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Testing transgenes for insect resistance using *Arabidopsis*

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Abstract

One possible strategy to delay the selection of resistant insect populations is the pyramiding of multiple resistance genes into a single cultivar. However, the transformation of most major crops remains prohibitively expensive if a large number of transgene combinations are to be evaluated. *Arabidopsis thaliana* is a potentially good plant for such preliminary evaluations. We determined that four major agricultural pests, *Spodoptera exigua*, *Helicoverpa zea*, *Pseudoplusia includens*, and *Heliothis virescens* grew as well when feeding on 'Landsberg Erecta' *Arabidopsis* as they did on plants of 'Cobb' soybean. Landsberg Erecta was then transformed with either a synthetic *Bacillus thuringiensis cryIA(c)* gene, or the cowpea trypsin inhibitor gene. Transformed plants were crossed to produce plants transgenic for both genes. Following quantification of transgene expression, the four caterpillar species were allowed to feed on wild-type plants, plants expressing either *cryIA(c)* or the cowpea trypsin inhibitor gene, or plants expressing both. Both genes reduced growth of the species tested, but *cryIA(c)* was more effective in controlling caterpillar growth than the cowpea trypsin inhibitor gene. The resistance of plants with both transgenes was lower than that of plants expressing the *cryIA(c)* gene alone, but higher than that of plants expressing the only the *CpTI* gene. This could be due to a lower concentration of Cry protein in the hemizygous F_1 plants. Thus, if the cowpea trypsin inhibitor had any potentiation effect on *cryIA(c)*, this effect was less than the *cryIA(c)* copy number effect. Alternatively, expression of the trypsin inhibitor gene could be antagonistic to the function of the *cryIA(c)* gene. Either way, these results suggest that the combined use of these two genes may not be effective.

Abbreviations: ANOVA, analysis of variance; BAPNA, benzoyl-DL-arginine-p-nitroanilide hydrochloride; BCIP, bromo-4-chloro-3-indolylphosphate; CTAB, hexadecyltrimethylammonium bromide; 2,4-D, 2,4-dichlorophenoxyacetic acid; EDTA, ethylenedinitrilotetraacetic acid; IAA, 1H-indole-3-acetic acid; LE, Landsberg Erecta; MES, 2-N-morpholinoethanesulfonic acid; NAA, 1-naphthaleneacetic acid; NBT, nitro blue tetrazolium; PCR, polymerase chain reaction; PVP, polyvinylpyrolidone; SDS, sodium dodecyl sulfate; Tris-HCl, Tris(hydroxy)methylaminomethane hydrochloride

Introduction

It is estimated that ca. 37% of all crop production worldwide is lost to pests and diseases annually, with 13% accounted for by insects alone [11]. Such is the case despite an estimated $7.5 billion annual cost to combat the problem [12]. One limitation in addressing crop loss due to pests and diseases is the almost exclusive reliance on chemical compounds. Prolonged and intensive selection pressure in large monocultures has brought about the evolution of insect resistance against a variety of commonly used insecticides.

Both conventional breeding and direct gene transfer to develop host plant resistance are promising ways

of offering protection against insect pests. These technologies can greatly reduce dependence on chemical control, and thus, reduce the cost of production, chemical contamination of the environment, and damage to non-target insects [5]. However, host plant resistance involving the transfer of antibiosis-related genes, such as those from the soil bacterium, *Bacillus thuringiensis* (*Bt*), encoding for insecticidal crystal proteins, are still protections based on lethal toxicants and can promote the evolution of resistance if allowed to exert consistent selection pressure on the target insects [20].

Insecticidal crystal proteins have the advantage of being extremely toxic to certain pests, and yet harmless to humans, most beneficial insects, and non-target organisms [29]. Insecticidal crystal proteins, also known as 'Cry' proteins or delta-endotoxins, are dissolved by a combination of high pH and proteases in the midgut of susceptible target insects. Toxic protein fragments, resulting from proteolytic cleavage of the endotoxins, bind to glycoprotein receptors on the brush border membrane of midgut epithelial cells. Subsequent toxin insertion causes an alteration of the permeability of the brush border membrane resulting in osmotic lysis, midgut paralysis, and insect death [1]. Certain studies involving external applications of *Bt* formulations have demonstrated the ability of some pests, such as the diamondback moth, *Plutella xylostella* L., to rapidly evolve resistance against *Bt* [29, 30, 32, 35]. Utilization of appropriate agricultural practices or cultural controls, including the pyramiding of various resistance genes into a cultivar, is one preventive strategy against the build up of resistance in insects [18].

Another resistance gene that can be utilized in a wide variety of crops is the cowpea trypsin inhibitor gene (*CpTI*) [15]. This gene encodes an 80 amino acid protein belonging to the Bowman-Birk type of double-headed serine protease inhibitor family. The inhibitor binds competitively to the binding site of a target enzyme, and subsequently renders the enzyme incapable of binding to and cleaving peptide bonds of proteins [8]. Serine proteinases (i.e., trypsin and chymotrypsin) are rarely found in plants, but are commonly detected in animals and microorganisms [6, 8]. This leads to the conclusion that serine proteinase inhibitors, such as *CpTI*, probably have no regulatory function in the plant, but act only as defensive agents against herbivores and pathogens by inhibiting their primary proteinases [6]. An insect's ingestion of proteinase inhibitors, however, does not necessarily eliminate proteolytic digestion in the insect's midgut. Instead, hyperproduction of proteolytic enzymes follows after the ingestion of inhibitors, which in turn can lead to reduced availability of essential amino acids for protein synthesis, leading to reduced growth and development [7, 11].

There have been several proposals to utilize genes encoding for proteinase inhibitors in combination with *Bt cry* genes, as a gene pyramiding strategy for crop protection [4, 16, 17]. Nevertheless, there is conflicting information as to the behavior of these two compounds when combined together, as previous studies using mixtures of proteinase inhibitors and *Bt* incorporated into artificial diets have reported both synergistic interactions [19] and no interactions [31]. Hoffman *et al.* [16] did a field evaluation of tobacco that was transgenic for either *Bt* or *CpTI*. They did not coexpress the two genes in a single plant to actually test if *Bt* insecticidal activity would be enhanced by the presence of the trypsin inhibitor. Hua *et al.* [17] successfully co-transformed tobacco with genes for a *Bt* Cry protein and a tomato proteinase inhibitor II. However, no further tests to investigate the effect of the gene combination on susceptible insects were reported. MacIntosh *et al.* [19] used a fusion protein consisting of a truncated *Bt* var. *kurstaki* insecticidal protein fragment and a trypsin inhibitor protein sequence from *Cucurbita maxima* (*CMTI*) in studying the effectiveness of this particular *Bt*-proteinase inhibitor gene combination. The protein fusion, which was expressed in tobacco, showed an enhanced insecticidal activity against target insects when incorporated into synthetic diets. However, no work was done to rule out the possibility that the increased insecticidal activity was simply the result of enhanced biological activity of the fused Cry protein instead of synergy, since trypsin inhibition capability of the fusion protein was not detected. In the same report, mixing very low concentrations of protease inhibitors (4 μM) with minimally effective amounts of purified *Bt* proteins increased the insecticidal activity of *Bt* proteins by 2- to 20-fold in synthetic diets. These results, however, cannot be extrapolated to *in planta* activity of transgenes for insecticidal proteins, since it is not known how additional compounds present in the plant might alter the efficacy of the toxins. For example, tannins in cotton decrease the effectiveness of *Bt* [23], while tannic acid in the bark of *Taxus baccata* (L.) increase it [14]. Thus, the effect of the combined use of proteinase inhibitor and *Bt* on insects feeding directly on plants remains untested.

This work was conducted to examine if the *CpTI* and *Bt* gene combination is potentially useful in gene pyramiding strategies against lepidopteran species, and to determine if this could be tested in arabidopsis. Given that transformation of arabidopsis is considerably easier than that of most major crops, arabidopsis may offer a system in which to efficiently test large numbers of gene combinations for use in breeding strategies. This work describes the use of *Arabidopsis thaliana* as the model system to test the relative effectiveness of the *CpTI* and *Bt* transgenes for insect control, and to investigate any potential synergism between the transgenes.

Materials and methods

Growth of soybean pests feeding on Arabidopsis

To ascertain the feasibility of using *Arabidopsis* as a useful plant for testing insect resistance genes, it was first necessary to determine if agriculturally important lepidopteran pests could feed and grow on *Arabidopsis* plants. Accordingly, four lepidopteran pests were tested on plants of either *Arabidopsis* 'Landsberg Erecta', 'Cobb' soybean, a cultivar susceptible to defoliation by these lepidopteran species, or GatIR81-296, a soybean breeding line with some insect resistance [3]. The lepidopteran species used in the experiment were corn earworm [*Helicoverpa zea* (Boddie)], soybean looper [*Pseudoplusia includens* (Walker)], beet armyworm [*Spodoptera exigua* (Hübner)], and tobacco budworm [*Heliothis virescens* (Fabricius)]. The original plan included the velvetbean caterpillar, [*Anticarsia gemmatalis* (Hübner)], but this species would not feed on *Arabidopsis* in a preliminary feeding test. Each of the four chosen lepidopteran species were used to infest six 5-cm pots of 3-week old *Arabidopsis* plants (each pot was enclosed in GA-7 box (Magenta Corp., Chicago, IL) with screened ventilation holes on top), six enclosed pots (styrofoam cups encased in sheer florist's conical mesh) of 2-week old Cobb, and six enclosed pots (same as for Cobb) of 2-week old GatIR81-296. For *Arabidopsis*, plant density was 6 to 8 plants per pot containing 1:1 sand/loam mixture (Hyponex loam, Hyponex, Maryville, OH). For the soybean lines, plant density was one first-trifoliolate-stage plant per cup of fumigated soil. Infestation was done the day eggs (USDA Insect Laboratory, Stoneville, MI) hatched after two days of incubation at room temperature, using five first-instar larvae per pot. The experimental design was randomized complete block design with six replicates. All plants were placed in the same controlled-environment room at 24 °C, 16 h photoperiod, 60 μE m^{-2} s^{-1} light intensity, and were kept for 7 days. Surviving larvae in individual pots were counted, weighed, and the capsule width and larval length measured. Analysis of variance (ANOVA) was performed for each insect type to determine any significant differences among treatments with respect to the four growth parameters mentioned. Fisher's LSD test, at $\alpha = 0.05$, was used to discriminate between treatments that had significantly different effects with respect to any of the four growth parameters. All statistical analyses were done using SAS PC (SAS Institute, Cary, NC).

Plasmid constructs

Two independent transformations were done using either pROK (*CpTI*+5) [15] or pH602-S.*Bt* [27]. Of these, pROK (*CpTI*+5) has the cowpea trypsin inhibitor gene driven by the 35S cauliflower mosaic virus (CaMV) promoter. The coding region for the mature trypsin inhibitor is 240 bp in length [25]. A 214 bp leader sequence and an untranslated 96 bp stretch at the 3′ end flank the coding region. The neomycin phosphotransferase II gene (*NPTII*), with a nopaline synthase promoter and polyadenylation terminal sequence, is located upstream of the *CpTI* expression cassette. The plasmid was mobilized into *Agrobacterium* strain LBA4404 via triparental mating.

The second plasmid, pH602-S *Bt* has the 1.8-kb toxin region of *cryIA(c)*, a synthetic *Bacillus thuringiensis* var. *kurstaki* gene that had been optimized for eukaryotic expression [27]. This is the same *Bt* construct that was used by Stewart *et al.* [27, 28] in transgenic soybean and canola. The *cryIA(c)*-coding sequence is 1853 bp long and is flanked by a 35S CaMV promoter and the Orf25 polyadenylation sequence. The entire *cryIA(c)* expression cassette is a 3.4 kb *Bgl*II fragment, ligated into pH602 using the vector's unique *Bgl*II site [26]. This vector is derived from pH575, but instead of a kanamycin resistance marker, it has a hygromycin phosphotransferase (HPT)-coding region driven by a CaMV 35S promoter and linked to an Orf25/26 polyadenylation sequence [22]. This plasmid was mobilized into *Agrobacterium* strain GV3850 via triparental mating.

Root culture transformation

The transformation procedure was based on a modified Valvekens *et al.* [34] protocol, using *Arabidopsis* ecotype Landsberg Erecta (LE). Seeds of *Arabidopsis* were surface-sterilized in 70% ethanol for 30 s, followed by immersion in a solution of 20% (v/v) commercial bleach (5% NaOCl) for 10 min, and three consecutive 1 min washes with sterile water. Around 50 to 100 surface-sterilized seeds were germinated on half-strength Murashige-Skoog (MS) medium [21]. To generate long root growth that was well suited for subsequent transformation steps, the seeds were concentrated in one area near the edge of 100 mm $\times$ 200 mm Petri dishes. The plates were propped up at a 45° angle, with seeds germinating from the top edge of the plates. Cultures were placed at 24 °C, with 23 h photoperiod, 60 μE m^{-2} s^{-1} light intensity from fluorescent lamp light source. Two to three weeks after germination, healthy root masses were collected from the plates. Roots were cut into 8-cm sections and plated on callus inducing medium (B5C at pH 5.8: B5 salts, B5 vitamins, 0.5 g l^{-1} MES, 1 mg l^{-1} 2,4-D, 0.05 mg l^{-1} kinetin, 20 g l^{-1} glucose, 2 g l^{-1} Gel-Rite (Merk, South Plainfield, NJ)). Each root strand was placed in contact with the medium to promote adventitious root growth. After 4 to 5 days of incubation on B5C, the root explants were ready for infection. The 8 cm sections were further cut into smaller pieces to create more wound sites for *Agrobacterium* entry. The newly cut sections were immersed in a diluted solution containing 1 part *Agrobacterium* YEP broth (10 g l^{-1} peptone, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl, Bacto agar 15 g l^{-1}) culture grown overnight from a single culture plate stab at 28 °C ($OD_{600} > 0.6$) to 10 parts liquid B5C medium for 30 min at room temperature. The explants were subsequently placed on sterile brown paper towels to blot off excess liquid, and were plated afterwards on fresh B5C medium. Four to five days later, the explants were transferred to shoot-inducing medium (MS9 at pH 5.8: MS salts, B5 vitamins, 0.5 g l^{-1} MES, 0.15 mg l^{-1} IAA, 20 g l^{-1} sucrose, 2 g l^{-1} Gel-Rite, and 2 mg l^{-1} filter-sterilized zeatin added last after the solution was autoclaved) supplemented with 100 mg l^{-1} cefoxitin, and 50 mg l^{-1} kanamycin sulfate or 35 mg l^{-1} hygromycin as a selection agent. One to two weeks later, bright green calli started appearing. These were transferred to fresh MS9 plates until the first buds emerged. When several shoots originated from one cluster, only one or two shoots from the cluster were taken for regeneration to avoid multiple clones of the same transformation event. Green shoots that were well-formed were transferred to root-inducing medium (B5R at pH 5.8: B5 salts, B5 vitamins, 0.5 g l^{-1} MES, 0.035 mg l^{-1} NAA, 20 g l^{-1} glucose, 2 g l^{-1} Gel-Rite) that contained 100 mg l^{-1} cefoxitin and the appropriate antibiotic for selection as described before. Culture periods on B5R were varied from 3 to 7 days. However, this variation in culture time did not appear to have any significant effect on root growth. Nonetheless, they were individually transferred to autoclaved, moistened, potting soil enclosed in GA-7 boxes after exposure to B5R, regardless of root growth progress. The GA-7 box lids were gradually opened 4–5 days after transferring the plants to soil to harden off the plants.

DNA extraction for Southern blot analysis

The procedure followed was a modification of Doyle and Doyle's CTAB DNA isolation protocol [10]. Ca. 0.2 g of leaf material from each putative transformant was ground in 500 μl of CTAB buffer (2% CTAB, 100 mM Tris-Cl, 20 mM EDTA, 1.4 M NaCl, 1% PVP). The solution was incubated in a 65 °C dry-bath for an hour. Two consecutive extractions using 1 volume chloroform/isoamyl alcohol (24:1 v/v) were done after incubation to minimize possible contamination of the final DNA solution with deleterious amounts of DNAse. The resulting aqueous phase in the second extraction was further processed according to standard protocol established by Doyle and Doyle [10].

Southern blot procedure

A modified protocol from Sambrook *et al.* [25] was followed. Ca 0.5 to 1 μg of genomic DNA per sample was digested overnight. DNA from putative pROK ($CpTI + 5$)-transformed plants was digested with *Eco*RI or *Hin*dIII. The *Eco*RI excises a 550 bp fragment containing a portion of the 240 bp *CpTI*-coding region. Alternatively, *Hin*dIII, recognizes only one cutting site within the T-DNA. For putative pH602-S.*Bt*-transformed plants, DNA was digested with either *Bgl*II or *Xba*I. While *Bgl*II excises a 3.4 kb fragment that contains the entire synthetic *Bt* expression cassette, *Xba*I recognizes a single cutting site within the T-DNA. After digestion, each set of samples cut with the same enzyme was loaded to a 1% agarose (w/v) gel prestained with 0.01% ethidium bromide (v/v). The gels were run overnight at 25 V, and were further treated according to standard Southern blotting

protocol. For putative pROK (*CpTI*+5) transformed plant DNA samples, the 550 bp *Eco*RI fragment containing a portion of the *CpTI*-coding region was used as probe. For putative pH602-S.*Bt*-transformed plant DNA samples, a 590 bp PCR product generated from within the 3.4 kb synthetic *Bt* cassette was used as probe. Autoradiograph exposure of the resulting filters (GeneScreen, Du Pont, Wilmington, DE) was done from 1 to 7 days, depending on the signal intensity, in a -80 °C freezer.

RNA extraction for northern blot analysis

A modified procedure based on Ausubel *et al.*'s lab manual protocol for extracting RNA from *CpTI*-expressing plants was used [2]. *Arabidopsis* seedlings grown on half-strength MS under kanamycin selection for two weeks were harvested. Ca. 1 g of leaf tissue was gathered and subsequently ground in liquid N_2. A 5 ml portion of homogenization buffer (0.1 M LiCl, 50 mM Tris pH 9, 10 mM EDTA, 2% SDS, 0.1 mg ml^{-1} proteinase K) were added. The resulting slurry was incubated at 37 °C for a maximum of 30 min. After incubation, two phenol/chloroform extractions were performed, followed by one chloroform extraction. To the final extract, 1/3 volume of 8 M LiCl was added, and was the incubated at -4 °C overnight. Subsequent isolation of poly(A)$^+$ RNA was performed as described in Sambrook *et al.* [25], using oligo(dT)-cellulose pellets (Gibco, Gaithersburg, MD) and disposable RNA isolation columns (Dispocolumn, BioRad, Richmond, CA).

Northern blot procedure

A modified protocol from Sambrook *et al.* [25] was followed. A formaldehyde gel was made as described in the manual [25]. Approximately 10 μg poly(A)$^+$ RNA were loaded per lane. The gel was run at 125 V for 3 h. RNA transfer to nitrocellulose filter (GeneScreen, DuPont, Wilmington, DE) and overnight filter hybridization using a 550 bp *Eco*RI fragment from *CpTI* as probe were performed as described in the manual [29]. Autoradiograph exposure was 7 days in a -80 °C freezer.

Immunostaining procedure

Immunostaining was done only on pH602-S.*Bt*-transformed plants due to the unavailability of antibodies against *CpTI*, the pROK ($CpTI + 5$) gene product of interest. Fifty mg of leaf material per sample were ground up in 100 μl of 0.1 M NaOH. The slurry was incubated on ice for 30 min, then neutralized with 20 μl of Tris-HCl pH 4.5. The slurry was spun to separate the clear supernatant protein extract which was transferred to a new tube. A 20 μl portion of the protein extract was used to determine the total crude protein content of the extracts through a Bradford assay kit (BioRad, Richmond, CA). Up to 20 μg of total crude protein per sample was loaded on to a 0.75 mm SDS slab gel of 15% acrylamide. The electrophoresis was performed on a vertical minigel system (BioRad, Richmond, CA) at room temperature, at 50 V, for ca. 3 h. Subsequent steps were performed according to the procedure devised by Pratt *et al.* [24]. Antibodies used for immunostaining were the following in sequence: rabbit anti-*Bt* serum (1:6000), goat anti-rabbit, rabbit anti-goat/alkaline phosphatase.

Cowpea trypsin inhibitor enzymatic assay

The trypsin inhibitor level was determined using a modified protocol by Geiger and Fritz [13]. Ca. 2 g of leaf material from pROK ($CpTI + 5$)-transformed plants and from wild-type arabidopsis were collected for protein extraction. Leaf material from each plant group was macerated in 25 ml phosphate buffer solutions (25 mM sodium phosphate, 10 mM EDTA-free acid, 1% Sarkosyl, 1% Triton X-100). A small amount from each extract was used in a Bradford assay to determine the total protein concentration. An assay solution consisting of the following was made in 1 ml cuvettes: 50 μl bovine trypsin solution (from a 50 mg l^{-1} stock solution in 1 mM HCl), an extract solution containing ca. 100= μg of soluble protein, and an appropriate amount of phosphate buffer to bring up the reaction volume to 800 μl. After briefly mixing the solution in the cuvettes, 200 μl BAPNA substrate solution (from a 1.74 mg/ml stock solution in phosphate buffer) was added to each cuvette. A *blank* solution for each sample extract was made. These blanks did not contain any substrate (BAPNA). Concurrently, a standard and its appropriate blank were also made. The cuvettes were incubated at room temperature for 10 min after the addition of all reagents. Absorbance readings at 410 nm were then gathered. This information was then used to calculate the moles of trypsin inhibited by 1 mg of total soluble protein extracted from the transgenic plants.

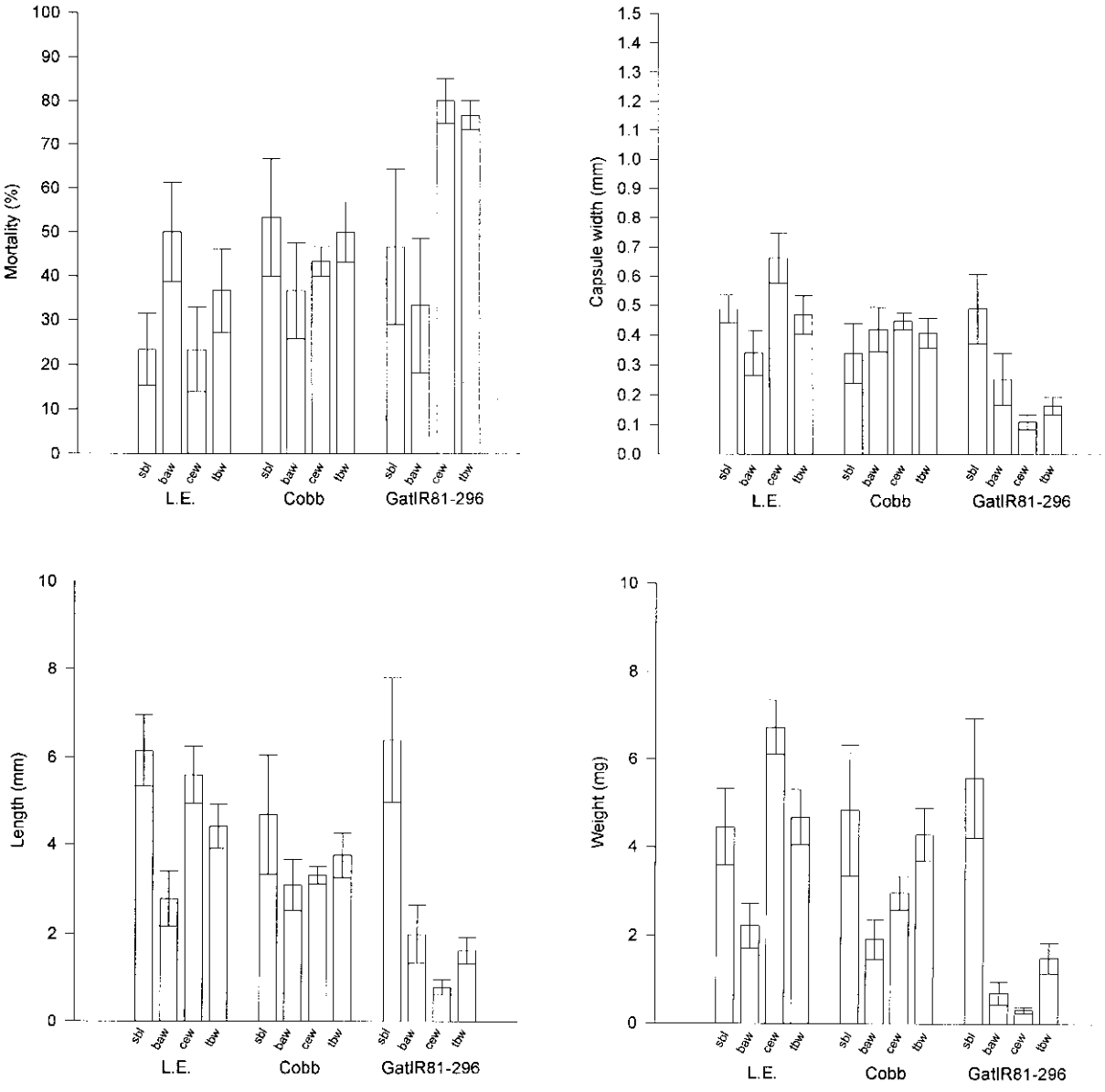

Figure 1. Weight, capsule width, length, and mortality of soybean looper (SBL), beet armyworm (BAW), corn earworm (CEW) and tobacco budworm (TBW) larvae feeding on *Arabidopsis* vs. two soybean genotypes. L.E. = *Arabidopsis* ecotype Landsberg Erecta; Cobb = susceptible soybean; GatIR81296 = resistant soybean. Bars represent standard errors.

Development of homozygous transgenic plants

Each putative transformant beginning from the T_0 generation was grown to maturity. Seeds from individuals were collected, surface-sterilized, and germinated on selection plates (100 × 20 mm) at 20 seeds per plant per plate (half-strength MS with either 50 mg l^{-1} kanamycin sulfate or 35 mg l^{-1} hygromycin). Depending on the number of seeds which successfully germinated, up to 12 seedlings were transferred individually to soil. Enough leaf material was gathered from each maturing plant so as to be able to do either a Southern analysis or a diagnostic PCR (*NPTII* primers were: 5′-AGAGGCTATTCGGCTATGACT [forward], 5′-CCATTTTCCACCATGATATTCC. The Syn.*cryIA(c)* primers were 5′-ATTTGGGGAATGTTTGGTCC [forward], 5′-ACAGTACGGATTGGGTAGCG) for confirmation of the presence of the genes of interest. For

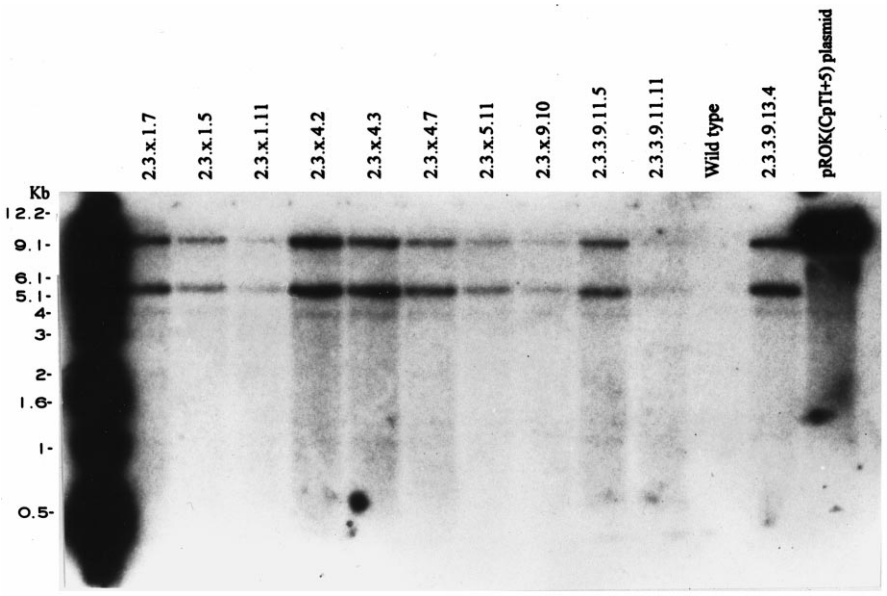

Figure 2. Southern blot of *CpTI*-positive T_3 plants. Genomic DNA digested with *Hin*dIII. *Hin*dIII cuts a single internal site within the T-DNA.

NPTII amplification, PCR reaction tubes had the following composition: 1 mM $MgCl_2$, 25 pmol of each primer, 2.5 units of *Taq* polymerase, and 100 mM dNTP. Thermocycler settings were 5 min at 95 °C (T_{melt}), 1 min at 53 °C ($T_{annealing}$), 1 min at 71 °C ($T_{extension}$). The expected amplified product for this reaction was a 550 bp fragment. For *Bt* fragment amplification, PCR reaction tubes had the same composition as above. However, the annealing temperature used was 54 °C. The amplified product for this reaction was a 590 bp fragment. Individual plants which were positive on either a Southern blot or a diagnostic PCR were allowed to mature. Individuals were tested for homozygosity of the transgene by germinating 25 to 30 progeny seeds from each individual on plates with the appropriate antibiotic. This approach in identifying homozygous lines was carried on until the T_3 generation for pROK($CpTI + 5$)-transformed plants and the pH602-S.*Bt*-transformed plants. At these particular generation stages, lines homozygous for the specific gene of interest had been identified. From these generation stages onwards, seeds coming from plants within a particular homozygous line were bulked.

Generation of F_1 hybrids

Reciprocal crosses were performed between *Bt* and *CpTI* homozygous lines. Partially opened flower buds of 3- to 4-week old plants were emasculated, after which their stigma were gently exposed for cross pollination. After cross pollination, the plants were allowed to mature and bear hybrid seeds, which were germinated on selection plates with both kanamycin and hygromycin at concentrations previously mentioned. Leaf material from three randomly selected seedlings that successfully germinated under selection was gathered for a western blot analysis and a trypsin inhibition assay to verify the presence and functionality of the transgenes.

Bioassay of transgenic plants

When all the needed plant materials were available, a second bioassay was conducted to detect the effects of the transgenes on the target insects. A randomized complete block design, blocked by week, was used. A total of six replicates, at a schedule of three replicates per week, was done. Each replicate contained 16 randomly distributed lepidopteran-type $\times$ diet treatment combinations, with each combination appearing once in every replicate. The diet treatments were (1) untransformed LE; (2) pROK($CpTI + 5$)-transformed arabidopsis; (3) pH602-S.*Bt*-transformed arabidopsis; and (4) F_1 progeny of 2×3. The lepidopteran species, growth conditions, larval infestation, and data collection and analysis were as described originally.

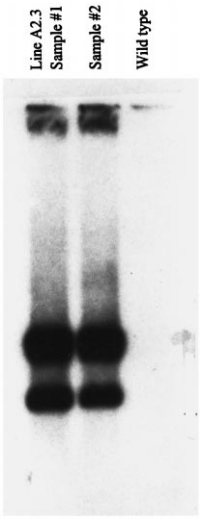

Figure 3. Northern blot of poly $(A)^+$ RNA from *CpTI*-positive T_3 plants.

Results and discussion

Previous studies using mixtures of proteinase inhibitors and *Bt* incorporated into artificial diets have reported both synergistic interactions [19] and no interactions [31]. Because there is interest in using these two types of genes in pyramiding strategies, we wanted to determine their effect on insects feeding directly on transgenic plants, as opposed to artificial diets.

Growth of soybean pests on Arabidopsis

The first goal was to determine the suitability of *Arabidopsis* as a host for lepidopteran pests of soybean. There was a significant interaction between insect species and diet for growth and development. For beet armyworm, soybean looper, and tobacco budworm feeding on *Arabidopsis*, growth and development were not significantly different from those of the same species feeding on Cobb soybean (Fig. 1), but corn earworm larvae feeding on *Arabidopsis* experienced greater growth and development than those feeding on Cobb soybean. With the exception of soybean looper, the remaining caterpillar species feeding on the insect-resistant GatIR81-296 soybean did poorly, as expected, when compared to those fed with either *Arabidopsis* or Cobb, across all fitness parameters considered.

In contrast to the growth and development parameters, there was no significant insect species by diet interaction for mortality, as all four species were affected in the same way by the plant they were feeding on. Regardless of species, insects that fed on GatIR81-296 had a significantly higher mortality rate than insects fed with either Cobb or *Arabidopsis*. There was no difference in mortality between insects fed on *Arabidopsis* and those fed on Cobb soybean (F-LSD, $\alpha = 0.05$). These results show that *Arabidopsis* is as good a host as soybean for various important lepidopteran agricultural pests, and in the case of corn earworm, it is better.

Transformants

Arabidopsis plants were transformed with either pROK($CpTI + 5$) or pH602-s.*Bt* via *Agrobacterium*-mediated gene transfer. Three individual lines containing pROK($CpTI + 5$) from separate transformation events were generated. One line did not produce viable seeds. Another line produced seeds which germinated under kanamycin selection, but had progeny which were physiologically smaller than their wild-type counterparts. The final line produced viable seeds which germinated under kanamycin selection and developed into plants with wild-type phenotypic appearance. Final verification of gene presence through Southern blot and adequate *CpTI* gene expression was done on the fourth generation. Southern blot analyses of the *Eco*RI-digested DNA showed a $<$ 500 bp truncated version of the *CpTI* gene in all samples tested. The blot with *Hin*dIII-digested samples revealed that there were two insertions of the *CpTI* gene in all the plants tested (Fig. 2). In testing for gene expression, two signals were detected using northern blot analysis, indicative of two populations of polyadenylated mRNA transcripts (Fig. 3). To verify the functionality of the *CpTI* gene products, a trypsin inhibition assay was done. For this, an analytical method employing BAPNA, a synthetic substrate that releases *p*-nitroaniline upon hydrolysis by bovine trypsin [13], was used. The average of two inhibition assay experiments indicated that 1 mg of total soluble protein from the selected transgenic line was able to inhibit 0.104 nM of trypsin which was almost twice the natural background activity of wild-type *Arabidopsis*.

For *Arabidopsis* transformation with pH602-S.*Bt*, three putative transformants were regenerated. Two of the three regenerants did not produce viable seeds. However, one line produced viable seeds that germinated on hygromycin. Diagnostic PCR of T_1 progeny using *Bt*-specific primers revealed the presence of the *Bt* gene fragment. The line was selfed to homozygosity until progeny, uniformly expressing the gene

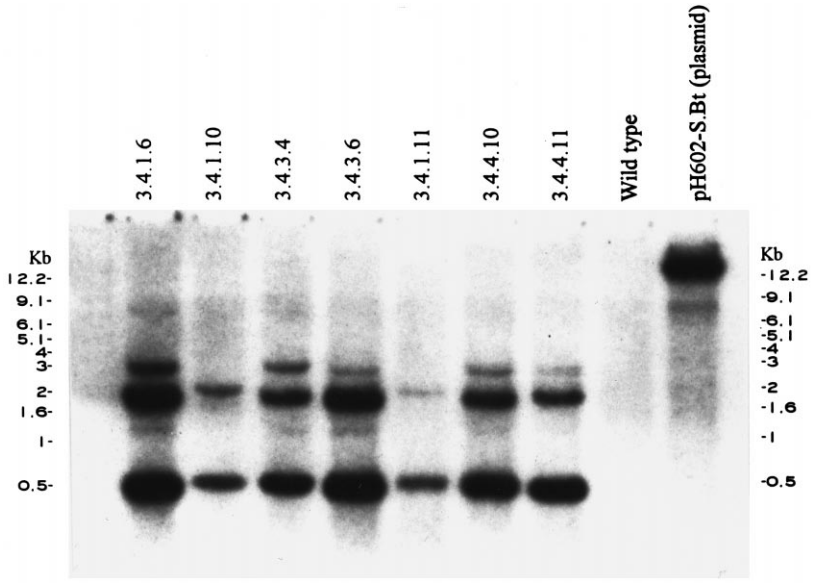

Figure 4. Southern blot of *Bt*-positive T_2 plants. Genomic DNA digested with *Xba*I. *Xba*I cuts a single internal site within the T-DNA, and is thus used to determine gene copy number.

were obtained. Southern blots of DNA samples digested with *Bgl*II showed that a duplicated version of the gene, based on observed length, was inserted. A Southern blot of the same batch of DNA samples, digested with *Xba*I, showed that the gene was inserted in at least three sites (Fig. 4). Western blots, using a polyclonal antibody, revealed the presence of a product of the expected size, 65 kDa, at a concentration of 0.005% of total soluble protein. This amount of *Bt* protein is enough to have a biological effect [33], but not so great as to overwhelm any effect of the trypsin inhibitor.

After the selected *CpTI* and *Bt* lines were crossed, the resulting progeny were subjected to a western blot analysis and a trypsin inhibition assay. The western blot revealed the presence of the crystal protein of the expected size (Fig. 5). However, expression was just about half the expression level of the homozygous *Bt* transformants (roughly 0.002% of total soluble protein). In the work by Stewart *et al.* on soybean [27] and canola [28], and by Carozzi *et al.* on tobacco [9], the same phenomenon was observed, in which individual plants hemizygous for the *Bt* gene contained half the amount of Cry protein that transgenic plants homozygous for *Bt* gene contained. In contrast, the F_1 plants showed a trypsin inhibition activity almost like that of homozygous *CpTI* transgenic plants. The average of two inhibition assays indicated that 1 mg of total soluble protein from the F_1 plants inhibited 0.124 nM

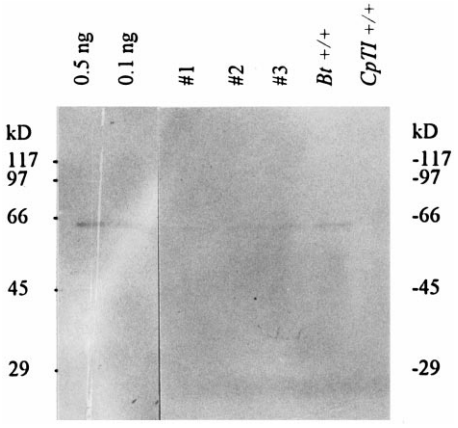

Figure 5. Western blot of F_1 hybrid progeny using CryIA(c) polyclonal antibody. The expected 66 kDa band is observed across all *Bt*-positive plants. Difference in expression levels between hybrid progeny and a *Bt*-homozygote is apparent. *CpTI* homozygote was tested as a negative control.

of trypsin, while the background activity of wild-type *Arabidopsis* was only able to inhibit 0.032 nM of trypsin.

Feeding trials with transgenic plants

A second bioassay was conducted to study the effects of resistance genes on selected lepidopteran species. ANOVA showed no insect by treatment interaction for

mortality and for all growth and development parameters ($p > 0.05$). Insects fed on wild-type arabidopsis performed consistently well across all fitness parameters, including mortality rate, as expected from the initial feeding trials (Fig. 6). All caterpillars fed transgenic plants grew significantly less than those fed wild-type plants. Caterpillars fed with arabidopsis homozygous for *Bt* were the least developed, followed by caterpillars fed with arabidopsis expressing both *Bt* and *CpTI*, then by caterpillars fed with arabidopsis homozygous for *CpTI*. Conversely, caterpillars fed with arabidopsis homozygous for *Bt* had the highest level of mortality, followed by caterpillars fed with arabidopsis expressing both *Bt* and *CpTI*, then by caterpillars fed with arabidopsis homozygous for *CpTI*.

The significant difference in growth between *Bt*-fed and *CpTI* or wild-type-fed caterpillars was clear across all growth parameters. However, the growth of caterpillars feeding on plants expressing both *Bt* and *CpTI* was inhibited to an extent intermediate to that of plants expressing only the *Bt* gene or only the *CpTI* gene. The trend for mortality levels across treatments was consistent with this observation. The mortality rate of caterpillars feeding on plants expressing both *Bt* and *CpTI* was intermediate to that of plants expressing only the *Bt* gene or only the *CpTI* gene. Although this outcome was initially unexpected, it is probably the result of the differences in the level of *Bt* expression due to *Bt* gene copy number. As mentioned previously, the level of *Bt* Cry protein in the F_1 hemizygotes was about half that of the *Bt*-homozygote parent. Also as mentioned previously, this same effect has been observed in transgenic tobacco, canola, and soybean. In these plants, the degree of growth suppression of caterpillars was correlated with the level of *Bt* Cry protein present [9, 27, 28]. In contrast, the *CpTI* gene deed not seem to be affected by copy number. Thus, if there is any effect of *CpTI* on the effectiveness of *Bt*, this effect is less than the effect due to *Bt* gene copy number.

Another plausible explanation for the observed results is that an antagonistic interaction exists between the products of the two resistance genes. *Bt* crystal proteins have to be cleaved into smaller fragments that possess the insecticidal activity in order to work. This processing requires the action of proteolytic midgut enzymes such as trypsin. The presence of proteins which can inhibit proteolytic activity of a key enzyme such as trypsin may lower the efficacy of *Bt*. This work does not rule out the possibility of this antagonistic relationship between the two resistance proteins. Expression of the two genes in *Arabidopsis* may have led to a lowered *Bt* efficacy.

The greater effectiveness of *Bt* over *CpTI* against corn earworm had been previously shown in transgenic tobacco [16]. We have confirmed these results for corn earworm when these two genes are expressed separately in LE, and extended this observation to other economically important pests: soybean looper, tobacco budworm, and beet armyworm. In all cases, *Bt* was significantly more effective than *CpTI* at inhibiting the growth of defoliating caterpillars, at the levels of expression tested.

Although *Bt* and *CpTI* have been coexpressed in the same plant before [17], this is the first report in which the combined effect of these two genes has been evaluated using caterpillars feeding directly on transgenic plants, as opposed to transgenic tissue incorporated into an artificial diet. Although the incorporation of these compounds into artificial diets had suggested there may be a synergistic interaction between these compounds [19], we were not able to detect any additive or synergistic effects. This reinforces the need for caution when extrapolating from artificial diet experiments to actual transgenic plants expressing resistance genes. Nevertheless, we cannot rule out that additive or synergistic effects may be present at different expression levels, different *Bt*/protein inhibitor combinations, or if expression had been in a different plant species.

Finally, arabidopsis can be a host plant for some economically important caterpillar species. It appears it can be a useful species in which to easily, effectively, and economically evaluate the potential effectiveness of transgenes for insect control. In this way, it may be possible to economically evaluate and preselect those genes or gene combinations showing the greatest potential for insect control. It has to be stressed, however, that it is difficult to entirely rely on results from diagnostic tests using *Arabidopsis* to predict the usefulness of resistance genes in actual crops. Firstly, not all insect pests will feed on arabidopsis (e.g. velvetbean caterpillar). Secondly, certain chemicals in some crops may be present in levels that can effect the efficacy of an insecticidal protein (e.g. cotton tannins and *Bt*). It is therefore, prudent to view results from such diagnostic tests merely as guides toward the preselection of useful resistance genes for deployment into actual crops.

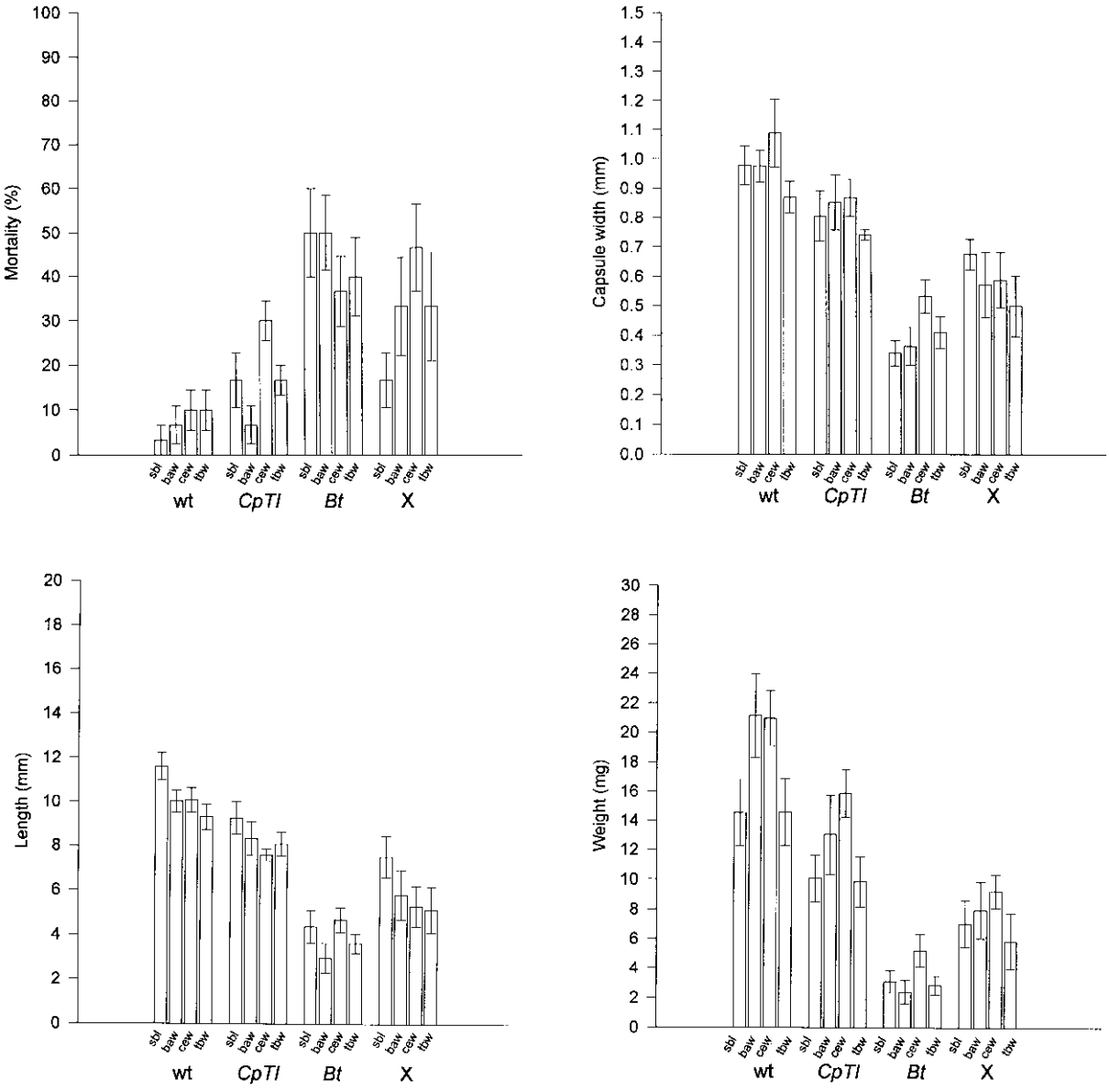

Figure 6. Weight, capsule width, length, and mortality of soybean looper (SBL), beet armyworm (BAW), corn earworm (CEW) and tobacco budworm (TBW) larvae feeding on Landsberg Erecta *Arabidopsis*. wt = wild-type; CpTI = expressing *CpTI*; Bt = expressing *Bt*; and x = expressing both. Bars represent standard errors.

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