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ORIGINAL PAPER

Effects of defoliating insect resistance QTLs and a *cry1Ac* transgene in soybean near-isogenic lines

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Abstract The crystal proteins coded by transgenes from *Bacillus thuringiensis* (Bt) have shown considerable value in providing effective insect resistance in a number of crop species, including soybean, *Glycine max* (L.) Merr. Additional sources of soybean insect resistance would be desirable to manage the development of tolerance/resistance to crystal proteins by defoliating insects and to sustain the deployment of Bt crops. The objective of this study was to evaluate the effects and interactions of three insect resistance quantitative trait loci (QTLs; QTL-M, QTL-H, and QTL-G) originating from Japanese soybean PI 229358 and a *cry1Ac* gene in a "Benning" genetic background. A set of 16 BC_6F_2-derived near isogenic lines (NILs) was developed using marker-assisted backcrosses and evaluated for resistance to soybean looper [SBL, *Pseudoplusia includens* (Walker)] and corn earworm [CEW, *Helicoverpa zea* (Boddie)] in field cage, greenhouse, and detached leaf assays. Both Bt and QTL-M had significantly reduced defoliation by both SBL and CEW and reduced larval weight of CEW. The antibiosis QTL-G had a significant effect on reducing CEW larval weight and also a significant effect on reducing defoliation by SBL and CEW in some assays. The antixenosis QTL-H had no main effect, but it appeared to function through interaction with QTL-M and QTL-G. Adding QTL-H and QTL-G further enhanced the resistance of the Bt and QTL-M combination to CEW in the field cage assay. These results should help guide the development of strategies for effective management of insect pests and for sustainable deployment of Bt genes.

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Introduction

The usefulness of pyramiding a Bt transgene with a gene that conditions insect resistance through a totally different mode of action has been recognized as a potential tool to slow the evolution of insect resistance to Bt crops (Gould et al. 1992; Roush 1998). Sachs et al. (1996) demonstrated that cotton isolines with natural terpenoid production and a Bt *cry1Ab* transgene were more resistant to tobacco budworm [*Heliothis virescens* (F.)] than isolines with either source of resistance alone. Douches et al. (2001) and Cooper et al. (2004) found that the combined resistance of natural leptine and a *cry3A* or *cry5* transgene provided better control of Colorado potato beetle [*Leptinotarsa decemlineata* (Say)] than either source of resistance alone in potato.

The above examples were facilitated by the knowledge of the compounds responsible for the host resistance and the relatively easy analysis for the presence of terpenoids or leptines. However, the use of Bt and host resistance gene combinations has been intractable when the nature of the host resistance is unknown, as has been the case for soybean. This limitation has been overcome through the use of

molecular markers and the discovery of quantitative trait loci (QTLs) that condition the insect resistance.

We have been investigating the potential for pyramiding QTLs for resistance to defoliating insects with a Bt *cry1Ac* transgene (Walker et al. 2004) in soybean [*Glycine max* (L.) Merr.]. These QTLs were found in a plant introduction (PI), PI 229358, from Japan (Rector et al. 1998, 2000). This PI shows both antixenosis (discouragement of insect colonization and/or feeding) and antibiosis (adverse effects on the insect life history) to several lepidoteran insects and a coleopteran pest (Lambert and Kilen 1984; Van Duyn et al. 1971). Rector et al. (1998, 2000) detected a major QTL (QTL-M) on linkage group (LG) M (Cregan et al. 1999, Song et al. 2004) of PI 229358 that conditions both antixenosis ($R^2 = 37\%$) and antibiosis ($R^2 = 22\%$) to corn earworm [CEW, *Helicoverpa zea* (Boddie)]. A QTL for antibiosis (QTL-G, $R^2 = 19\%$) was identified on LG G, and one QTL for antixenosis (QTL-H, $R^2 = 16\%$) was discovered on LG H. Narvel et al. (2001) tagged these QTLs with simple sequence repeat (SSR) markers, which Walker et al. (2004) used to transfer QTL-M and QTL-H from PI 229358 into Jack-Bt, a line with a *cry1Ac* transgene (Stewart et al. 1996). In both detached leaf assays and field studies, a BC_2F_3 line with the combined resistance of QTL-M and *cry1Ac* was more resistant to CEW and soybean looper [SBL, *Pseudoplusia includens* (Walker)] than lines with either source of resistance alone (Walker et al. 2004).

The objective of this study was to extend the research of Walker et al. (2004) by including a fourth resistance QTL, namely QTL-G along with QTL-M, QTL-H, and a *cry1Ac* transgene. Sixteen novel combinations of these resistance factors were evaluated for their effects and interactions, by using a set of BC_6F_2-derived near-isogenic lines (NILs) derived from "Benning". The results from this study should help guide the development of strategies for effective management of insect pests and for sustainable deployment of Bt genes in soybean integrated pest management programs.

Materials and methods

Plant materials

Sixteen BC_6F_2-derived NILs with a Benning genetic background and with all possible combinations of three PI 229358-derived defoliating insect resistance QTLs and a Bt *cry1Ac* transgene (Table 1) were developed using marker-assisted backcrosses. First, eight BC_6F_2-derived NILs with all possible combinations of the three resistance QTLs were developed from an initial cross between Benning and PI 229358. Concurrently, a BC_6F_2-derived NIL of Benning (Ben-Bt) was developed from an initial cross between Benning and Jack-Bt. NILs with different combinations of the three resistance QTLs and the Bt gene were then developed from a cross between Ben-MHG and Ben-Bt (Fig. 1). Benning is a Maturity Group VII soybean cultivar that is relatively susceptible to defoliating insects (Boerma et al. 1997). Ben-mhgbt is a BC_6F_2-derived NIL that does not contain PI 229358 genome in the QTL-M, QTL-H, and QTL-G intervals, nor does it carry the Bt gene.

Table 1 Benning soybean near-isogenic lines (NILs) with and without PI 229358 alleles at QTL-M, QTL-H, and QTL-G and a transgene *cry1Ac* (Bt)

NIL	Resistance QTLs or gene complement			
	QTL-M	QTL-H	QTL-G	*cry1Ac*
Ben-mhgbt	–	–	–	–
Ben-M	+	–	–	–
Ben-H	–	+	–	–
Ben-G	–	–	+	–
Ben-MH	+	+	–	–
Ben-MG	+	–	+	–
Ben-HG	–	+	+	–
Ben-MHG	+	+	+	–
Ben-Bt	–	–	–	+
Ben-MBt	+	–	–	+
Ben-HBt	–	+	–	+
Ben-GBt	–	–	+	+
Ben-MHBt	+	+	–	+
Ben-MGBt	+	–	+	+
Ben-HGBt	–	+	+	+
Ben-MHGBt	+	+	+	+

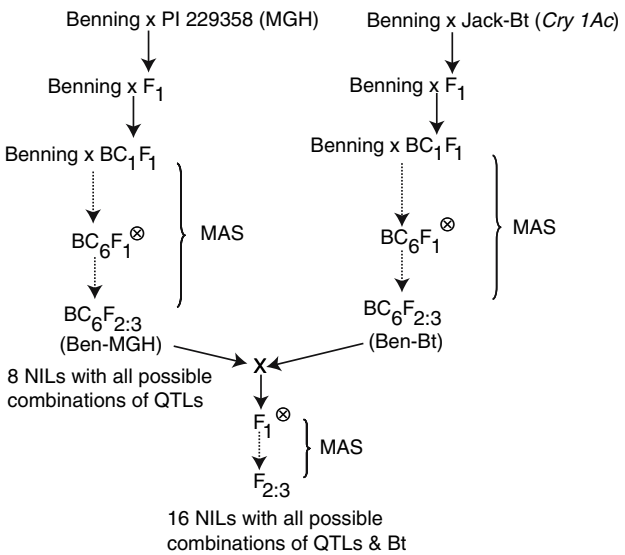


Fig. 1 Breeding scheme used to develop $BC_6F_{2:3}$ Benning near-isogenic lines (NILs). Marker-assisted selection (MAS) was used as indicated

Marker-assisted selection

QTL-M, QTL-G, and QTL-H were first listed as CEW 1–1, CEW 6–1, and CEW 1–2, respectively, in SoyBase (http://www.soybase.org, verified September 2007) since they were first described using feeding assays with CEW. The SSR markers linked to the three QTLs (Narvel et al. 2001) and Bt gene-specific PCR primers (Santos et al. 1997) were used to select individuals with specific combinations of the three QTLs and the Bt gene. The SSR markers used for each backcross generation and the final selection of each NIL were Satt220 and Satt175 flanking QTL-M, Sat_334 and Sat_118 flanking QTL-H, and Sct_199 and Satt191 flanking QTL-G, as well as Satt536, Sat_122, and Satt472 within the intervals of QTL-M, QTL-H, and QTL-G, respectively. The sequences of all SSR primers were obtained from the SoyBase website (soybase.org).

DNA isolation was conducted as described by Zhu et al. (2006). PCR reactions were prepared using a protocol modified from Li et al. (2002). Briefly, amplification was performed in a total volume of 10 µl, containing 40 ng template DNA, 1X thermophilic DNA polymerase buffer (10 m*M* Tris–HCl pH 8.0, 50 m*M* KCl, and 0.1% Triton® X-100), 2.5 m*M* $MgCl_2$, 100 μM each of dNTPs, 0.1 μM each of forward and reverse primers, and 0.5 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR was performed with a PTC-225 DNA Engine Tetrad (MJ Research, Waltham, MA, USA) thermal cycler. Thermal cycling conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 46°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 5 min. One of the SSR primers for each marker was labeled with either 6-FAM, HEX, or NED fluorescent tags to allow detection with an ABI Prism 377 DNA sequencer (PE-ABI, Foster City, CA, USA). Marker data were collected with GeneScan v. 2.1 (PE ABI, Foster City, CA, USA) and analyzed with Genotyper v. 2.5 software (PE ABI, Foster City, CA, USA).

Field studies

Evaluation of the sixteen NILs in the field screen cages was similar to that described by Walker et al. (2004). Experimental plots were planted on 27 June 2005 in two adjacent areas in a field at the University of Georgia Plant Sciences Farm near Athens, GA. Each experimental unit (plot) consisted of a 6-plant hill. Because Bt protein can be less toxic to late instar larvae, the plots were separated by 90 cm to reduce migration of older larvae from plot to plot. Drip irrigation lines were installed along each row of hills so that adequate moisture could be maintained during the test period.

Twelve replications of each genotype were planted in one area for CEW infestation. Nine replications of each genotype were planted in the other area for SBL infestation. The CEW and SBL were chosen for the assays because they represent important soybean pests known to differ in their sensitivity to the CRY1AC protein toxin (Stewart et al. 1996; Walker et al. 2000). Both species are also pests of cotton in the Southeast and Delta regions of the USA, where *cry1Ac*-expressing cotton cultivars are widely planted. A randomized complete block experimental design was used. After the plants had become established (1-week-old seedlings), a Quonset-shaped screen cage was constructed of lumber and PVC pipe over each area. The screen cage was covered with a 0.9 × 0.9-mm nylon mesh to prevent parasitoids, predators, and other pest insects from migrating into the test area, while confining the test insects.

When most plants were in the V3 stage of development (Fehr et al. 1971), infestations were initiated. Freshly hatched larvae were mixed with corn grits, which were then applied to the foliage in measured doses using a "bazooka" mechanical applicator (Wiseman et al. 1980). For SBL, eight infestations were applied during 2 weeks, and a total of 520 neonate larvae were placed on each hill of plants. The CEW cage received three infestations during 1 week, with a total of 270 larvae placed on each hill. Both SBL and CEW eggs were obtained from the Insect Biology and Population Management Research Laboratory (USDA-ARS, Tifton, GA, USA).

Visual estimates of defoliation were made over a 10-day-period, beginning 12 days after the initial infestation. Percent defoliation of each hill plot was estimated by four different individuals, and means from these estimates for each experimental unit were used in the data analysis.

Greenhouse tests

Evaluation of the 16 NILs under greenhouse conditions with CEW and SBL employed a procedure modified from All et al. (1989). Briefly, one seed was planted per 450-mL polystyrene foam cup with three holes punched in the bottom and filled with Fafard 2 mix (Conrad Fafard, Agawam, MA, USA). Only cups with healthy seedlings were chosen, and each experimental unit (plot) consisted of four seedlings of the same genotype. A randomized complete block design with 15 replications was used. The cups were placed in a stainless steel pan, measuring 4.9 m long × 1.2 m wide × 8 cm deep. When infestations were initiated, the pans were filled with 2 cm water for irrigation and to drown any larvae that fell from the plants. The plots were separated by about 30 cm so that the water trap would intercept larvae migrating from plot to plot. Four neonate (<5 h old) larvae were placed on an unexpanded trifoliolate leaf of

each plant using a 000-size camel's hair brush when plants were at the V2 stage. Defoliations were visually estimated by four different individuals 10 days after infestation, and means from these estimates for each experimental unit were used in the analysis of variance.

Detached leaf bioassay

Antibiosis of each of the 16 NILs to CEW was measured in a growth chamber using a procedure modified from Walker et al. (2002). The growth chamber was maintained at 27°C and 85% ambient humidity, and a 14-h photoperiod was maintained with fluorescent and incandescent lights providing ca. 40 µmol photons m^{-2} s^{-1}. The bioassays were set up as randomized complete block designs with 10 replications. Newly expanded trifoliolate leaves were collected from greenhouse-grown plants. One leaflet from a trifoliolate leaf was placed into a Petri dish (100 × 25 mm), and only one larva was placed in each dish to prevent cannibalism. Each experimental unit consisted of three larvae from the dishes containing leaflets from the same trifoliolate leaf. A fresh leaf was added to each dish after 4 days, and the feeding was stopped (typically 6 days after infestation) by moving all of the dishes to 4°C once the leaf tissue in any one of the dishes was completely consumed. An hour later, larvae were transferred to empty dishes, frozen at −20°C and weighed. The data were recorded as the average weight of surviving larvae.

Data analyses

Percent defoliation and larval weight were first tested for normality by using the Univariate Procedure (Proc Univariate) of SAS (SAS Institute Inc 1988). Data for percent defoliation and larval weight from all experiments were considered normally distributed because their skewness and kurtosis tests did not significantly deviate from zero. It was therefore unnecessary to transform the data prior to analyses of variance (ANOVA). The ANOVA for the traits were conducted with the General Linear Model Procedure (Proc GLM) of SAS (SAS Institute Inc 1990) to identify significant differences between lines ($P \leq 0.05$). Since all possible QTLs or gene combinations were tested, the treatment effects were considered a 2 × 2 × 2 × 2 factorial treatment design. This type of analysis revealed not only significant main effects of individual QTLs and Bt, but also significant interactions affecting defoliation by both SBL and CEW, or to larval weight of CEW (Table 2).

Results

The ANOVA of the factorial combinations of QTL-M, QTL-H, QTL-G, and Bt evaluated for CEW and SBL across field and greenhouse environments and in a growth chamber provided insight into the relative importance of the various resistance genes and their interactions (Table 2). Based on the magnitude and significance of the various *F*-tests, Bt had a major effect on resistance to CEW and SBL in all five testing environments. For QTL-M, the *F*-test values were somewhat smaller in magnitude than for Bt, but were also highly significant in all the testing environments.

Among the various two-way interactions, the QTL-M × Bt interaction was significant at the 0.01 probability level in all but the growth chamber antibiosis experiment.

Table 2 *F*-values for significance of QTL and Bt main effects and interactions on defoliation by corn earworm (CEW) and soybean looper (SBL) in field cage and greenhouse assays, or larval weight of CEW measured as antibiosis in a growth chamber

Sources	*df*	Field cage		Greenhouse		Antibiosis
		SBL	CEW	SBL	CEW	CEW
M	1	202.2**	156.4**	59.0**	90.5**	85.3**
H	1	ns	ns	ns	ns	ns
G	1	20.0**	ns	ns	6.2*	4.3*
Bt	1	318.0**	811.8**	101.8**	456.5**	506.6**
M × H	1	ns	4.6*	ns	ns	ns
M × G	1	ns	ns	ns	ns	ns
M × Bt	1	42.1**	99.0**	9.6**	37.3**	ns
H × G	1	ns	6.3*	ns	6.8*	ns
H × Bt	1	ns	ns	ns	ns	ns
G × Bt	1	8.0**	ns	ns	ns	ns
M × H × G	1	5.5*	28.4**	4.3*	ns	10.2**
M × H × Bt	1	6.7*	10.1**	11.7**	ns	ns
H × G × Bt	1	17.8**	14.6**	ns	ns	ns
M × G × Bt	1	ns	19.3**	ns	ns	ns
M × H × G × Bt	1	ns	ns	ns	ns	5.0*

ns not significant

*, ** *F* value significant at the 0.05 and 0.01 probability level

The remaining two- and three-way interactions were mostly non-significant ($P > 0.05$) or were generally of lower magnitude than the F values for Bt, QTL-M, and the QTL-M × Bt interaction. Hence these main effects and interactions were studied in more detail.

Effects on defoliation in the field

Mean defoliation by SBL and CEW in the field cage studies is shown in Fig. 2. In general, the Bt NILs sustained significantly less defoliation from both SBL and CEW than the non-Bt lines. The mean defoliation by SBL (25.1 vs. 13.5%) and CEW (53.6 vs. 22.6%) on the non-Bt NILs was about twice as high as that on the Bt NILs (Table 3). QTL-M also had a significant main effect on reducing defoliation by both SBL and CEW (Table 2). The mean defoliation by SBL on the NILs with QTL-M was nearly one-half (14.7 vs. 23.9%) that on the NILs without QTL-M (Table 3). A similar result was obtained with CEW (31.3 vs. 45.0%). The combination of Bt and QTL-M led to significantly less defoliation by SBL than did Bt or QTL-M alone (Fig. 2A; Table 3). The QTL-M × Bt interaction was due to a difference in the magnitude of response, rather than a lack of response of either resistance gene (Table 3).

Based on previous results (Rector et al. 2000; Narvel et al. 2001), the antibiosis QTL-G was not expected to have a significant main effect on reducing defoliation by SBL. However, while the difference between the mean defoliation (17.8%) on the QTL-G NILs and that (20.7%) on the NILs without QTL-G was small, it was nevertheless statistically significant (Fig. 2A; Table 2). The QTL-H NIL experienced significantly less defoliation by both SBL and CEW than the NIL Ben-mhgbt, which lacks resistance genes to insect defoliation. However, QTL-H had no significant main effect, as evidenced by the fact that the average defoliation on the NILs with QTL-H was essentially the same as that on the NILs without QTL-H. QTL-H was nevertheless involved in interactions with QTL-M, QTL-G, and Bt (Table 2). We do not know the molecular basis of these interactions, but the combination of QTL-H with QTL-M, QTL-G, and Bt resulted in significantly less defoliation by SBL (7.1 vs. 14.1%) and CEW (11.7 vs. 33.2%) on the NIL Ben-MHGBt than on the NIL Ben-MGBt, which lacks QTL-H (Fig. 2). Ultimately, the combination

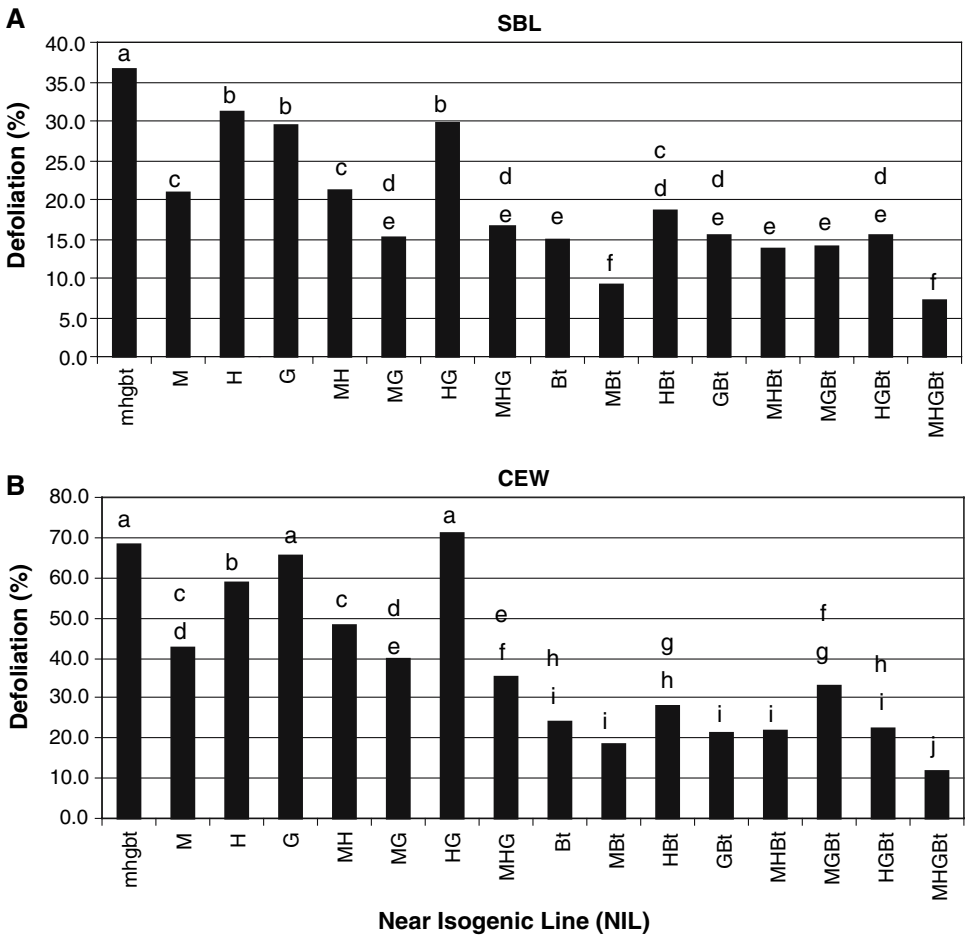


Fig. 2 Mean defoliation by (**A**) soybean looper (SBL) and (**B**) corn earworm (CEW) on 16 Benning soybean near-isogenic lines (NILs) in field cage assays. Defoliations of NILs with the same letters are not significantly different at the 0.05 probability level. An *upper case letter* indicates the presence of a resistance allele from a particular linkage group (*M*, *H*, or *G*) of PI 229358 or of a *cry1Ac* transgene from *Bacillus thuringiensis* (Bt). *Lower case letters* in the first line to the left indicate that no known resistance alleles were present

Table 3 QTL-M and Bt main effects and their interactions on percent defoliation by corn earworm (CEW) and soybean looper (SBL) in greenhouse and field cage assays

	-Bt	+Bt	Mean
CEW/Greenhouse			
-M[a]	36.1 a	12.5 c	24.3**
+M	22.7 b	9.6 d	16.2
Mean	29.4**	11.1	
CEW/Cage			
-M	65.9 a	24.0 c	45.0**
+M	41.4 b	21.2 c	31.3
Mean	53.6**	22.6	
SBL/Greenhouse			
-M	25.3 a	10.6 b	18.0**
+M	13.4 b	5.6 c	9.5
Mean	19.4**	8.1	
SBL/Cage			
-M	31.8 a	16.0 c	23.9**
+M	18.4 b	11.0 d	14.7
Mean	25.1**	13.5	

** Significantly different from its counterpart at the 0.01 probability level

[a] Numbers followed by the same letters are not significantly different at the 0.05 probability level

of QTL-M, -H, and -G, and Bt was less defoliated than all other combinations for CEW, but no additional resistance QTL was able to improve the resistance of QTL-M and Bt combination against SBL.

Effects on defoliation in the greenhouse

Mean defoliation by SBL and CEW in the greenhouse tests is shown in Fig. 3. As was the case in the field studies, the NILs having Bt were significantly less defoliated by both SBL and CEW than the non-Bt lines. The mean defoliation by SBL (19.4 vs. 8.1%) and CEW (29.4 vs. 11.1%) on the NILs without Bt was at least twice that on the NILs with Bt (Table 3). Likewise, QTL-M also had significant effect on reducing defoliation by both SBL and CEW (Table 2). The mean defoliation by SBL (9.5 vs. 18.0%) and CEW (16.2 vs. 24.3%) on the NILs carrying QTL-M was significantly lower than that on the NILs without QTL-M. Also, as was the case in the field, the Bt effect was enhanced by QTL-M (Table 3).

The antibiosis QTL-G had a significant main effect on reducing defoliation by CEW in the greenhouse test (Fig. 3; Table 2). This was unexpected since the greenhouse test was not designed to measure antibiosis. In contrast, QTL-H did not have a significant main effect on either CEW or SBL, but was involved in interactions with QTL-M, QTL-G, and Bt (Table 2). The combination of QTL-H with QTL-M, QTL-G, and Bt resulted in significantly less CEW defoliation on Ben-MHGBt compared with that observed on Ben-MGBt without QTL-H (Fig. 3). The combination of QTL-H with QTL-M and Bt also tended to have less defoliation by SBL on the NIL Ben-MHBt (4.0%) relative to that on the NIL Ben-MBt (7.2%), although this difference was not significant.

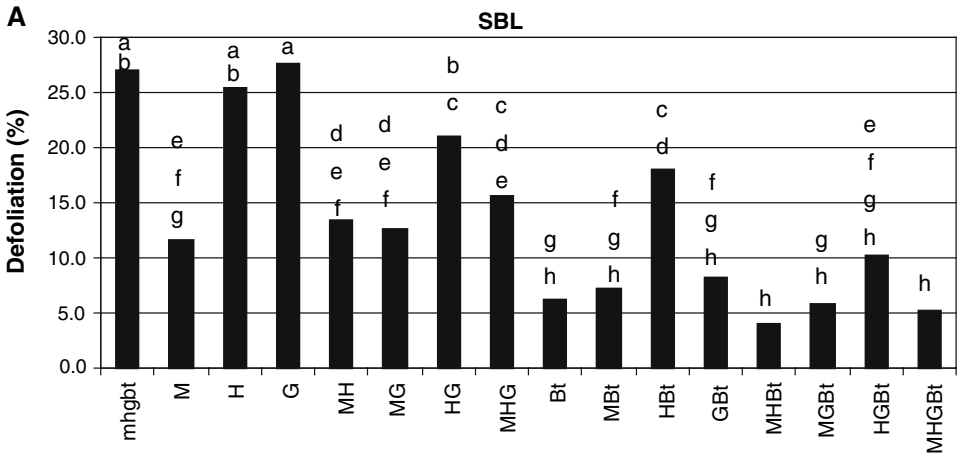
Effects on CEW larval weight in detached leaf assay

The mean larval weight (129.3 mg) produced on the NILs without Bt was over three times higher than that (36.0 mg) on the NILs with Bt (Fig. 4). QTL-M also had a significant main effect on reducing CEW larval weight (Table 2). The larvae fed on the NILs carrying QTL-M were 37% smaller (mean of 63.6 vs. 101.6 mg) than those fed on the NILs without QTL-M. The NIL with both QTL-M and Bt produced even smaller CEW (17.3 mg) compared with the NILs carrying QTL-M (99.1 mg) or Bt (43.3 mg) alone (Fig. 4). Thus the Bt effect was again enhanced by an endogenous insect resistance QTL in the Benning background.

The main effect of the antibiosis QTL-G resulted in a significant reduction of CEW larval weight (Fig. 4; Table 2). The mean larval weight (78.1 mg) from the NILs carrying QTL-G was significantly lower than that (87.2 mg) from the NILs without QTL-G. This effect of antibiosis QTL-G was not confirmed in a previous study (Zhu et al. 2006), but was detected here, likely because CEW cannibalism was largely prevented in this study. QTL-H did not have a significant main effect on reducing CEW larval weight (Table 2), as was expected since QTL-H is an antixenosis QTL.

Discussion

The comparisons among the Benning NILs possessing the various combinations of the three PI 229358-derived insect resistance QTLs provide valuable information on the confirmation of the QTL effects in the same elite background reported by Zhu et al. (2006). Consistent with their data for CEW, the current study found that QTL-M has the largest effect of the three QTLs on reducing defoliation by both SBL and CEW, and larval weight of CEW in the Benning genetic background. Both defoliation and larval weight were decreased by about one-third due to QTL-M, which is consistent with the effects for antixenosis and antibiosis originally estimated by Rector et al. (1998, 2000). The antibiosis QTL-G had a significant effect on reducing CEW larval weight as previously identified (Rector et al. 2000). Unexpectedly, this QTL was found here to have a significant effect on reducing defoliation by SBL and CEW in



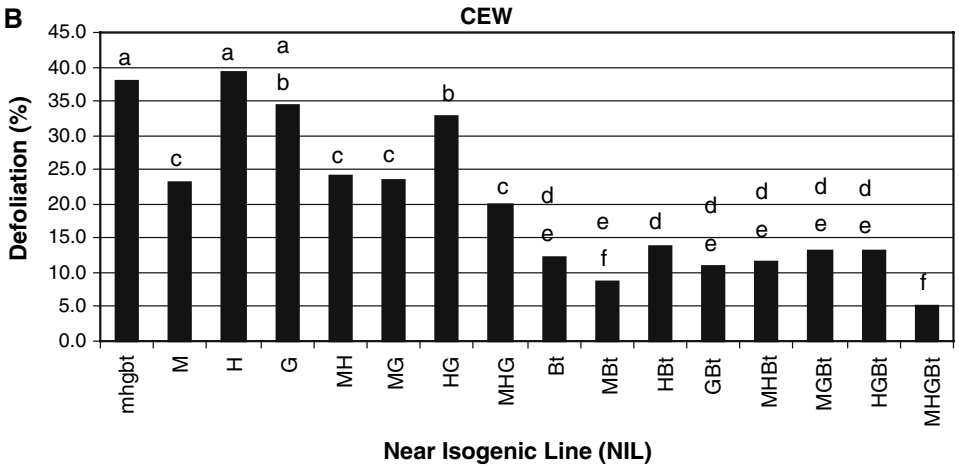


Fig. 3 Mean defoliation by (**A**) soybean looper (SBL) and (**B**) corn earworm (CEW) on 16 Benning soybean near-isogenic lines (NILs) in greenhouse assays. Defoliations of NILs with the same letters are not significantly different at the 0.05 probability level. An *upper case letter* indicates the presence of a resistance allele from a particular linkage group (*M*, *H*, or *G*) of PI 229358 or of a *cry1Ac* transgene from Bt *Lower case letters* in the first line to the *left* indicate that no known resistance alleles were present

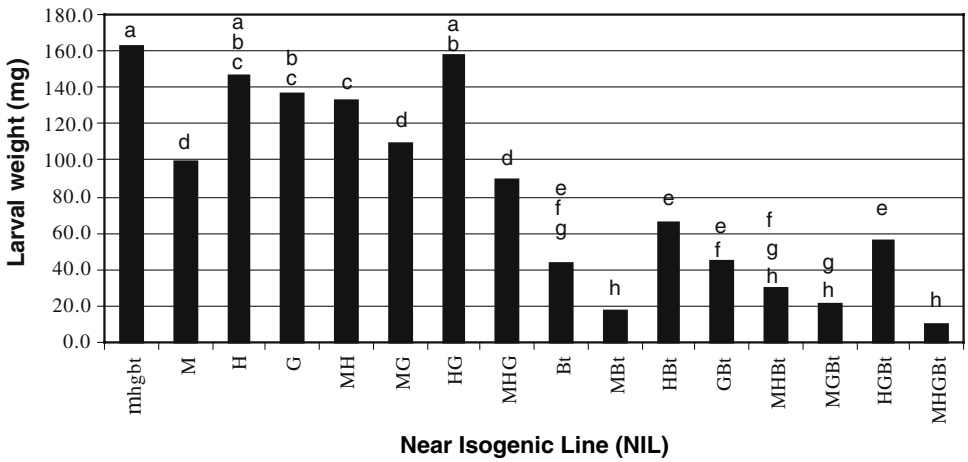


Fig. 4 Mean larval weight of corn earworm (CEW) fed on 16 Benning soybean near-isogenic lines (NILs) in growth chamber assays. Larval weights from NILs with the same letters are not significantly different at the 0.05 probability level. An *upper case letter* indicates the presence of a resistance allele from a particular linkage group (*M*, *H*, or *G*) of PI 229358 or of a *cry1Ac* transgene from Bt. *Lower case letters* in the first line to the *left* indicate that no known resistance alleles were present

some assays. This may be due to the possibility that in the field-cage and greenhouse tests each plot was separated, allowing both the antibiotic and the antixenotic effects to be expressed. Larvae stunted by antibiosis resistance factors are likely to consume less foliage. However, the larval weight was decreased only 10% due to QTL-G, and the effect of QTL-G was lower than that estimated in the original QTL analysis. The main effect of antixenosis QTL-H was not significant, which is consistent with previous studies (Walker et al. 2004; Zhu et al. 2006).

Consistent with the results of this study, previous work had determined that pyramiding Bt with QTL-M provided greater resistance than either Bt or QTL-M alone (Walker et al. 2004). One major goal of this work was to determine if adding additional QTLs would further enhance the resistance provided by the combination of Bt and QTL-M. Such

an enhancement was found for CEW under field cage conditions where the MHGBt NIL was the least defoliated of the NILs with various resistance QTL combinations (Fig. 2B). Although for SBL, and for CEW in the other assays, the use of additional QTLs for insect resistance did not provide statistically greater resistance than the use of the Bt and QTL-M combination, the Ben-MHGBt NIL showed the highest level of resistance in all tests except the greenhouse assay for SBL (Figs. 2, 3, and 4).

The field cage studies are likely more representative of actual field conditions than either the greenhouse or growth chamber assays, suggesting that pyramiding several insect resistance QTLs will be effective against important insect defoliators of soybean. Furthermore, while the addition of QTL-G and/or QTL-H did not provide greater effectiveness in the greenhouse or growth chamber assays, the possibility still remains that the use of these QTLs can contribute towards the durability of resistance, and thus enhance the "high dose/refugia" resistance management strategy (Walker et al. 2004) in the deployment of Bt soybean.

The use of pyramided resistance in soybean may be particularly relevant. A study by Kurtz et al. (2005) found that non-Bt soybean fields are important as a refuge for CEW and have contributed to the durability of the Bt effectiveness in Bt corn and cotton. Addition of soybean with a single Bt gene in production will increase the selection pressure that is already high from Bt corn and cotton. Therefore, soybean with a single Bt gene is unlikely to ever be approved for cultivation in the USA unless it carries additional sources of resistance pyramided with the Bt gene to supplement the resistance management strategy. Pyramiding a Bt gene with a gene such as a plant endogenous gene that controls pests through a totally different mode of action has shown to increase the resistance provided by only a Bt gene (Cooper et al. 2004; Douches et al. 2001; Sachs et al. 1996; Walker et al. 2004) and also has a potential to slow the evolution of insect resistance to Bt crops (Roush 1998).

Benning NILs carrying each individual QTL or all three QTLs have been released as germplasm for use as sources of single and multiple insect resistance QTLs in soybean breeding programs (Zhu et al. 2007). Historically, the use of insect resistance QTLs from PI 229358 was associated with linkage drag resulting in reduced seed yields (Lambert and Tyler 1999). However, QTL-M and -H were found not to have linkage drag on yield in the Benning NILs, but introgression of QTL-G did reduce yield (Warrington 2006; Zhu et al. 2007), indicating that markers closely linked to QTL-G are needed to identify the breakage of the linkage drag. In the meantime, the goal of breeding a high-yielding soybean with useful levels of insect resistance is finally achievable after decades of work through the use of marker-assisted selection to introgress QTL-M and -H. The combination of these QTLs with a Bt gene presents an additional strategy for the deployment of effective insect resistance in soybean.

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