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Insect Control and Dosage Effects in Transgenic Canola Containing a Synthetic *Bacillus thuringiensis cryIAc* Gene[1]

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Zygotic hypocotyls of canola (*Brassica napus* L.) cv Oscar, cv Westar, and the breeding line UGA188–20B were transformed with a truncated synthetic *Bacillus thuringiensis* insecticidal crystal protein gene (*Bt cryIAc*) under the control of the cauliflower mosaic virus 35S promoter using *Agrobacterium tumefaciens*-mediated transformation. Fifty-seven independently transformed lines were produced, containing 1 to 12 copies of the transgenes. A range of *cry* expressors was produced from 0 to 0.4% Cry as a percentage of total extractable protein. The *Brassica* specialists, the diamondback moth (*Plutella xylostella* L.) and the cabbage looper (*Trichoplusia ni* Hübner), were completely controlled by low-, medium-, and high-expressing lines. Whereas control of the generalist lepidopteran, the corn earworm (*Helicoverpa zea* Boddie), was nearly complete, the other generalist caterpillar tested, the beet armyworm (*Spodoptera exigua* Hübner), showed a dose response that had a negative association between defoliation and *cry* expression. These plants were produced as models for an ecological research assessment of the risk involved in the field release of naturalized transgenic plants harboring a gene (*Bt*) that confers higher relative fitness under herbivore-feeding pressure.

The seeds of a number of *Brassica* L. species are cultivated for the production of oil. Collectively, the oilseed *Brassica* sp., generally referred to as rapeseed, supply more than 13% of the world's supply of edible oils and rank third behind soybean and oil palm in importance. The term "canola" was adopted by the Canadians in 1979 and used to describe oilseed *Brassica* cultivars that produce oils containing less than 2% erucic acid and to describe defatted seed meals with less than 30 μmol g^{-1} of aliphatic glucosinolates. *Brassica napus* L. canola cultivars are currently dominant in U.S. production, although canola-quality *Brassica rapa* (synonymous with *Brassica campestris*) cultivars also exist (Raymer et al., 1990).

Production of canola in the U.S. has grown at a modest rate during the last 10 years from virtually 0 in 1985 to 165,000 ha in 1995 (C. Boynton, U.S. Canola Association, personal communication). As production of canola continues to grow, insect problems are expected to become more serious (Lamb, 1989). This may be particularly true as canola production expands in the southeastern United States and California, where mild winter temperatures are likely to lead to increased herbivory, as compared with the much cooler areas (e.g. Canada), where canola has historically been produced. Ubiquitous lepidopteran *Brassica* specialists, such as the DBM and the CBL, and generalist lepidop- terans, such as the BAW and CEW, may increase in importance where canola is grown in warmer regions (Buntin and Raymer, 1994). This scenario may be especially viable with regard to generalist herbivores, since glucosinolates, a hypothesized antiherbivorant to generalist insects (Giamoustaris and Mithen, 1995), have been bred out of canola-quality rapeseed. Transgenic canola cultivars with insecticidal properties will certainly play a major role in integrated pest management strategies for canola pests (Talekar and Shelton, 1993; Evans and Scarisbrick, 1994).

The objectives for this study were 2-fold. (a) To determine the effect of *Bt* expression in *B. napus* on antibiosis for several lepidopteran insects. Unlike the related *Bt* soybean study (Stewart et al., 1996), in which only few, low-expressing synthetic *Bacillus thuringiensis* insecticidal crystal protein (*Bt cryIAc*) plants were produced, the likelihood of obtaining a wide range of *Bt* expression is greater using a species that is more amenable to genetic transformation, such as *B. napus*. (b) To develop biological tools to test models pertaining to biotechnological risk assessment. It is possible that a plant species, such as *B. napus*, that is able to persist in nonagricultural environments could become more weedy in a transgenic form if the transgene confers an increment of fitness and the plant is naturalized in areas of its cultivation. Thus, we developed the *Bt* canola to ultimately test population-level ecological hypotheses.

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Abbreviations: BAW, beet armyworm (*Spodoptera exigua* Hübner); *Bt*, *Bacillus thuringiensis*; CBL, cabbage looper (*Trichoplusia ni* Hübner); CEW, corn earworm (*Helicoverpa zea* Boddie); DBM, diamondback moth (*Plutella xylostella* L.); *HPH*, hygromycin phosphotransferase gene; HSD, honestly significant difference; MS, Murashige-Skoog; T_1, progeny from primary transgenic plants.

MATERIALS AND METHODS

Vector, Construct, and *Agrobacterium tumefaciens* Strain

We used the same synthetic *Bt cryIAc/HPH* construct as described by Stewart et al. (1996). This was placed in the vector pH602 (Firoozabady et al., 1987), which contains a hygromycin-selectable marker under control of the cauliflower mosaic virus 35S promoter (renamed pH602/Bt; Singset et al., 1996; Fig. 1). pH602/Bt was electroporated into the *Agrobacterium tumefaciens* strain GV3850, a disarmed C58 derivative.

Tissue Culture and Genetic Transformation

The tissue culture and transformation procedures were modified from Mehra-Palta et al. (1991). Seedlings and tissue cultures were maintained in a growth room under cool-white fluorescent lights (30 μE irradiance), 23-h photoperiods, and 25°C. Seeds of *Brassica napus* cv Westar, cv Oscar, and the breeding line UGA188–20B were surface-sterilized in 10% commercial bleach for 20 min and germinated on MS basal medium (Murashige and Skoog, 1962, as modified by Mehra-Palta et al., 1991) containing MS salts, 40 mg L^{-1} $FeNa_2$ EDTA, 100 mg L^{-1} *myo*-inositol, 0.1 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} pyridoxine HCl, 0.02 mg L^{-1} thiamine HCl, 0.4 mg L^{-1} Gly, 30 g L^{-1} Suc, and 0.5% agarose (SeaKem, FMC Products, Rockland, ME). Hypocotyls were excised from 5-d-old seedlings and preconditioned for 24 h on basal medium containing 1 mg L^{-1} 2,4-D. Hypocotyls were treated with an *Agrobacterium* solution (10^8–10^9 cells mL^{-1} in liquid MS basal medium) for 30 min and co-cultivated for 3 d on basal medium with 1 mg L^{-1} 2,4-D. After co-cultivation, the hypocotyl segments were transferred to the same medium containing 500 mg L^{-1} Mefoxin (Merck, West Point, PA) and 10 mg L^{-1} hygromycin to select transformed cells. After 1 week, the hypocotyl segments were transferred to basal medium containing 4 mg L^{-1} 6-benzylaminopurine, 2 mg L^{-1} zeatin, 5 mg L^{-1} silver nitrate, and selection agents at the above rates. Transfers to fresh medium were made every 3 weeks. After shoot formation (in 3–6 weeks), shoots were transferred to basal medium containing 0.05 mg L^{-1} 6-benzylaminopurine plus Mefoxin for shoot elongation. After 2 to 4 weeks, shoots were transferred to basal medium containing 0.1 mg L^{-1} indole butyric acid plus Mefoxin to initiate root formation. Alternatively, root initiation was accomplished by placing shoots rooted in a sand/bark mixture in 2-inch pots subsequent to dipping in Rootone (Rigo, Buckner, KY) containing 0.1% indole butyric acid. After plants were rooted, they were hardened-off and transferred to 35-cm-long × 10-cm-diameter conical plastic pots. Primary transgenic plants were grown in a growth chamber with 500 μE irradiance, 16-h photoperiods, and thermoperiods of 21 and 16°C. Seeds were harvested, and T_1 plants were grown in 400-mL Styrofoam cups under the same conditions as above but under 12-h photoperiods to maintain plants in a vegetative state for the insect bioassays.

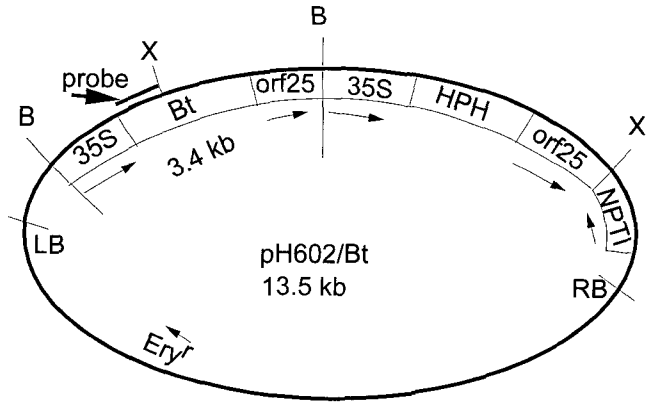

Figure 1. Plasmid pH602/Bt. The *Agrobacterium* transformation vector contains the same *Bt/HPH* construct as reported by Stewart et al. (1996). B, *Bgl*II; X, *Xba*I; LB, left border; RB, right border; orf25, open reading frame 25; Ery^r, erythromycin resistance; NPTI, neomycin phosphotransferase I. The PCR fragment used as a probe within the *cry*-coding region is denoted.

DNA and Protein Blot Analyses

DNA blot analysis and protein blot analysis (Stewart et al., 1996) were used with 10 μg of total protein in each sample lane of the protein gels. In addition, we tested for an association between transgene copy number and expression level using a Spearman rank-correlation test (SAS Institute, 1990).

Insect Bioassays

Detached leaf-insect bioassays were performed on T_1 plants using previously described containers and methods (Parrott et al., 1994). These containers held single, detached leaves and 10 neonate larvae (except for 15 DBM larvae) that were approximately 2 h old. The following insects were tested singularly: DBM, CBL, CEW, and BAW. Plant class was determined by transgenic line (Westar or Oscar) and *Bt* status (±). Five response variables were measured. Percentage of defoliation and insect survivorship data were collected at the end of each 6-d trial. Insect body length, head capsule diameter, and fresh mass were measured after insects were collected and frozen at the end of the trial. The completely random factorial design included eight plant classes, which consisted of different independent T_1 *Bt*-transformants, and T_1 *Bt*-null segregants × eight plants per class (some lines had fewer plants tested) × two replicate leaves per plant = n = 120 to 180 data points for each of the five response variables per each insect tested. Analysis of variances were performed by insect. Multiple comparisons were made using Tukey's HSD (SAS Institute, 1990). We tested for an association between transgene expression and antibiosis response variables by performing Spearman correlations (SAS Institute, 1990).

RESULTS

Genetic Transformation and Primary Transformants

We recovered 57 independently transformed lines of *Bt* canola. Whereas most canola transformation work has been

Table I. *Summary of genetic transformation efficiency of Agrobacterium-mediated transformation of three canola cultivars with a synthetic Bt cryIAc gene*

Cultivar	Start[a]	Callus[b]	Shoots[c]	Rooted[d]	Transgenic[e]	Fertile[f]
Oscar	560	161	155	98	40	32
UGA 188-20B	800	199	28	22	7	5
Westar	720	278	82	20	10	9

[a] Number of hypocotyl segments co-incubated in *Agrobacterium* solution. [b] Number of hypocotyl segments producing callus during hygromycin selection. [c] Number of total shoots from callus. [d] Number of rooted shoots, either in vitro or ex vivo. [e] Number of transgenic plants as shown by DNA blot analysis. [f] Number of fertile transgenic plants determined by seed set.

performed using cv Westar, we achieved the highest efficiencies using cv Oscar, in which 7% of hypocotyl segments that were exposed to *Agrobacterium* harboring the *Bt* construct produced transgenic plants (Table I). For this reason, we chose to focus most of our T_1 studies primarily on the recovered Oscar lines.

Transgenes were stably integrated as shown by DNA blot analysis. The mean and median transgene copy numbers were 5.15 and 5, respectively. The mean and median *cry* expressions were 554 and 312 ng mg^{-1}, respectively. We recovered only four transformants with single-copy inserts (9% of fertile transformed lines).

Molecular Analyses of T_1 Transgenic Plants

We germinated 10 to 20 seeds from a range of Oscar (o) and Westar (w) *Bt* expressors for formal analyses. The small number of seeds germinated was insufficient to determine segregation patterns except in the one- or multicopy lines, in which the transgene copies were obviously linked (Fig. 2). Of these lines (namely o52, o56, and o68) segregation patterns were Mendelian (3:1) using a χ^2 test at $P < 0.05$ (Fig. 2). Expression was low (o68, w58; less than 50 ng CryIAc mg^{-1} protein); moderate (o3, o56; about 270 ng CryIAc mg^{-1} protein); moderately high (o52; 586 ng CryIAc mg^{-1} protein); and high (o96, w53; more than 1300 ng CryIAc mg^{-1} protein) (Table II; Fig. 3). Segregating nontransgenics were used for negative controls (the "no" classification). Sample sizes for Westar lines were small because of resource limitations.

Insect Bioassays Performed on T_1 Transgenic Plants

The Cry-susceptible *Brassica* specialists DBM and CBL, assayed against transgenic plants using detached leaf-infestation assays, were completely inhibited by transgenic plants, regardless of *cry* expression level. All DBM and CBL were killed by transgenic plants except for w58, a low-expressing Westar line in which insect survivorship and defoliation were less than 5% than that of nontransgenic. The other low-expressing line (o68) also allowed a few CBL larvae to survive during the assay.

Similarly, the results of trials using the generalist CEW showed significant differences ($P = 0.05$) only between nontransgenics and transgenics. However, on both low-expressing transgenic lines (o68 and w58) and moderate expressors (o3 and o56) a few larvae survived the assay (Table III). There were no significant differences in the growth characteristics of those insects that survived (Table III). There were slight amounts of defoliation, except in the o56 line, in which no defoliation was noted (Table III).

In contrast, there was a noticeable gradation of insect survivorship, defoliation, and insect growth among transgenics in the trials using Cry-tolerant BAW (Table IV). Here, both analysis of variance and Spearman correlations showed a negative association between insect survivorship, growth, defoliation, and Cry expression. Insect survivorship was more than 3 times higher on nontransgenic plants as compared with a high-expressing transgenic line. Also, nontransgenic plants suffered more than 25 times the defoliation (Table IV). Correlation analysis showed a good logarithmic fit between expression and survivorship ($r = -0.51$ for nontransformed data; $r = -0.73$ for log transformed data) and defoliation ($r = -0.52$ for nontransformed data; $r = -0.74$ for log transformed data) (Fig. 4). Correlation analysis also showed a significant negative association between expression and surviving insect cap-

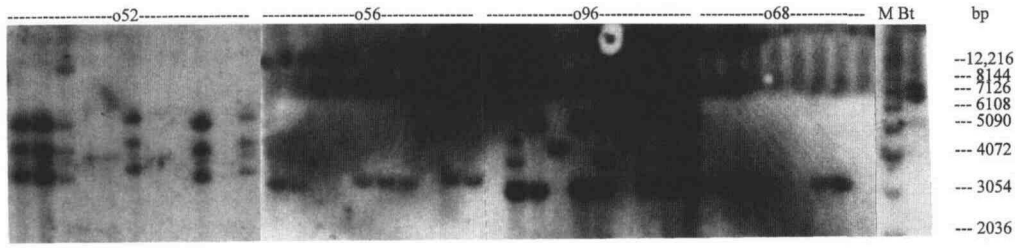

Figure 2. DNA blot analysis of four T_1 transgenic lines. Ten micrograms of genomic DNA and 50 pg of pH602/Bt (Bt) were digested with *Xba*I, electrophoresed in a 1% agarose gel, blotted onto a nylon filter, and hybridized to a ^{32}P-labeled *cry*-specific probe. M, 1-kb DNA ladder (GIBCO-BRL). Genomic DNA was isolated from leaves of T_1 plants (1-copy *Bt* lines o56 and o68 and multicopy lines o52 and o96). The copies are obviously linked in o52. See Table II for line characteristics.

Table II. *Summary of T_1 Bt canola molecular and expression data*

Different letters indicate significant differences using Tukey's HSD at $\alpha = 0.05$.

Line	Transgene Copy No.	n^a	CrylAc Synthesis
			ng mg^{-1}
o3	6	5	269 ± 86 a
o52	2	7	586 ± 356 ab
o56	1	7	267 ± 130 a
o68	1	6	5 ± 0 a
o96	4	5	1379 ± 341 c
w53	2	3	1463 ± 271 c
w58	3	3	40 ± 10 a

[a] n = number of 2-week-old leaves/plants sampled for immunoblot analysis.

sule head diameter ($r = -0.35$), body length ($r = -0.54$), and weight ($r = -0.45$). In all cases $P < 0.0001$, except for the insect head capsule diameter, for which $P = 0.004$.

DISCUSSION

Transgenic Plants

The transformation method used was effective in producing a large number of stably transformed canola plant lines. Oscar, a blackleg (*Leptosphaeria maculans*)-resistant Australian cultivar recently introduced into the southeastern United States for use in blackleg-infested areas (Raymer et al., 1995), had the highest transformation efficiencies. This is the first report, to our knowledge, of transgenic Oscar. The high relative transformation frequencies (compared to that of Westar) coupled with the blackleg resistance makes Oscar a superior candidate for canola transformation studies.

The maximum level of *Bt* expression reported here (0.4%, Cry production as a percentage of total extractable protein) is among the highest reported in the literature, owing to at least some degree to an efficient recoding of the endotoxin gene and the large number of independent transformants produced. For example, researchers who have transformed plants with synthetic *Bt* genes under the control of a constitutive promoter have recovered plants expressing a wide range of maximums: 0.025 and 0.1% of CryIIIA in potato (Adang et al., 1993; Perlak et al., 1993); 0.03% of CryIAb in tobacco (Perlak et al., 1991); 0.05% of CryIAb in rice (Fujimoto et al., 1993); and 0.4% of CryIAb in maize (Koziel et al., 1993). A recently published paper by McBride et al. (1995) demonstrated an alternative to transgenics with synthetic *Bt* genes by engineering a native *CryIAc* into tobacco chloroplasts, yielding transgenic plants with 3 to 5% Cry. Although the expression level was very high and no gene reconstruction was needed, transgenic chloroplasts will continue to segregate, providing the possibility of irregular inheritance.

Insect Control

We tested a suite of lepidopterans ranging in Cry susceptibility, including both *Brassica* specialists and polyphagous species. From most susceptible to least susceptible to Cry toxins, the insects rank CBL > DBM = CEW > BAW (MacIntosh et al., 1990; Moar et al., 1990; Tabashnik et al., 1994). BAW, along with other *Spodoptera* sp., are known to be tolerant to *Bt* toxins (Moar et al., 1990). Feeding was prevented by three of the species by even the lowest expressors, whereas the generalist BAW, although negatively affected by high doses of endotoxin, survived on all transgenics (Table IV). The gradation of *cry* expressors provided an opportunity to test the effect of expression level on antibiosis of a *Bt*-tolerant insect species (BAW). Dose effects have only been rarely examined and documented using *Bt* transgenics. A priori, one would intuitively expect a positive correlation between *Bt* dose (expression) and antibiosis. Adang et al. (1993) found a positive association between *cryIIIA* expression and antibiosis of Colorado potato beetle on transgenic potato. Other researchers who have generated large numbers of independent transformants have not reported this association (Perlak et al., 1991; Koziel et al., 1993). However, in these studies, either the research involved insect species that are very sensitive to *Bt* toxin, such that all individuals were killed regardless of expression, or the experimental design did not test explicitly for the association of expression and antibiosis. Dose effects will likely play an important role in two ecological problems: the evolution of insect resistance to *Bt* and the evolution of weedier *Brassica*.

Insect Resistance to *Bt*

Perhaps the greatest potential problem with the commercialization and environmental release of *Bt* transgenic plants is that of the evolution of insect resistance to *Bt*. Wild populations of DBM have been collected that show high levels of resistance to CryI toxins (Tabashnik, 1994). Perhaps even more disconcerting is the fact that three different *Bt*-resistant colonies of *Heliothis virescens* have been collected, and all three lines have mapped to three different genetic loci on a *H. virescens* genetic linkage map (Heckel, 1994). Thus, with no strategy in place for manag-

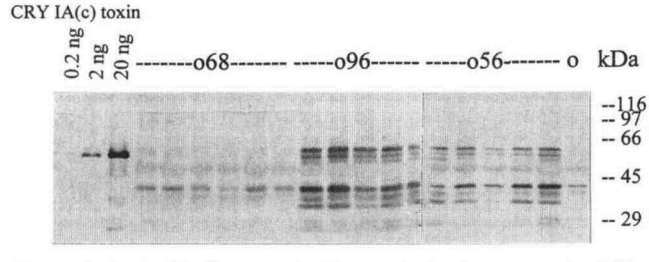

Figure 3. T_1 Bt CrylAc protein blot analysis. Immunostained blot shows typical progeny expression from low (o68), high (o96), and medium (o56) *cry* expressors. See Table II for line characteristics. o, Untransformed Oscar. Densitometric measurements were performed on the two top bands of each lane. These two bands represent the size anticipated (that of the standard) and a slightly larger protein. The three lower, specifically immunostained bands were assumed not to be biologically active and therefore not used in the estimate of expression.

Table III. *Summary of detached-leaf insect bioassays of CEW, a generalist lepidopteran that is somewhat tolerant to CrylAc*

The means and SDs are presented. Different letters within columns denote significant differences at P = 0.05 (Tukey's HSD). * denotes no insect survivors.

Line	*n*	Live Larvae	Head Capsule	Body Length	Larval Weight	Defoliation
			mm	*mm*	*mg*	*%*
no	14	4.71 ± 2.23 a	0.75 ± 0.23 a	6.38 ± 2.26 a	6.12 ± 5.33 a	26.8 ± 26.6 a
o3	18	0.22 ± 0.55 b	0.32 ± 0 a	2.70 ± 0.52 a	0.50 ± 0.26 a	0.28 ± 0.96 b
o52	14	0 b	0.30 ± 0 a	4.00 ± 0 a	0.20 ± 0 a	0 b
o56	16	0.06 ± 0.25 b	0.30 ± 0 a	2.40 ± 0 a	0.20 ± 0 a	0 b
o68	14	0.21 ± 0.43 b	0.43 ± 0.12 a	3.37 ± 1.51 a	4.33 ± 1.0 a	0.36 ± 0.63 b
o96	16	0 b	*	*	*	0 b
w53	5	0 b	*	*	*	0 b
w58	4	0.50 ± 0.58 b	0.39 ± 0.13 b	3.15 ± 0.21 b	0.60 ± 0.14 b	0.50 ± 0.58 b

ing resistance, some insect populations will evolve resistance very quickly through strong selective pressures and the presence of multiple resistance genes. Although various strategies to manage resistance have been proposed, there is little empirical data showing that one strategy is superior to another. However, to manage *Bt* resistance, mechanisms of insect resistance must be elucidated to make predictive models of insect evolution. Transgenic plants with a gradation of *cry* expression provide research tools allowing researchers to select for *Bt*-resistant insects in controlled settings for the purpose of studying modes and dynamics of the evolution of *Bt* resistance. Furthermore, Cry-tolerant insect species such as BAW, which may be affected by Cry toxins at relatively high doses, could be useful as part of that system to study dose effects of *Bt* toxins.

Bt Transgenes May Increase Weediness of *Brassica*

Another risk in deploying *Bt* transgenic plants that has been discussed very little is that of increasing the fitness of the host. For insecticidal transgenic plants, and for any transgenic plants engineered with a gene that may confer an increment of fitness to its host, there is the possibilitythat host plants may become more weedy. Theoretically, this scenario could occur under only two conditions: (a) the host plant is already naturalized in a nonagricultural environment and/or it is sexually compatible with a species that is naturalized, and (b) there is selection pressure in the nonagricultural environment. The combination of *Bt* and canola fulfills these requirements, thus making it a good model to test some basic hypotheses. We will be using *Bt* canola in a field study to investigate the effect that insect-feeding pressure may have on population replacement of nontransgenic plants with transgenic plants with *Bt*. Such a study will provide data that can be used to assess the risk involved in deploying naturalized transgenic plants with a transgene conferring fitness to its host. In addition, and perhaps most important, it is becoming clear that transgenes in canola will be introgressed rapidly into weedy relatives such as *B. campestris* (synonymous with *B. rapa*) and *Raphanus raphinistrum* (Jørgensen and Andersen, 1994; Scheffler and Dale, 1994; Mikkelsen et al., 1996). Such genes, such as *Bt cryIAc*, at threshold levels of insect-feeding pressure, will likely increase the relative fitness of its host. However, it is not known whether these effects will translate into increased weediness. Additional research is needed to address these concerns, and it seems that the *Bt* canola produced in this study will be a useful tool to this end.

Table IV. *Summary of detached-leaf insect bioassays of BAW, a generalist lepidopteran that is very tolerant to CrylAc*

The means and SDs are presented. Different letters within columns denote significant differences at P = 0.05 (Tukey's HSD). Feeding data are from bioassays of BAW, a generalist lepidopteran that is resistant to CrylAc.

Line	*n*	Live Larvae	Head Capsule	Body Length	Larval Weight	Defoliation
			mm	*mm*	*mg*	*%*
no	12	6.50 ± 1.57 a	0.97 ± 0.14 a	8.38 ± 1.57 a	13.4 ± 6.1 a	70.8 ± 16.5 a
o3	13	3.62 ± 1.66 bc	0.45 ± 0.11 b	3.52 ± 1.06 bcd	1.55 ± 1.39 b	5.85 ± 6.67 c
o52	12	2.00 ± 1.60 c	0.36 ± 0.09 b	2.54 ± 0.74 e	0.49 ± 0.32 b	2.75 ± 1.76 c
o56	14	3.50 ± 1.79 bc	0.41 ± 0.08 b	3.01 ± 0.62 d	0.88 ± 0.55 b	7.21 ± 6.57 c
o68	10	4.50 ± 2.92 ab	0.57 ± 0.27 b	4.81 ± 2.33 bc	4.75 ± 5.67 b	29.2 ± 24.7 b
o96	14	3.07 ± 1.94 bc	0.50 ± 0.51 b	2.56 ± 0.53 e	0.53 ± 0.35 b	3.86 ± 3.78 c
w53	6	4.00 ± 3.03 bc	0.37 ± 0.10 b	2.72 ± 0.82 e	0.46 ± 0.27 b	6.17 ± 6.85 c
w58	4	6.00 ± 2.83 ab	0.63 ± 0.18 a	5.21 ± 2.28 b	4.79 ± 3.66 b	40.8 ± 29.0 b

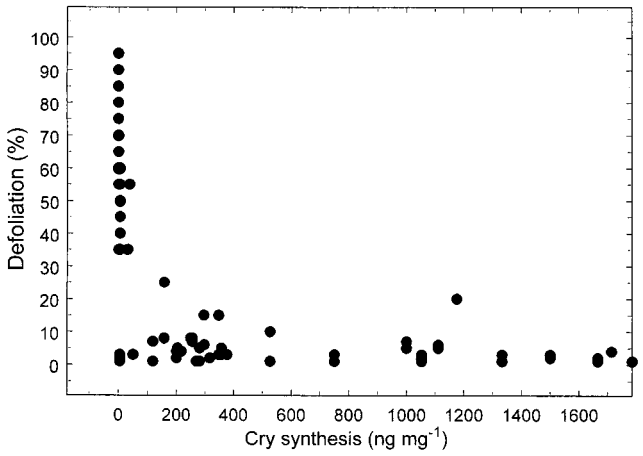

Figure 4. Defoliation versus Cry synthesis. Detached-leaf bioassays were performed in which 10 BAW were applied to each 2-week-old leaf. Defoliation after 6 d was negatively associated with *cry* expression at $P > 0.0001$.

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