level of resistance in different plant structures were similar in Bt and Bt+CpTI cotton. Both types of transgenic cotton interfered with development of sixth-stage larvae to adults, and no offspring was produced by *H. armigera* that fed on Bt or Bt+CpTI cotton from the sixth stage onward. First-, second-, and third-stage larvae spent significantly less time feeding on transgenic cotton than on conventional cotton, and the reduction in feeding time was significantly greater on Bt+CpTI cotton than on Bt cotton. Food conversion efficiency was lower on transgenic varieties than on conventional cotton, but there was no significant difference between Bt and Bt+CpTI cotton. In 3-yr field experimentation, bollworm densities were greatly suppressed on transgenic as compared with conventional cotton, but no significant differences between Bt and Bt+CpTI cotton were found. Overall, the results from laboratory work indicate that introduction of the CpTI gene in Bt cotton raises some components of resistance in cotton against *H. armigera*, but enhanced control of *H. armigera* under field conditions, due to expression of the CpTI gene, was not demonstrated.

KEY WORDS cotton, *Bacillus thuringiensis*, cowpea trypsin inhibitor, resistance, *Helicoverpa armigera*

Transgenic resistance to insects, based on toxin genes derived from *Bacillus thuringiensis* (Berliner) (Bt), has become the mainstay of caterpillar control in corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) in the Americas, Asia, and Australia (Naranjo et al. 2008). However, there is concern regarding the narrow genetic basis of this host plant resistance and the potential vulnerability to resistance development in target pests (Tabashnik et al. 2003, 2008; Tabashnik 2008). There is a continued need for genes that can broaden the basis of plant resistance to insects.

Hilder at al. (1987) expressed a gene coding for cowpea trypsin inhibitor (CpTI) into tobacco (*Nicotiana* spp.) and demonstrated that this conferred resistance to tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae). CpTIs are enzymes that occur naturally in cowpeas, *Vigna unguiculata* L. They interfere with the activity of trypsin, which catalyzes the hydrolysis of proteins, and play a key role in food digestion in vertebrates as well as invertebrates. Trypsin appeared early in evolution and occurs in all phyla (Muhlia-Almazán et al. 2008). Thus, CpTIs should be expected to confer very broad-spectrum antiherbivore activity. Cowpeas, which contain CpTIs naturally, are used for human consumption after cooking, suggesting that the CpTI gene might be safely expressed in food crops (Boulter et al. 1989).

In the 1990s, Chinese scientists engineered the Bt Cry1Ac *gene* into locally adapted Chinese cotton varieties (Xie et al. 1991, Cui and Guo 1996). Later, they engineered the CpTI gene into varieties that already expressed the Cry1Ac gene (Li et al. 2000). To date, >50 transgenic varieties have been bred and released into production. These varieties demonstrated high resistance to the main cotton pest in China, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), commonly known as the cotton bollworm, greatly

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reducing pesticide usage to the benefit of farmers and the environment (Pray et al. 2001, Huang et al. 2002). The varieties currently on the Chinese market include both single gene (Cry1Ac) and double gene (Cry1Ac+CpTI) genotypes (Wu and Guo 2005).

Limited information is available on the effectiveness of the CpTI gene in making crops resistant to insects (Hilder and Boulter 1999), and in particular whether the resistance conferred by CpTI and Bt genes is additive. Han et al. (2006, 2007, 2008) demonstrated resistance of Cry1Ac+CpTI rice varieties to major pests, such as *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae), *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae), and *Sesamia inferens* (Walker) (Lepidoptera: Noctuidae). The contribution of the CpTI gene to the demonstrated resistance in Bt+CpTI rice is not clear, however, because the experiments did not include a single gene Bt variety. Sun et al. (2003) reported that in a field trial, the resistance of single gene Bt cotton and double gene cotton to *H. armigera* did not differ significantly during the second insect generation. However, during the third generation the bollworm density was suppressed more on the double gene variety than on the single gene Bt cotton, indicating that expression of the CpTI gene had raised the level of plant resistance as compared with the level of resistance expressed in the Bt variety. More studies are needed to substantiate this first indication.

The level of Bt toxin, and the associated resistance varies seasonally and between different plant parts (Xia et al. 2001, Kranthi et al. 2005, Wan et al. 2005, Kranthi 2006, Llewellyn et al. 2007). The level of resistance to insects in Bt varieties declines in the course of the season, with the leaves diminishing substantially in resistance level, whereas the reproductive structures remain comparatively well protected. No information has been published on the seasonal and within plant variation in resistance to insects in Bt+CpTI cotton.

Herbivore mortality is an important indicator for host plant resistance. More subtle indicators for resistance include sublethal effects, such as a lower efficiency of food use (Prütz and Dettner 2005), modified larval behavior on resistant plant genotypes (Zhang et al. 2004, Yang et al. 2008), and increased duration of development. Effects on reproduction are also important. Ultimately, resistance, or the lack thereof, is expressed at the level of the population, and the final proof of resistance to insects in field crops must be given in the field. We have striven to obtain a broad set of indicators to characterize the effect of Bt and CpTI genes on resistance of cotton to *H. armigera.*

Here, we report on laboratory and field studies in which resistance to cotton bollworm of two transgenic lines, one Bt and a second Bt+CpTI, was compared with that of a nontransgenic variety. In the laboratory, we quantified bionomic parameters—survival, development, and growth—when larvae were fed on different plant structures at different times of the season. Development of sixth-stage larvae into pupae and adults is described quantitatively, and an analysis is made of the efficiency of food use in fifth-stage larvae. In three field experiments, we quantified population dynamics of cotton bollworm on three cotton varieties, a Bt cotton, a Bt+CpTI cotton, and a conventional variety. The null hypothesis for this work is that CpTI does not raise the level of resistance of Bt cotton to *H. armigera,* whereas the alternate hypothesis is that presence of the CpTI gene enhances resistance as compared with the Bt variety.

Materials and Methods

Cotton Genotypes. Cotton varieties CRI23 (conventional), CRI32 (Bt cotton; Cry1Ac), CRI41 (Cry1Ac +CpTI cotton), and CRI44 (Cry1Ac cotton) were bred at the China Cotton Research Institute, Chinese Academy of Agricultural Sciences, Anyang, Henan, China. The transgenic variety CRI32 was generated from nontransgenic CRI17. The transgenic variety CRI41 was generated by importing the plant express carrier pGBI121S4ABC into CRI23, by using pollen tube channels injection (Guo et al. 2002). The construct pGBI121S4ABC includes an artificial GFM Cry1Ac gene and a modified CpTI gene (Guo et al. 1999). Laboratory trials were conducted with varieties CRI23, CRI32, and CRI41. Field trials were done with varieties CRI23, CRI32, and CRI41 in 2002 and with CRI23, CRI44, and CRI41 in 2007 and 2008.

Insects. *H. armigera* were reared on artificial diet (Shen and Wu 1995: 93) in the laboratory. The culture was started from late instars collected in conventional cotton fields near the China Cotton Research Institute (Anyang, Henan) in autumn 1999. The collected larvae were overwintered as pupae, and the first generation of offspring were used in laboratory experiments during summer 2000.

Survival of the Six Larval Stages of *H. armigera* on Leaves and Reproductive Structures of Bt Cotton and Bt+CpTI Cotton. Survival of all six larval instars of *H. armigera* was determined on six plant structures, collected from cotton varieties CRI23, CRI32, and CRI41 from May to September 2000. During collection of the plant material in the field, the plant structures were operationally defined as follows: 1) leaves: newly full-grown leaves; 2) bracts: supporting bracts of medium-aged squares (the flower bud is called a square); 3) squares: flower buds without supporting bracts; 4) petals: petals of open flowers (the flowers are open for only 1 d); 5) flower bases: all parts of the flower except bracts and petals; and 6) bolls: the young fruit, without the supporting bracts. Each structure was tested at the peak of its relative abundance in the field. Thus, leaves were tested in May, squares and square-supporting bracts in June, flowers and flower petals in July, and bolls in August.

Newly molted larvae (0–24 h) of each of the six larval stages (L1–L6) were collected from the culture, and reared for 6 d on the collected plant structures. Three replicate batches of 150 (L1) or 30 insects (L2–L6) were used for measuring survival on each plant structure. Larvae were reared individually in 10- by 2-cm test tubes, except the L1s, which were kept with five per tube. Tests were conducted in an incubator (27 ± 0.5°C, 70–80%

RH, and a photoperiod of 14.10 [L:D] h). Survival was scored on day 6 of the test.

In initial analysis, means for each replicate were calculated and analyzed using pairwise *t*-tests. As pairwise *t*-tests on raw data yielded mostly insignificant effects, a meta-analysis was carried out after pooling data from replicates. For each of 108 ($6 \times 6 \times 3$) combinations of larvae stage (six levels), plant structure (six levels), and cotton genotype (three levels), the overall proportion surviving larvae, *s*, was calculated and transformed to logits, $\text{logit}(s) = \ln(s/1 - s)$, to stabilize variance and linearize responses to the three treatment factors. General linear models were fitted in SPSS version 17.0.3 (SPSS Inc., Chicago, IL), procedure general linear model–univariate, assuming normal variance. Plant structure and cotton genotype were included as fixed effects, whereas larval stage was include either as a fixed factor (six levels) or as a covariate coded as the vector [1 2 3 4 5 6] (1 df). Main effects and two-way interactions were included in the fitted analysis of variance (ANOVA) models, whereas the three-way interaction was included in the error main square.

Seasonal Trends in Survival of First-Stage *H. armigera* Larvae on Leaves and Reproductive Structures of Bt Cotton and Bt+CpTI Cotton. From May to September 2000, leaves and reproductive structures were collected monthly from conventional cotton CRI23, Bt cotton CRI32, and Bt+CpTI cotton CRI41, to determine seasonal trends in survival of neonate *H. armigera* larvae. Trials were conducted between the 10th and 20th day of each month. Neonate larvae (0–24 h old) were collected from the culture and fed in groups of five in test tubes (10 by 2 cm) with ad libitum food of one of the following kinds: 1) cotton leaves, 2) bracts, 3) squares, 4) petals, 5) flower bases, and 6) bolls. Larvae were kept in an incubator (27 ± 0.5°C, 70–80% RH, and a photoperiod of 14:10 [L:D] h). Survival was scored on day 6 of the test.

Logits of survival were calculated for each replicate and analyzed with regression by using procedure general linear model–univariate in SPSS. Variety (three levels), plant structure (six levels) and month of the year (five levels) were used as fixed factors in the regression model.

Behavior of First-, Second-, and Third-Stage *H. armigera* Larvae on Bt Cotton and Bt+CpTI Cotton. Cotton plants of CRI23, CRI32, and CRI42 were grown in pots in the glasshouse. When the plants were 25 cm tall, one first-, second-, or third-stage larva was released on each plant, and observations were made every 15 min during 6 h of the larva's behavior: feeding, walking, hanging on a thread (spinning down), or resting on the plant. Observations were made on 10 larvae of each instar per trial, and the trial was replicated three times.

Results were analyzed with a full factorial three-way ANOVA, by using cotton variety and instar as fixed factors, and replicates as random factor. A suitable transformation of the data was chosen to stabilize variance, by using Levene's test and residual plots as criteria. Times feeding, walking, and resting were converted to proportions of the total time and then transformed using the arc tangent of the square root. Time spinning down was log transformed. Insignificant interactions were omitted to obtain the final model.

Effect of Bt Cotton and Bt+CpTI Cotton on Growth and Development of Sixth-Stage *H. armigera* Larvae. Growth and development into adults of sixth-stage larvae, when fed different plant structures, was studied in July 2000. Newly molted 0–24-h-old sixth-stage larvae were individually reared in 12- by 3.5-cm test tubes in a climate cabinet, until the adult molt. We determined the pupation ratio (pupae/initial number of sixth-stage larvae), average duration of the pupal stage, fledging ratio (adults/initial number of pupae), and the average life span of adults. The sixth-stage larvae were weighed during the second day, and pupae on the third day of the pupal stage. The experiment was replicated three times, with 10 larvae for each cotton variety per replicate.

Results were analyzed with a full factorial three-way ANOVA, by using cotton variety and plant structure as fixed factors, and replicates as random factor. Insignificant interactions were omitted to obtain the final model. Because Levene's test and plots of residuals did not point to departures from the ANOVA assumptions of normality and homoscedasticity, untransformed data were used in the analysis.

Effect of Bt Cotton and Bt+CpTI Cotton on Food Use by Sixth-Stage *H. armigera* Larvae. Fifth-stage larvae were used to determine the effect of Bt cotton and Bt+CpTI cotton on larval growth rate and the efficiency of food use. Newly molted 0–24-h-old sixth-stage larvae were fed during 48 h on six plants structures of three cotton types (CRI23, CRI32, and CRI42) in the laboratory. Before and after the trial the larvae were weighed. Food was refreshed at 24 h. Fresh weights of the eaten leaf material and produced feces were determined after 24 and 48 h. Five indices were calculated: approximate digestibility (AD, as percentage), food conversion efficiency (ECI, as percentage), relative consumption rate (RCR, as milligrams per milligram per day), relative metabolization rate. (RMR, as milligrams per milligram per day), and relative growth rate (RGR, as milligrams per milligram per day) (Waldbauer 1968, Tang et al. 1996):

$$\text{AD} = (\text{food intake} - \text{weight of excrements})/(\text{food intake}) \times 100\%$$

$$\text{ECI} = (\text{weight increase})/(\text{food intake}) \times 100\%$$

$$\text{RCR} = (\text{food intake})/(\text{average weight} \times \text{days})$$

$$\text{RMR} = (\text{food intake} - \text{weight of excrements} - \text{weight increase})/(\text{average weight} \times \text{days})$$

$$\text{RGR} = (\text{weight increase})/(\text{average weight} \times \text{days})$$

Allowance was made for transpirational weight loss of leaves. Three replicates were made, with 20 larvae for each cotton variety per replicate. Data were analyzed with three-way ANOVA, by using genotype (three levels) and plant structure (six levels) as fixed factor and replicate (three levels) as random factor. Homoscedasticity of the data were checked using residual and spread versus level plots.

Population Dynamics of *H. armigera* on Bt Cotton and Bt+CpTI Cotton in the Field. In 3 yr (2002, 2007, and 2008), field plots of ≈0.7 ha were laid out in cotton fields near the China Cotton Research Institute, Anyang, Henan, China (36° 03′ N and 114° 29′ E). In each year, all observations were made in a single field, which was subdivided into three plots, one for each cotton variety. The large size of the plots minimizes inter plot interference, but the lack of replication at the plot level means that treatment effects in any 1 yr may be confounded with site effects. Replication over years is therefore necessary. In each year, observations were made every 5 d of the number of bollworm eggs and larvae on 150 plants per plot. No pesticides were used.

For statistical analysis, the total count of eggs and larvae was determined per plot in each year. A two-way ANOVA was conducted, by using year and cotton genotype as fixed factors and the interaction for estimating mean square error. Counts were square root transformed to stabilize variance.

Results

Survival of the Six Larval Stages of *H. armigera* on Leaves and Reproductive Structures of Bt Cotton and Bt+CpTI Cotton. Survival was lowest on leaves (36% average over all varieties and stages tested), highest on bolls (58%), and intermediate on the other structures (44–50%). Survival was much lower on transgenic varieties than on the conventional cotton (Fig. 1). Averaged over all larval stages and plant structures, survival was 80% per stage on conventional cotton, 40% on Bt cotton, and 34% on Bt+CpTI cotton. Average survival across plant structures was 2.8, 1.9, 2.3, and 1.2 times greater on Bt cotton than on Bt+CpTI cotton in the L1–L4 stages, respectively. Survival on the two transgenics was similar in the L5 and L6, however. Across all larval stages, the difference was a factor 1.6 (geometric mean) higher survival per stage on Bt cotton, compared with Bt+CpTI cotton.

Three-way ANOVA of these survival data resulted in highly significant main effects of larval stage ($F_{5,50} = 91.8$; $P < 0.001$), plant structure ($F_{5,50} = 7.6$; $P < 0.001$), and cotton genotype ($F_{2,50} = 195$; $P < 0.001$) as well as significant two-way interactions: larval stage × plant structure ($F_{25,50} = 1.86$; $P = 0.03$), larval stage × cotton genotype ($F_{10,50} = 4.02$; $P < 0.001$), and plant structure × cotton genotype ($F_{10,50} = 3.38$; $P = 0.002$). Thus, larval survival was affected by cotton genotype, by the plant structure fed, and it differed among stages, with all three factors interacting. Focusing on main effects, marginal mean logit survival was 1.728 ± 0.155 on the conventional variety, −0.746 ± 0.155 on the Bt cotton, and −1.319 ± 0.155 on the Bt+CpTI cotton (SED = 0.22; least significant difference $[LSD]_{5\%} = 0.44$), translating to 85, 32, and 21% survival, with clear separation according to LSD at the 5% level. The marginal mean logit survival for the six larval stages was −2.08 ± 0.22 in L1, −1.51 ± 0.22 in L2, −0.49 ± 0.22 in L3, 0.17 ± 0.22 in L4, 1.24 ± 0.22 in L5, and 2.01 ± 0.22 in L6 (SED = 0.31; $LSD_{5\%} = 0.62$), translating to 11, 18, 38, 54, 77, and 88% survival in the six larval stages, respectively, with significant separation in mortality between stages, except between the L1 and L2. The marginal mean logit survival in ANOVA was −0.85 ± 0.22 on leaves, −0.29 ± 0.22 on petals, −0.11 ± 0.22 on bracts, −0.05 ± 0.22 on squares, 0.11 on flower bases, and 0.52 ± 0.22 on bolls (SED = 0.31; $LSD_{5\%} = 0.62$), translating to 30, 43, 47, 49, 53, and 63% survival on these six structures, respectively, with significant separation at 5% level between leaves and any structure except petals, and between bolls on the one hand, and leaves, petals or bracts at the other hand. If larval stage was entered into the general linear model as a covariate, coded as the vector [1 2 3 4 5 6], in interaction with the variety, the slopes for the effect of stage on logit survival was estimated as 0.56 ± 0.075 on conventional cotton, 0.86 ± 0.075 on Bt cotton, and 1.10 ± 0.075 on Bt+CpTI cotton (SED = 0.11; $LSD_{5\%} = 0.22$), with significant pairwise differences between the varieties. These regression coefficients indicate that the odds of survival increase with a factor $\exp(0.56) = 1.7$ per stage on conventional cotton, with a factor $\exp(0.86) = 2.4$ per stage on Bt cotton and with a factor $\exp(1.10) = 3.0$ on Bt+CpTI cotton. These regression results underscore the marked increase in survival with larval stage in *H. armigera*, especially on transgenic varieties.

Seasonal Trends in Survival of First-Stage *H. armigera* Larvae on Leaves and Reproductive Structures of Bt Cotton and Bt+CpTI Cotton. Bt cotton and Bt+CpTI cotton showed similar trends in the level of resistance to first-stage larvae through the season. Survival on leaves increased from 0% in May until ≈50% in August and September (Fig. 2). Three-way ANOVA resulted in highly significant main effects of plant structure ($F_{5,12} = 20.2$; $P < 0.001$), and month of the year ($F_{4,12} = 21.5$; $P < 0.001$), but there was no significant difference between Bt cotton and Bt + CpTI cotton ($F_{1,12} = 0.13$; N.S.). None of the two-way interactions were significant. plant structure × month of the year ($F_{12,12} = 2.1$; N.S.), plant structure × cotton genotype ($F_{5,12} = 1.8$; N.S.), and month of the year × cotton genotype ($F_{4,12} = 0.4$; N.S.). After dropping interactions from the models, the main effect of plant structure was characterized by marginal means of logit survival of −4.7 ± 0.33 for bracts, −4.5 ± 0.33 for squares, −4.2 ± 0.39 for petals, −3.4 ± 0.39 for bolls, −2.5 ± 0.39 for flower bases, and −1.9 ± 0.27 for leaves, corresponding to survival percentages of 1, 1, 1.5, 3, 8, and 15%, respectively. The order of resistance rating was therefore, from more to less resistant, bracts ≈ squares ≥ petals ≥ bolls ≥ flower bases ≥ leaves. The main effect of month of the year was characterized by marginal means of logit survival of −6.7 ± 0.67 for May, −4.1 ± 0.37 for June, −2.0 ± 0.25 for July, −2.6 ± 0.25 for August, and −2.2 ± 0.25 for September, corresponding to survival percentages of 0, 2, 13, 7, and 11%, respectively, and indicating greater resistance during May and June than in July, August, and September.

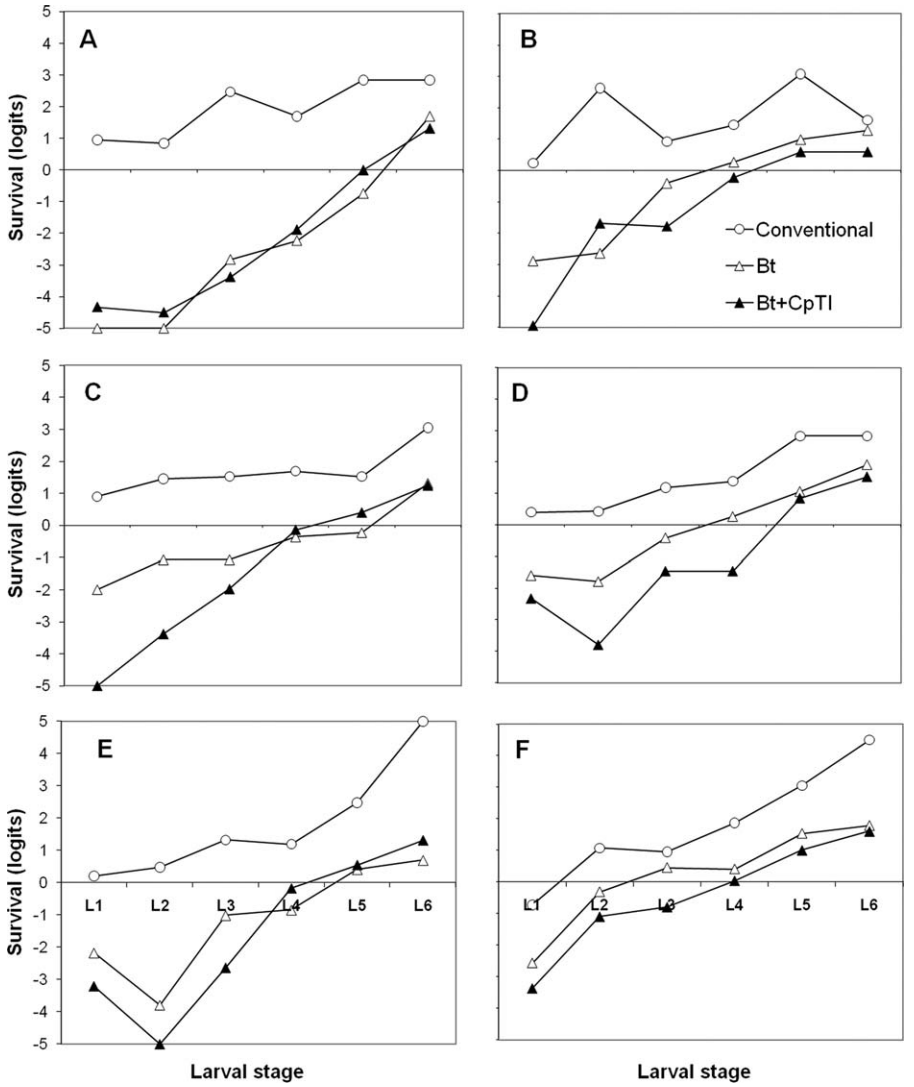

Fig. 1. Survival (logit transformed; y-axis) in six larval stages (L1–L6) (x-axis) of *H. armigera* on six different plant structures (panels) from (○) conventional cotton, (△) Bt cotton, and (▲) Bt+CpTI cotton. (A) Leaves (May). (B) Squares (June). (C) bracts (June). (D) Flower bases (July). (E) Flower petals (July). (F) Bolls (August). Logit values −5, −4, . . . , 4, 5 correspond to survival of 0.7, 1.8, 4.7, 11.9, 26.9, 50.0, 73.1, 88.1, 95.3, 98.2, and 99.3%. The logit scale stretches values near 0 and 100% survival, and thus brings out more clearly the relative differences in survival near the ends of the percentages scale (i.e., when survival is either very low or very high).

Behavior of First-, Second-, and Third-Stage *H. armigera* Larvae on Bt Cotton and Bt+CpTI Cotton. L1s, L2s, and L3s spent substantially more time feeding on conventional cotton than on Bt cotton, whereas the time spent feeding on Bt+CpTI cotton was further reduced compared with Bt cotton in each of the three instars tested (Fig. 3). ANOVA indicated significant effects of genotype ($F_{2,18} = 137$; $P < 0.001$), larval stage ($F_{2,18} = 14.0$; $P < 0.001$), and the genotype × stage interaction ($F_{4,18} = 8.7$; $P < 0.001$). The effect of replicate was not significant ($F_{2,3.18} = 0.11$; N.S.). Feeding times (marginal means from the ANOVA at angular scale) were 0.461 ± 0.013 on conventional cotton, 0.315 ± 0.013 on Bt cotton, and 0.147 ± 0.013 on Bt+CpTI cotton, with all three pairwise differences significant at $\alpha = 0.001$.

ANOVA of resting time showed a significant effect of genotype ($F_{2,18} = 25.8$; $P < 0.001$), a borderline significant effect of larval stage ($F_{2,18} = 3.0$. $P = 0.077$), and a significant interaction between genotype and stage ($F_{4,18} = 8.2$; $P < 0.001$). Replicate had no significant effect ($F_{2,1.53} = 0.33$; N.S.). Resting times (marginal means from the ANOVA at angular scale) were 0.611 ± 0.009 on conventional cotton, 0.613 ± 0.009 on Bt cotton, and 0.695 ± 0.009 on Bt+CpTI cotton, with a significant difference between Bt+CpTI cotton and the other two varieties at $\alpha = 0.001$. There was no significant difference in

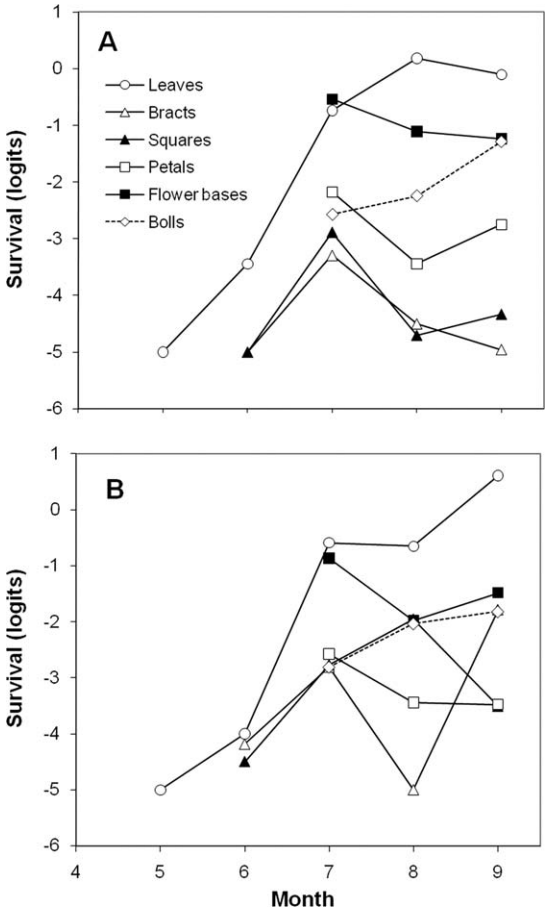

Fig. 2. Seasonal trends in survival of first-stage larvae of *H. armigera* on six plant structures of Bt cotton (A) and Bt+CpTI cotton (B).

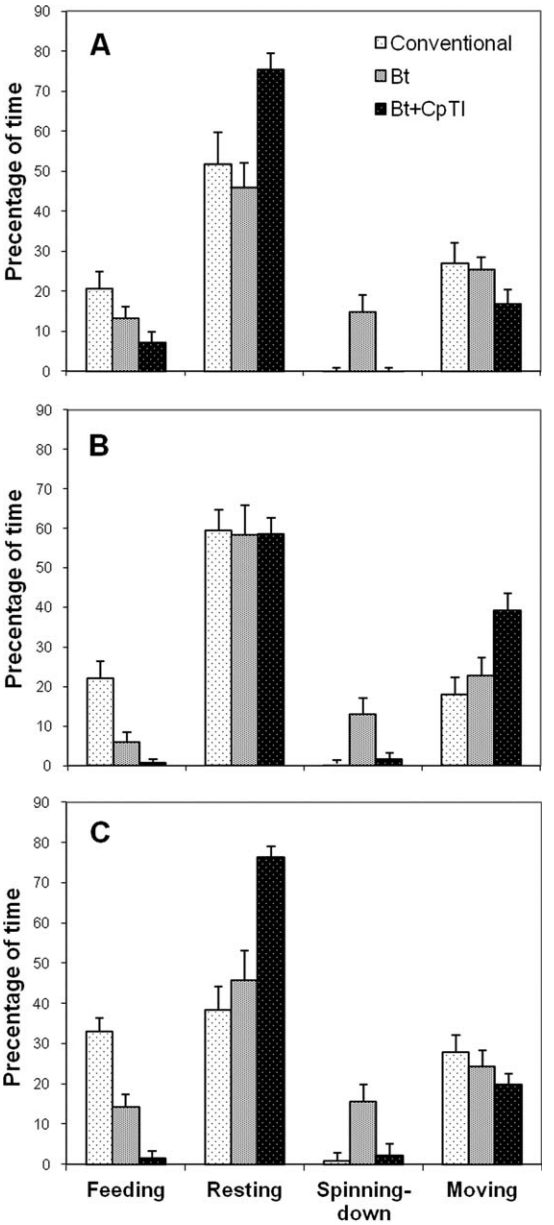

Fig. 3. Influence of Bt cotton and Bt+CpTI cotton on behavior of (A) first-, (B) second-, and (C) third-stage larvae of *H. armigera*. Behavior of a larva was classed each 15 min as feeding, resting, hanging from the plant on a silk thread (spinning down), or moving in 6-h-long observation sessions. Bars and whiskers indicate means ± SEM of three replicate trials, each involving 10 larvae of each stage.

resting time between Bt cotton and conventional cotton.

On Bt cotton, larvae were observed hanging from silks ≈10% of the time, a behavior indicative of host rejection, and rarely observed on the other two varieties. ANOVA showed significant effects of genotype ($F_{2,18} = 410$; $P < 0.001$), stage ($F_{2,18} = 44.9$; $P < 0.001$), and the genotype × stage interaction ($F_{4,18} = 16.4$; $P < 0.001$). Times spinning down (marginal means from ANOVA at logarithmic scale) were -0.368 ± 0.039 on conventional cotton, 1.148 ± 0.039 on Bt cotton, and -0.031 ± 0.039 on Bt+CpTI cotton, with highly significant pairwise differences between genotypes ($t_{18} = 27.1$; $P < 0.001$ for conventional versus Bt cotton; $t_{18} = 6$ for conventional versus Bt+CpTI cotton; and $t_{18} = 21.1$; $P < 0.001$ for Bt cotton versus Bt+CpTI cotton).

There were no significant main effects in the ANOVA of the time spent moving (genotype: $F_{2,18} = 0.007$; N.S.; and stage: $F_{2,18} = 1.09$; N.S.), but the interaction between genotype and stage was significant ($F_{4,18} = 15.2$; $P < 0.001$), indicating different genotype effects between stages as illustrated in Fig. 3. Overall, the data show behaviors indicating reduced feeding and host rejection both in Bt cotton and in Bt+CpTI cotton but more strongly in the latter.

Effect of Bt Cotton and Bt+CpTI Cotton on Growth and Development of Sixth-Stage *H. armigera* Larvae. Weight of L6 larvae on day 2 was significantly affected by the cotton variety ($F_{2,34} = 92.3$; $P < 0.001$), with largest weight on the conventional variety and

lower weight on both transgenics (Fig. 4A). Which of the two transgenics resulted in the greatest larval weight depended on the structure that was offered (significant interaction of variety and structure: $F_{10,34} = 9.5$; $P < 0.001$). Significantly greater weight was achieved on the squares and bolls of Bt cotton, compared with Bt+CpTI cotton, whereas the opposite difference was significant when the larvae were fed leaves, flowers, or petals (main effect $F_{5,34} = 9.1$; $P < 0.001$; pairwise *t*-tests at $\alpha = 0.05$; LSD = 33.4 g).

Duration of the sixth stage was generally shortest on the conventional cotton (Fig. 4B), and there was a significant main effect of variety ($F_{2,34} = 28.2$; $P < 0.001$) and structure ($F_{5,34} = 27.7$; $P < 0.001$) as well as a significant interaction ($F_{10,34} = 7.0$; $P < 0.001$). Averaged over plant structures, there was a significant separation ($LSD_{5\%} = 0.43$ d) between larval duration on conventional cotton (3.60 ± 0.105 d), Bt cotton (4.72 ± 0.105 d), and Bt+CpTI cotton (4.16 ± 0.105 d).

Pupation success was greatest (82.7 ± 1.6%) on the conventional cotton, with a significant overall difference with both Bt cotton (44.3 ± 1.6%) and Bt+CpTI cotton (43.9 ± 1.6%) ($F_{2,24} = 199$; $P < 0.001$). Differences between the cotton genotypes varied with plant structure (Fig. 4C) (interaction $F_{10,24} = 22.3$; $P < 0.001$), reflecting patterns seen in the weight of L6 (Fig. 4B).

Pupal weight varied in parallel with pupation success. Main effects of variety ($F_{2,34} = 120.6$; $P < 0.001$), plant structure ($F_{5,34} = 18.8$; $P < 0.001$), and the interaction ($F_{10,34} = 9{,}96$; $P < 0.001$) were significant. Greatest pupal weight (averaged over plant structures) was attained on conventional cotton (233 ± 3.5 g), compared with 161 ± 3.5 g on Bt cotton and 173 ± 3.5 g on Bt+CpTI cotton ($LSD_{5\%} = 10.1$ g). Differences in duration of the pupal stage were relatively small (Fig. 4E), but main effects of variety ($F_{2,34} = 9.7$; $P = 0.001$), plant structure ($F_{5,34} = 4.1$; $P = 0.005$), and the interaction ($F_{9,34} = 3.6$; $P = 0.003$) were significant.

There were large differences between cotton genotypes ($F_{2,32} = 151$; $P < 0.001$) in the success of the adult molt, which was highest when larvae had been fed on conventional cotton (average success rate across plant structures of 70 ± 1.7%) versus 42.4 ± 1.9% on Bt cotton and lower still, 28.9 ± 1.7%, on Bt+CpTI cotton. Plant structure ($F_{5,32} = 10.3$; $P < 0.001$) and the interaction between cotton variety and plant structure ($F_{9,32} = 13.4$; $P < 0.001$) had significant effects on molting success. The effects were similar to the pattern observed with weight of L6 larvae and pupae, and pupation success.

There was a significant difference in adult life span ($F_{2,32} = 13.3$; $P < 0.001$) when larvae had been feeding on conventional cotton (7.94 ± 0.20 d) compared with when they had been feeding on Bt cotton (8.57 ± 0.20 d) or Bt+CpTI cotton (8.86 ± 0.20 d) ($LSD_{5\%} = 0.57$ d). Adults from larvae that had been reared on conventional cotton were highly fecund. Most of the moths from larvae fed on transgenic cottons were deformed and had difficulty emerging from the pupal exuvium, but those that emerged successfully did not mate nor lay eggs.

Effect of Bt Cotton and Bt+CpTI Cotton on Food Use by Sixth-Instar *H. armigera* Larvae. Statistical analysis of parameters for food utilization efficiency showed significant effects of plant genotype and plant structure (Table 1). Food conversion efficiency of L6, averaged over plant structures, was 36% in the control, 21% on Bt cotton, and 16% on Bt+CpTI cotton, with significant pairwise differences between the control and both transgenic cottons in ANOVA (Table 1). There was no significant difference between the two transgenic varieties. Digestibility was 80% on conventional cotton, 54% on Bt cotton, and 61% on Bt+CpTI cotton, with significant pairwise differences between the control and the two transgenic varieties, but not between the two transgenic varieties. Consumption rate was reduced substantially on the two transgenic varieties, compared with the control, and the reduction was greater in Bt cotton than in Bt+CpTI cotton. The greater food intake in Bt+CpTI cotton compared with Bt cotton was offset by greater metabolic losses, characterized by a significant difference in metabolization rate between Bt+CpTI cotton and conventional or Bt cotton. As an end result, relative growth rate was substantially reduced on the two transgenic varieties, from 0.17 d^{-1} on conventional cotton, to 0.10 d^{-1} on Bt cotton, and 0.08 d^{-1} on Bt+CpTI cotton. The difference between conventional cotton and the two transgenics was significant, but the two transgenics did not differ significantly (Table 1). These data show that food intake and food conversion is reduced on transgenic cottons. Comparing the two transgenic varieties, larvae feeding on Bt cotton ate less but had lower metabolic losses, and the final growth rate on the two transgenic varieties was similar.

There were several differences between plant structures in parameters for food utilization efficiency (Table 1). The greatest overall growth rate, as an integrative parameter, was attained on leaves and bolls.

Population Dynamics of *H. armigera* on Bt Cotton and Bt+CpTI Cotton in the Field. There were three distinct generations of larvae in 2002 and 2007, and two in 2008. Densities of larvae were smaller on Bt cotton or Bt+CpTI cotton than on conventional cotton, but densities of eggs were similar among the three varieties (Fig. 5). Statistical analysis showed significant differences between years in the number of eggs ($F_{2,4} = 16$; $P = 0.012$), with 2007 having a significantly lower egg count than the other 2 yr in pairwise *t*-tests at $\alpha = 0.05$, but there was no difference in total egg count between varieties ($F_{2,4} = 2.1$; N.S.). The number of larvae did not differ between years ($F_{2,4} = 0.24$; N.S.), but there was a significant difference in total larvae count between varieties ($F_{2,4} = 64$; $P = 0.001$). This genotype effect is entirely due to the difference between conventional cotton and the two transgenic varieties (significant at $\alpha = 0.05$ in pairwise *t*-tests). There was no significant difference between the two transgenic varieties in a pairwise *t*-test at $\alpha = 0.05$. The number of larvae counted per 150 plants, and totaled

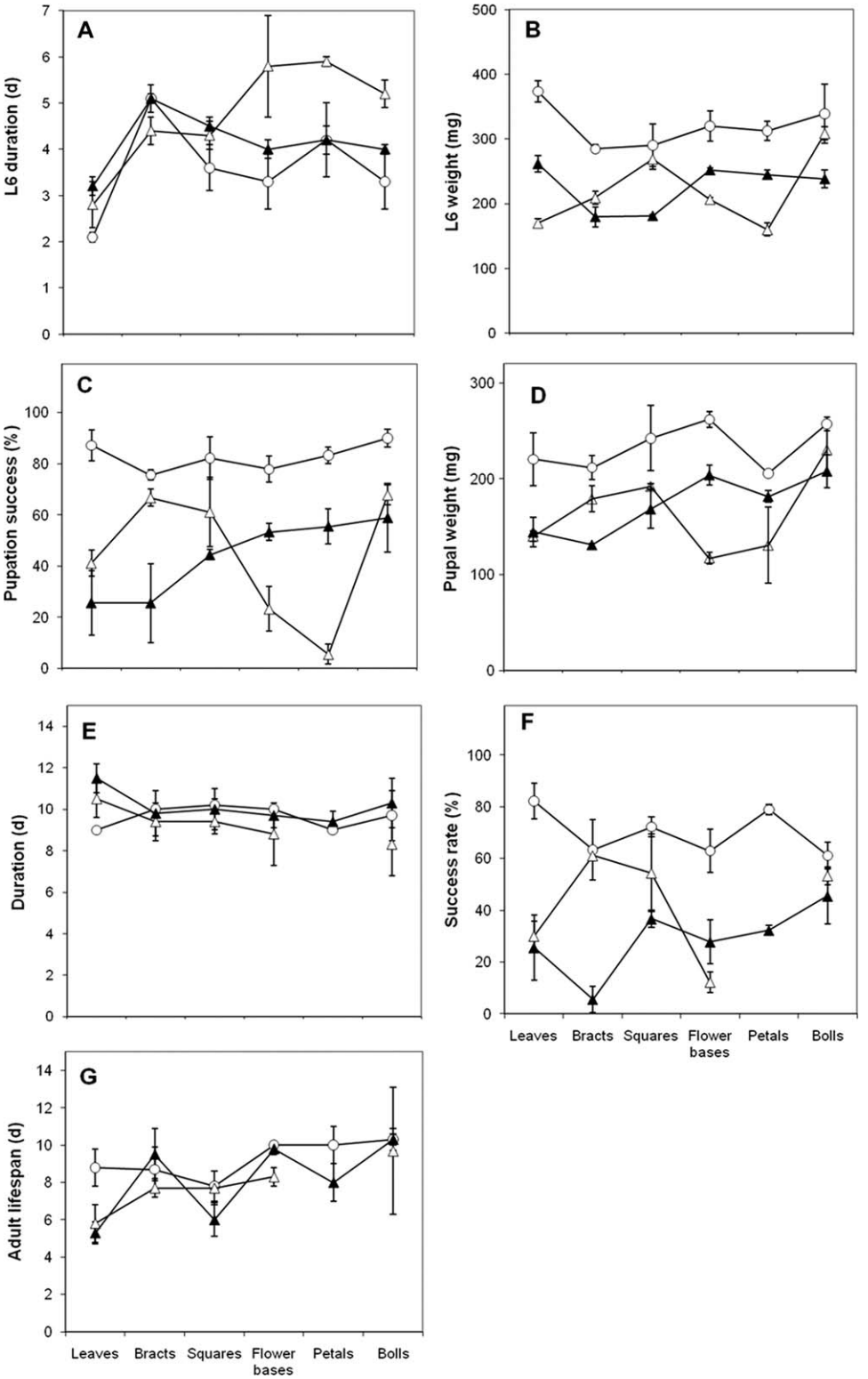

Fig. 4. Effects of Bt cotton and Bt+CpTI cotton on growth and development of sixth-stage larvae (L6) into adults. (A) Weight of L6. (B) Duration of L6 stage. (C) Pupation success. (D) Pupal weight. (E) Duration of pupal stage. (F) Success rate of adult molt. (G) Adult life span.

Table 1. Indices for efficiency of food utilization of *H. armigera* fifth-stage larvae, feeding on six different structures of conventional, Bt and Bt+CpTI cotton plants

Parameter	Digestibility (AD), %	Conversion efficiency (ECI), %	Consumption rate (RCR), g/g/d	Metabolization rate (RMR), g/g/d	Growth rate (RGR), g/g/d
Treatment effects					
Genotype	$F_{2,36} = 26.2$; $P < 0.001$	$F_{2,36} = 39.9$; $P < 0.001$	$F_{2,36} = 37.8$; $P < 0.001$	$F_{2,36} = 18.6$; $P < 0.001$	$F_{2,36} = 15.3$; $P < 0.001$
Structure	$F_{5,36} = 5.9$; $P < 0.001$	$F_{5,36} = 16.7$; $P < 0.001$	$F_{5,36} = 21.6$; $P < 0.001$	$F_{5,36} = 25.9$; $P < 0.001$	$F_{5,36} = 7.0$; $P < 0.001$
Interaction	$F_{10,36} = 1.5$; N.S.	$F_{10,36} = 6.2$; $P < 0.001$	$F_{10,36} = 3.4$; $P = 0.003$	$F_{10,36} = 3.7$; $P = 0.002$	$F_{10,36} = 3.8$; $P = 0.001$
Marginal means					
Genotype					
Conventional	80.4 ± 2.7a	36.3 ± 2.4a	0.60 ± 0.04a	0.16 ± 0.03b	0.166 ± 0.012a
Bt	54.0 ± 2.7b	20.5 ± 2.4b	0.25 ± 0.04c	0.16 ± 0.03b	0.099 ± 0.012b
Bt+CpTI	61.5 ± 2.7b	15.5 ± 2.4b	0.40 ± 0.04b	0.32 ± 0.03a	0.079 ± 0.012b
Structure					
Leaves	74.7 ± 3.8a	40.5 ± 2.4a	0.32 ± 0.04b	0.11 ± 0.03c	0.168 ± 0.016a
Bracts	72.1 ± 3.8a	12.0 ± 2.4e	0.66 ± 0.04a	0.48 ± 0.03a	0.102 ± 0.016b
Squares	48.9 ± 3.8c	18.1 ± 2.4de	0.36 ± 0.04b	0.10 ± 0.03c	0.091 ± 0.016b
Petals	62.0 ± 3.8ab	25.8 ± 2.4bc	0.19 ± 0.04c	0.07 ± 0.03c	0.062 ± 0.016b
Flower bases	62.0 ± 3.8b	28.4 ± 2.4b	0.36 ± 0.04b	0.23 ± 0.03b	0.098 ± 0.016b
Bolls	67.4 ± 3.8ab	19.8 ± 2.4cd	0.64 ± 0.04a	0.27 ± 0.03b	0.168 ± 0.016a

Table provides *F* values and significance of main effects and interaction in ANOVA. Marginal means are given for the main effects. Letter codes indicate significance of pairwise differences (LSD) at $\alpha = 0.05$.

over the season, illustrate this. This number was 58 in Bt cotton versus 52 in Bt+CpTI cotton in 2002, 40 in Bt cotton versus 17 in Bt+CpTI cotton in 2007, and 21 in Bt cotton versus 40 in Bt+CpTI cotton in 2008. Given the low counts on both transgenics, and the lack of consistency between years, we conclude that there is no consistent improvement in resistance as a result of the CpTI gene identified at the field level.

Discussion

The main finding of this study is that cotton varieties with a double gene modification, expressing both Cry1Ac and CpTI, exhibit subtly modified, and overall greater resistance to cotton bollworm than cotton with a single Cry1Ac gene under laboratory conditions. The effect of CpTI was most clearly expressed in the response of early instars of *H. armigera.* Early instars showed greater mortality on Bt+CpTI cotton than on Bt cotton. The proportion survival differed by a factor 2.8 in the L1, 1.9 in the L2, and 2.3 in the L3 stage. In the other instars, there was no clear difference in the level of host plant resistance. The responsiveness of early stages to CpTI also was demonstrated in the behavioral experiment, which showed reduced feeding duration on Bt+CpTI cotton, compared with Bt cotton. In later instars, such as the L6, interactive effects were found, with some structures from Bt+CpTI cotton being more resistant than the same structures from Bt cotton, but the reverse phenomenon for other structures. Likewise, food utilization efficiency in L5 did not markedly differ between larvae feeding on Bt or on Bt+CpTI cotton. The level of resistance in leaves of Bt cotton and Bt+CpTI cotton declined during the season, and the patterns were similar for both transgenic varieties. We did not find a significant difference in resistance between Bt cotton and Bt+CpTI cotton in the field.

To our knowledge, this is the first comprehensive study that indicates that inclusion of the CpTI gene enhances resistance of a Bt crop, and has in this sense direct added value for avoiding crop loss due to insect feeding. The value of CpTI as a building block of host plant resistance has been debated, and after initial interest (Hilder et al. 1987, Boulter et al. 1989), CpTI has not attracted widespread interest for commercial use because the size of effects was deemed insufficient (Hilder and Boulter 1999). Our findings are in agreement with this assessment as the resistance of Bt+CpTI cotton is only subtly greater than that of Bt cotton under laboratory conditions, whereas results in the field are inconclusive. However, in practical field experience, we can repeatedly find late-stage *H. armigera* larvae in Bt cotton, whereas we are unable to find late-stage larvae in Bt+CpTI cotton. In the United States, the level of resistance of cotton with Cry1Ac genes has been raised by expressing additional genes from *B. thuringiensis,* in particular the Cry2Ab and Cry1F genes (Tabashnik et al. 2009). Generally, these double gene varieties have significantly higher levels of resistance than single-gene varieties expressing Cry1Ac only (Adamczyk et al. 2001, Gore et al. 2001, Stewart et al. 2001). The comparatively strong effect of the additional gene on the resistance level in these double gene varieties would suggest that these genes might complement the effect of the Cry1Ac gene more effectively than the CpTI gene does. Such a conclusion is precocious, however, because the level of resistance is specific for the combination of crop, genes, and insect species (Adamczyk et al. 2001). Direct comparisons between varieties with different resistance genes, by using specific insect pest species, would be needed to substantiate such a hypothesis.

Laboratory studies have shown that selection for resistance in cotton bollworm populations in the laboratory is slower on Bt+CpTI cotton than on Bt cotton (Zhao et al. 1999). Likewise, Zhao et al. (2003) showed that selection for resistance was slower when greenhouse populations of the diamond back moth, *Plutella xylostella* (L.), were exposed to a double gene broc-

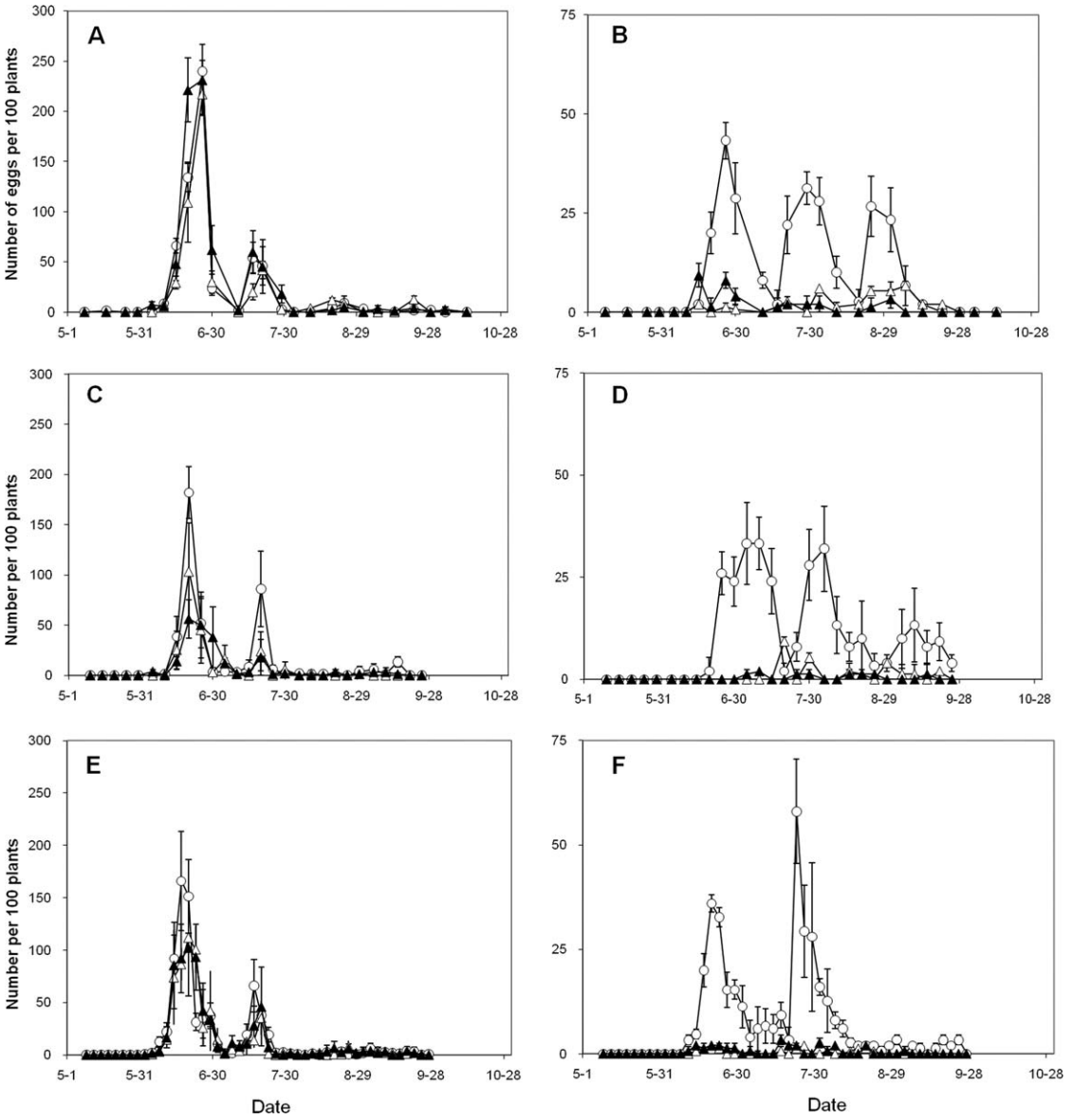

Fig. 5. Densities of eggs (left) and larvae (right) of *H. armigera* in 3 yr on conventional cotton (○), Bt cotton (△), and Bt+CpTI cotton (▲). A and B, 2002; C and D, 2007; and E and F, 2008.

coli, expressing Cry1Ac and Cry1C, than when they were exposed to single gene broccoli expressing either Cry1Ac or Cry1C. Double gene cotton varieties expressing Bt toxins with a different mode of action are widely used in the United States and elsewhere, and it is thought this will raise control, broaden the action spectrum, and enhance the durability of crop resistance to insects (Bates et al. 2005). The work of Zhao et al. (2003) indicates that the pyramiding of the Cry1Ac and CpTI genes will make a valuable contribution to the durability of transgenic resistance in China, even if results presented here indicate that the immediate benefit in crop protection is modest.

A decline in resistance level with age of the cotton crop has been consistently found in cottons modified with the Cry1Ac gene (Kranthi et al. 2005, Olsen et al. 2005, Wan et al. 2005, Kranthi 2006, Llewellyn et al. 2007). To our knowledge, ours is the first report that a similar decline occurs in Bt+CpTI cotton. Likewise, we found that resistance expression profiles among plant organs are similar among Bt (Cry1Ac) cotton and Bt+CpTI cotton. The subtlety of the difference in resistance level in combination with the similarity in temporal and within plant patterns of resistance between Bt and Bt+CpTI cotton may suggest that the expression of

the Cry1Ac protein accounts for a major part of the resistance of Bt+CpTI cotton. Further work is needed to test this hypothesis.

Differences in resistance level between plant organs reported here are difficult to compare with results of Kranthi et al. (2005) and Olsen et al. (2005) because different operational definitions of plant structures were used. For example, Kranthi et al. (2005) made distinction between sepals, anthers and ovaries, whereas we lumped these flower parts in one category for practical reasons. In a broad sense, our results are in agreement with those of Kranthi et al. (2005) and Olsen et al. (2005) in that there are significant differences between structures. In contrast to Kranthi et al. (2005), we find that leaves are less resistant than reproductive structures. Here, we also show that the differences among structures vary over time, which poses another difficulty when comparing among studies.

Results of behavioral observations and food utilization studies indicate that expression of CpTI confers additional toxicity and deterrence to Bt cotton plants. Results of the behavioral observations contrast, however, with those of Zhang et al. (2004) who found that *H. armigera* L1 consumed greater quantities of leaf material on a Bt+CpTI cotton variety (SGK321) than on a Bt variety (Zhong30). The difference in finding could be due to a range of factors, e.g., the use of leaves versus whole plants, differences in varieties used, or the difference in experimental setup (choice versus no-choice). This contrast accentuates that many factors affect the level of resistance exhibited by transgenic cotton varieties, and need to be taken into account when assessing resistance. Similar to results of Zhang et al. (2004), we found a major difference in acceptance between conventional cotton on the one hand and the two transgenic varieties, in agreement with results in Bt cotton in other studies (e.g., Benedict et al. 1992, Li et al. 2007, Yang et al. 2008).

Currently, almost all cotton that is grown in China is genetically modified for resistance to insects. Acceptance of these varieties is very high because are effective for the farmer. Three significant benefits have been recorded: economic, farmer health, and environmental (Pray et al. 2001, Marra et al. 2002, Wu and Guo 2005). Economic benefits accrue from reduced farmer expenditure on pesticides. The reduced use of pesticides, which in practice are applied using knapsack sprayers, has greatly reduced exposure to pesticides, resulting in a reduction in reported cases of pesticide poisoning (Pray et al. 2001). Finally, the reduced use of pesticides for bollworm control in transgenic cotton alleviates the environmental burden of pesticides. Genetic modification of crops for herbivore resistance is often compatible with biological control (Romeis et al. 2006, 2008). Impacts on nontarget species and arthropod biodiversity in cropping systems are minor and much smaller than when pesticides are used. Therefore, the overall balance of genetically engineered resistance to pests is positive. However, the reduction in pesticide use associated with the use of transgenic cotton can release other pests, e.g., mirid bugs (Lu et al. 2010). Therefore, as noted by Tabashnik (1994) and Wu et al. (2008), integration of transgenic resistance with other methods for pest management remains essential.

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