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Author(s): Huarong Li, Lawrent L. Buschman, Fangneng Huang, Kun Yan Zhu, Bryony Bonning, and Brenda Oppert
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DiPel-Selected *Ostrinia nubilalis* Larvae Are Not Resistant to Transgenic Corn Expressing *Bacillus thuringiensis* Cry1Ab

HUARONG LI,[1,2] LAWRENT L. BUSCHMAN,[1,3] FANGNENG HUANG,[4] KUN YAN ZHU,[1] BRYONY BONNING,[2] AND BRENDA OPPERT[5]

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ABSTRACT The survival of KS-SC DiPel-resistant and -susceptible European corn borer, *Ostrinia nubilalis* (Hübner), was evaluated on different tissues from corn, *Zea mays* L., hybrids, including a nontransgenic and two transgenic corn plants (events MON810 and Bt11) expressing high doses of *Bacillus thuringiensis* (Bt) Cry1Ab. The survival of Bt-resistant and -susceptible third instars was similar after a 5-d exposure to transgenic plant tissues. Survivors eventually died when returned to Bt corn tissues, but many were able to continue development when transferred to non-Bt corn tissues. Survival of resistant and susceptible larvae also was evaluated in bioassays with dilutions of leaf extracts from the three corn hybrids incorporated in an artificial diet. In these assays, survival was significantly higher for resistant *O. nubilalis* neonates at three of the five dilutions compared with the susceptible strain, but the resistance ratio was only 2.2- and 2.4-fold for MON810 and Bt11, respectively. The data demonstrate that Bt-resistant and unselected control *O. nubilalis* larvae were similar in susceptibility to MON810 and Bt11 event corn hybrids. Although we were unable to evaluate the Cry1Ab protein that larvae were exposed to in the transgenic tissue because of company restrictions, Cry1Ab protoxin produced in *Escherichia coli* was incubated with extracts from non-Bt corn leaves to simulate the in planta effect on the transgenic protein. Cry1Ab protoxin was hydrolyzed rapidly by enzymes in the corn extract into peptide fragments with molecular masses ranging from 132 to 74 kDa, and eventually 58 kDa. Overall, these data suggest that plant enzymes hydrolyze transgenic toxin to one that is functionally activated. Therefore, resistant insect populations with reduced proteinase activity do not seem to pose a threat to the efficacy of commercial MON810 and Bt11 corn hybrids.

KEY WORDS *Ostrinia nubilalis*, *Bacillus thuringiensis*, resistance mechanisms, resistance management, proteinase-mediated resistance

The European corn borer, *Ostrinia nubilalis* (Hübner), is one of the most damaging corn, *Zea mays* L. pests in North America, and it is a major target of insecticidal *Bacillus thuringiensis* (Bt) sprayable preparations, such as DiPel ES (Valent BioSciences Corporation, Walnut Creek, CA), and transgenic Bt corn hybrids. The KS-SC strain of *O. nubilalis* was selected in the laboratory with DiPel, and it is 254-fold resistant to one of its constituent Cry toxins, Cry1Ab protoxin (Huang et al. 1997, Li et al. 2005a). However, in greenhouse experiments, KS-SC resistant insects did not survive on transgenic corn plants expressing Cry1Ab (Huang et al. 2002). The inability of the resistant strain to survive on transgenic corn implied that the plants were toxic either because the dose was high enough to suppress the resistance, or that cofactors were present in the plant that negated resistance.

Resistance to Bt toxins in insects is primarily due to changes in insect proteinases or toxin receptors (for review, see Ferré and Van Rie 2002). Bt toxin mode of action is mediated by 1) solubilization of the crystallized protoxins, 2) activation of the solubilized protoxins, and 3) toxin binding to the lining of the midgut (Gill et al. 1992). More recently, an additional step involving toxin oligomerization has been proposed (Bravo et al. 2004). Alternatively, a new mode of action of Bt toxins suggested that toxin mode of action is conveyed through toxin monomer-receptor signaling via second messenger (Zhang et al. 2006). In both models, for Bt protoxins to be toxic they must be solubilized and hydrolyzed by insect gut proteinases.

Resistance to Bt toxins can be mediated by reduced proteinase activity in the insect midgut, presumably because it reduces the quantity of activated toxin present to poison the midgut (Oppert et al. 1997;

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[1] Department of Entomology, 123 Waters Hall, Kansas State University, Manhattan, KS 66506.

[2] Department of Entomology, 418 Science II, Iowa State University, Ames, IA 50011-3222.

[3] Kansas State University Southwest Research and Extension Center, 4500 E. Mary St., Garden City, KS 67846-6028.

[4] Department of Entomology, 404 Life Sciences Bldg., Louisiana State University Agricultural Center, Baton Rouge, LA 70803.

[5] Corresponding author: Grain Marketing and Production Research Center, USDA–ARS, 1515 College Ave., Manhattan, KS 66502 (e-mail: bso@ksu.edu).

Oppert, 1999; Li et al. 2004b, 2005a). Resistance also is mediated by a reduction in the number of toxin binding sites, a reduction in toxin affinity to receptors in the insect midgut epithelial membrane, or both (Ferré et al. 1991, Lee et al. 1995, Zhao et al. 2000, González-Cabrera et al. 2003, Morin et al. 2003). Resistance to Cry1Ab and Cry1Ac in the KS-SC strain is associated with reduced protoxin activation due to reduced trypsin-like proteinase activity in the gut (Huang et al. 1999a; Li et al. 2004b, 2005a), but not with a loss of midgut receptor binding sites or reduced binding affinity for toxin (Li et al. 2004a). Resistance to Cry1Ab can be reduced >95% if KS-SC insects are fed activated toxin, indicating that KS-SC insects lack enzymes to efficiently hydrolyze Cry1Ab protoxins (Li et al. 2005a).

The objectives of this study were to determine whether the survival of DiPel-resistant *O. nubilalis* larvae differs on intact Bt corn plants compared with different corn tissues or extracts. Our data demonstrate that the resistant strain of *O. nubilalis* was almost equally as sensitive to MON810- and Bt11-event corn plants as the unselected control insect strain. Our experiments further demonstrate that enzymes in corn extracts can hydrolyze transgenic Cry1Ab protein to a functionally activated form that is able to overcome the resistance mediated by reduced protoxin activation in the resistant strain.

Materials and Methods

Insects. The laboratory strain of *O. nubilalis* was started from egg masses collected near St. John, KS, in 1995, reared on a meridic diet (Reed et al. 1972). A DiPel-resistant strain (KS-SC) was selected from this susceptible strain by exposing neonates to low doses of DiPel ES (*Bacillus thuringiensis* subsp. *kurstaki* HD-1, Abbott Laboratories, Abbott Park, IL) incorporated into the diet at rates causing 80–95% mortality (Huang et al. 1997). When this study was conducted, the susceptible strain had been in culture for ≈40 generations without exposure to any chemical insecticides or Bt toxins, and the resistant strain had been exposed to 35 cycles of selection. The resistant strain was 30- to 70-fold resistant to DiPel and 205- to 254-fold resistant to Cry1Ab protoxin (Li et al. 2005a, 2005b).

The *O. nubilalis* voucher specimens (voucher no. 079) are located in the Kansas State University Museum of Entomological and Prairie Arthropod Research, Department of Entomology, Manhattan, KS.

Bioassays on Corn Tissues. The corn hybrids used in this study were obtained from commercial seeds: Cargill 7770 (non-Bt), Cargill 7821 Bt (YieldGard, event MON810, Cargill, Minneapolis, MN) and NK N4242 Bt (YieldGard, event Bt11, Syngenta, San Diego, CA). Cargill 7770 is a near isogenic line to Cargill 7821 Bt corn. Both Bt corn hybrids expressed Cry1Ab protein (Agbios 2002). The corn plants were grown in the greenhouse in a standard soil mixture in 18.9-liter pots (two plants per pot). At the whorl stage (10–12 leaf), the stalks, leaf sheaths, expanded leaves (nodes 5–7), and unexpanded new (nongreen) leaves were excised and placed in resealable plastic bags to avoid desiccation. At the tassel stage, leaf sheath, husk, stalk, and ears were excised from the middle part of three-six of the same hybrid plants.

Four pieces of each of the corn tissues were cut into 5-cm-long sections to fit into plastic dishes (90 by 80 by 70 mm) (Bio-RT-8 & Bio-CV-16, CD International, Pitman, NJ) with four layers of filter paper moistened with distilled water. The stalks and ears were split in half to expose the inner tissues to the larvae. Larvae reared on meridic diet to the third instar were added to each of the tissue samples (10 of each strain), and they were maintained in a growth chamber at 25°C, 60% RH, and a photoperiod of 16:8 (L:D) h. Larval survival was recorded after 5 d. Larvae were considered dead when they looked darkened or did not move when touched. There were three to six replications, and a single plant provided all the tissues for a single replication. Some larvae were still alive after 5-d exposure to the Bt corn tissues, and survivors were collected and divided into two groups. One group was reared on the same type of Bt corn, whereas the other was reared on non-Bt corn. The survival of larvae was recorded after an additional 6-d exposure.

Bioassays on Diets Containing Corn Leaf Extracts. To facilitate dilution and an even distribution into the diet, leaf extracts from Bt and non-Bt corn plants also were used in laboratory bioassays. Leaf extracts of Bt and non-Bt corn plants were obtained from another set of plants from the same three corn hybrids listed previously. At the 10–12-leaf stage, leaf blades were collected, cut into 1–2-cm sections, and frozen at −80°C for 1 or 2 d. Frozen leaf tissue (100 g) was placed in a heavy-duty blender (model CB15, Waring Laboratory, Torrington, CT) with 200 ml of cold distilled water and blended for 5 min at room temperature. The suspension was then filtered through a layer of nylon screen (324 mesh). The filtered liquid was blended again with another 100 g of leaves (to concentrate the leaf proteins in the liquid). The final filtrate was centrifuged at $160 \times g$ for 10 min at 4°C to remove tissue debris and chlorophyll. The supernatant was further concentrated by lyophilizing at approximately −50°C and $2–3 \times 10^{-3}$ Mbar vacuum. Five dilutions were prepared for each of the leaf extracts (1-, 2-, 4-, 8-, and 24-fold) by using phosphate-buffered saline (PBS), pH 7.4, containing 0.1% (wt:vol) bovine serum albumin. Four milliliters of each dilution were added to 20 ml of standard diet (45°C) and incorporated by drawing into a syringe and expelling several times (Huang et al. 1997, Li et al. 2005b). Each diet was dispensed into 48 cells on a 128-cell bioassay tray (Bio-Ba-128, C-D International, Pitman, NJ). A control diet was prepared using 4.0 ml of PBS. After the diet solidified, one neonate (<24 h old) was placed on the diet surface in each cell, and the cells were sealed with perforated plastic covers (C-D International), and diet trays were placed in the growth chamber. Larval survival and body weight were recorded at 7 d after insect inoculation, and the weight of larvae reared on test diets were calculated as a percentage of the average weight of larvae reared on control diet.

For each *O. nubilalis* strain, there were three replications for each dilution, and 16 neonates for each replication in each bioassay. The bioassay was independently repeated for both MON810 and Bt11 Bt corn toxin preparations. To determine the effect of leaf extracts from non-Bt corn plants, one bioassay was conducted with three replicates using non-Bt corn leaf extract.

Hydrolysis of Cry1Ab Protoxin by Corn Extracts. To determine whether enzymes in corn leaf tissues can hydrolyze Cry1Ab protoxin during the extraction process or in planta, 3 g of fresh corn leaf tissues from a whorl-stage greenhouse-grown non-Bt corn plant (Cargill7770) was finely ground in 6 ml of deionized water with a pestle and mortar on ice. The ground mixture was filtered through two layers of cheesecloth and then centrifuged at 20,000 $\times$ *g* for 10 min at 4°C. Total protein concentration of the leaf extract (supernatant) was 7.6 mg/ml as determined by the Bradford method (Bradford 1976). Cry1Ab protoxin was obtained from recombinant *Escherichia coli* (ECE54 isolate, *Bacillus* Genetic Stock Center, The Ohio State University, Columbus, OH), by using a procedure described by Li et al. (2005a). Ten milligrams of Cry1Ab protoxin crystal was solubilized in 1 ml of 50 mM Na_2CO_3, pH 10 at 37°C for 3 h and then centrifuged at 10,000 $\times$ *g* for 10 min at 23°C. The supernatant was dialyzed overnight by using a 10,000 molecular mass exclusion (Slide-A-Lyzer Dialysis Cassette, Pierce Chemical, Rockford, IL) in deionized water at 4°C with constant stirring. The protein concentration of the dialyzed Cry1Ab protoxin solution was 4.7 mg/ml. Twenty-five microliters of corn leaf extract (190 μg of total protein) was incubated with 5 μl of protoxin solution (23.5 μg of total protein) at 23°C for 5 and 15 s, and for 1, 5, and 30 min. In a second experiment, 25 μl of corn leaf extract was incubated with 5 μl of protoxin under similar conditions at 4, 23, and 37°C for 3 h. Each experiment included negative controls of 25 μl of leaf extract plus 5 μl of water, 5 μl of protoxin solution plus 25 μl of water at 23°C for 30 min and 3 h, and 5 μl of protoxin solution plus 25 μl of water without incubation before heating. Positive control was a bovine trypsin-activated Cry1Ab (Li et al. 2004b). After the designated time, reactions were terminated by heating the samples at 95°C for 10 min. After cooling, sodium dodecyl sulfate (SDS) sample buffer was added, and samples were reheated at 95°C for 5 min. Samples were subjected to 8% SDS-polyacrylamide gel electrophoresis (Laemmli 1970), and then they were transferred to polyvinylidene difluoride membranes. Western blot analysis was performed using rat polyclonal antiserum against Cry1Ac (1:2,000) and enhanced chemiluminesce (Pierce Chemical).

Data Analysis. The survival of third instars of *O. nubilalis* was analyzed in a three-way analysis of variance (ANOVA) with corn hybrid, insect strain, and corn stage as the three main factors, and with data for tissues pooled for each corn hybrid (PROC GLM, SAS Institute 1990). The survival of third instars also was analyzed separately for whorl and tassel stages in a three-way ANOVA, with corn hybrid, insect strain, and corn tissue as the three main factors. There was a significant interaction between corn hybrid and corn tissue, so these data were reanalyzed separately for each corn hybrid, by using a two-way ANOVA with insect strain and corn tissue as the two main factors. The second analysis provided mean separations using a least significant difference (LSD) test across insect strain and corn tissue within each corn hybrid and growth stage.

The survival and growth inhibition data for larvae feeding on diets treated with different dilutions of corn leaf extracts from each corn hybrid were analyzed in three two-way ANOVA, with dilution and insect strain as the main factors (PROC GLM, SAS Institute 1990), similar to a previous analysis (Tabashnik et al. 1987). Larval survival was corrected for control mortality using Abbott's method (Abbott 1925). Probit analysis of these data were not possible because we were unable to obtain permission to determine Cry1Ab concentrations in the transgenic plants.

Results

Bioassay on Corn Tissues. The survival of third instars of *O. nubilalis* larvae on different corn hybrids was analyzed in a three-way ANOVA, averaged across tissues (Fig. 1). The main effect of insect strain was not significant ($P = 0.0719$), but corn hybrid and corn stage were highly significant ($P < 0.0001$). The data were reanalyzed separately for the two corn stages, and the main effect of insect strain again was not significant ($P = 0.0599$ for whorl stage and $P = 0.2335$ for tassel stage), whereas corn hybrid ($P < 0.0001$ for both stages) and corn tissue ($P < 0.0001$ for both stages) were highly significant. The interaction between corn tissue and corn hybrid again was significant ($P < 0.0001$ for whorl stage and $P = 0.0029$ for tassel stage), apparently due to the relatively high larval survival on all non-Bt corn tissues versus the low and variable survival of larvae on tissues from the Bt hybrids. There were slight variations in survival across the different tissues, but in only one case was the mean survival of the two insect strains significantly different, and that was with new leaves of whorl stage-Bt11 corn plants. This observation suggests that new leaves may express higher levels of natural resistance factors.

Among the third instars of *O. nubilalis* that survived 5 d of exposure on MON810 tissues, all that were transferred to new MON810 tissues died during an additional 6-d exposure (Fig. 2A). In contrast, 27.5% of resistant and 22.5% of susceptible larvae survived on non-Bt corn tissues after 6 d and continued to develop to the pupal stage. Among the third instars that survived 5 d of exposure on Bt11 tissues, $\approx$8% of both resistant and susceptible larvae returned to Bt11 corn tissues were alive after 6 d (Fig. 2B), but these larvae did not gain weight, and all died in a few days. In contrast, 55.2% of resistant and 44.0% of susceptible larvae transferred to non-Bt corn were alive after 6 d and developed to the pupal stage.

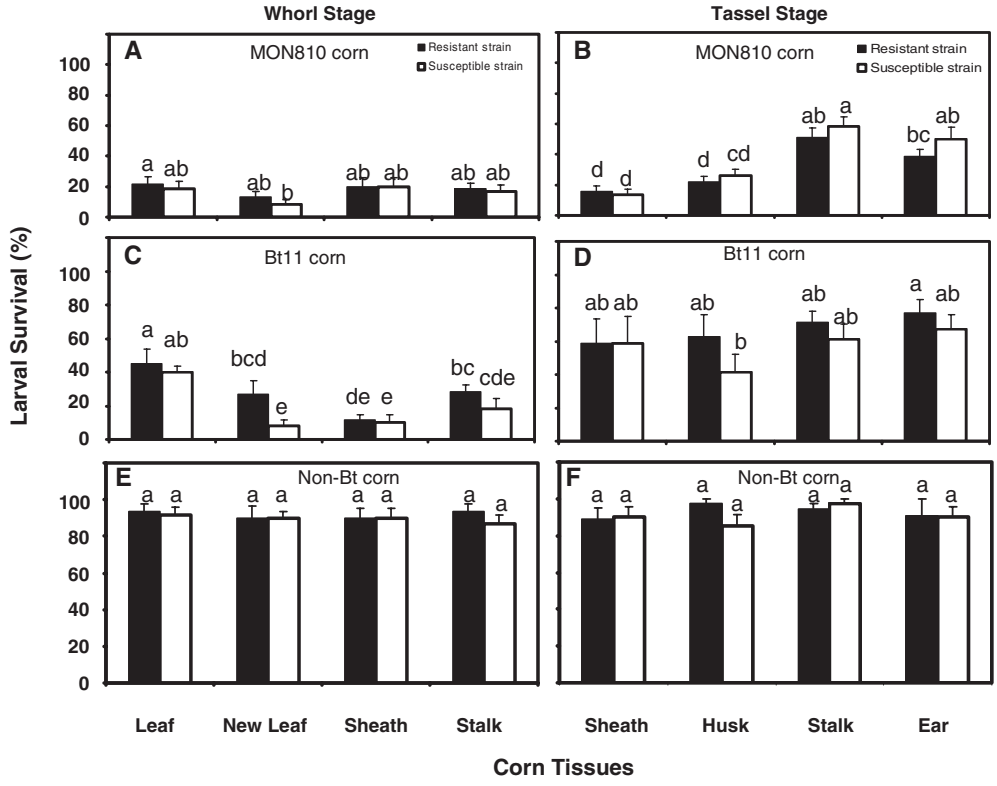

Fig. 1. Percentage of survival of third instars of KS-SC-resistant and -susceptible *O. nubilalis* after 5-d exposure to different corn tissues. In each panel. the means with a same letter are not significantly different at $P = 0.05$ level (ANOVA, LSD test).

Bioassays on Diets Containing Corn Leaf Extracts. In the more precise Bt-corn extract bioassays, only marginal (approx. two-fold) resistance was detected in the KS-SC strain. In a two-way ANOVA of survival of *O. nubilalis* neonates across the five dilutions of Bt corn leaf extracts, the main effect of insect strain was highly significant for both MON810 ($P < 0.0001$) and Bt11 ($P < 0.0001$) extracts. The interaction between insect strain and dilution was highly significant, because there was no mortality of resistant larvae at the two lowest concentrations. Survival of *O. nubilalis* neonates on diets containing MON810 Bt corn leaf extract increased from 25 to 100% for the resistant strain and from 10 to 93% for the susceptible strain across the five dilutions (Fig. 3). Survival of *O. nubilalis* neonates on diets containing Bt11-corn leaf extract increased from 15 to 100% for the resistant strain and from 5 to 95% for the susceptible strain with

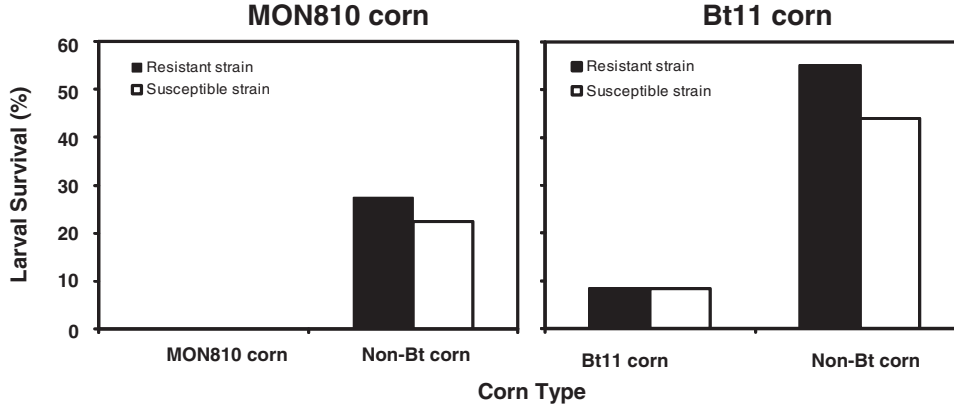

Fig. 2. Percentage of survival of the third instars of *O. nubilalis* exposed to Bt corn tissues for 5 d and then transferred to the same Bt corn tissues or to non-Bt corn tissues for another 6 d. Means were calculated from two replications.

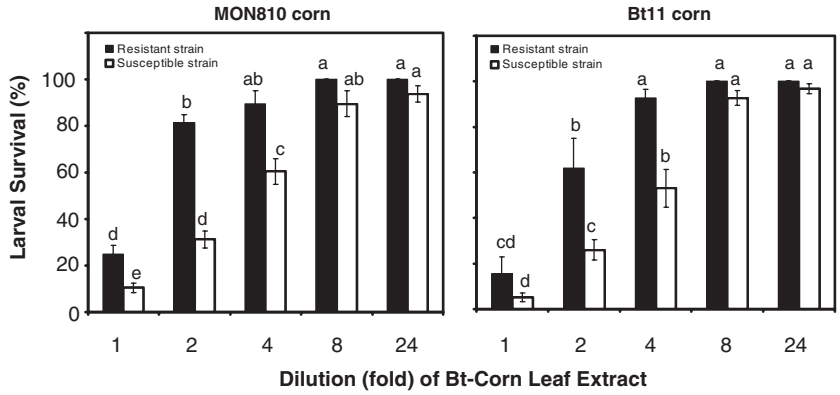

Fig. 3. Percentage of survival of neonate *O. nubilalis* after 7-d exposure to artificial diets containing Bt corn leaf extracts at variety of dilutions. In each panel the means with a same letter are not significantly different at $P = 0.05$ level (ANOVA, LSD test).

increasing concentrations. The mean comparisons indicated that the survival was significantly higher for the resistant strain at the first three dilutions of MON810 and second and third dilutions of Bt11. The resistance ratios (survival for resistant/susceptible) were 2.2- and 2.4-fold for the first three dilutions of MON810 and Bt11, respectively.

Mortality and growth inhibition of *O. nubilalis* neonates placed on diets containing non-Bt corn leaf extracts was minimal, except at the two highest concentrations (Table 1, dilutions 1 and 2). At the highest concentration, the survival of neonates was 95.8% for resistant and 87.5% for susceptible strains, and the relative weights were 32.0 and 29.9% of control weights for the resistant and susceptible strains, respectively.

Hydrolysis of Cry1Ab Protoxin by Corn Extracts. Cry1Ab protoxin incubated with non-Bt corn leaf extracts was hydrolyzed to several peptide fragments, with molecular masses ranging from ≈132–74 kDa (Fig. 4A). The ≈74-kDa fragment was larger than the stable fragment obtained with bovine trypsin, ≈58 kDa (Li et al. 2004b). Protoxin hydrolysis by the leaf extract enzymes occurred quite rapidly, within 5 s. When the incubation of protoxin and leaf extract was prolonged to 3 h, the ≈58-kDa fragment was obtained, and nearly all of the protoxin was hydrolyzed (Fig. 4B). Negative controls of Cry1Ab protoxin only indicated some minor hydrolysis of the protoxin without enzyme treatment.

Table 1. Effect of leaf extract from non-Bt corn plants on larval survival and growth of KS-SC-resistant and -susceptible *O. nubilalis*

Dilution (-fold)	*n*	Survival (%)		Relative wt per larva (%) (mean ± SE)[a]	
		Resistant strain	Susceptible strain	Resistant strain	Susceptible strain
Control	288	100	100	100a	100a
24	288	100	100	86.5 ± 11.8ab	89.7 ± 5.09ab
8	288	100	100	106 ± 1.17a	84.2 ± 16.0ab
4	288	100	100	93.2 ± 15.4ab	78.3 ± 15.7ab
2	288	100	95.8	66.4 ± 16.8bc	47.8 ± 7.33dc
1	288	95.8	87.5	32.0 ± 5.56d	29.9 ± 5.66d

[a] Means not followed with a same letter within a column are significantly different at $P = 0.05$ (ANOVA, LSD = 30.32).

Discussion

In a previous study, both KS-SC and Bt-susceptible *O. nubilalis* third instars died after 12–15 d of exposure to intact Bt corn (events MON810 and Bt11) (Huang et al. 2002). However, there were a few larvae that survived 12–15 d on event Bt176 (expressing Cry1Ab) and event DBT418 (Cry1Ac). In all cases, the rates of survival were similar for resistant and susceptible larvae. In this study, a shorter period of exposure was selected to increase the sensitivity for detecting differences in larval survival between the two strains. Individual corn tissues were compared to determine whether specific tissues had a lower expression of Bt protein, as has been suggested in a previous study (Koziel et al. 1993). However, the rates of survival were similar for resistant and susceptible *O. nubilalis* on different tissues selected from the two hybrids. Thus, results from the current study with plant tissues and the greenhouse test with intact plants indicate that there are no significant differences in the survival of Bt-resistant and -susceptible *O. nubilalis* larvae on Bt corn.

The bioassay of Bt-corn leaf extract (events MON810 and Bt11) in an artificial diet demonstrated a statistically significant difference in the survival of resistant and susceptible *O. nubilalis* larvae. The bioassay with leaf extract provided better precision compared with that of intact corn plants or plant tissues because a broader range of concentrations of plant derived Cry1Ab was evaluated. However, the resistance ratio of resistant to susceptible insects was low (approx. two-fold).

The molecular mass of a full-length Cry1Ab protoxin is 132 kDa, and transgenic Bt corn hybrids express truncated forms, with molecular masses of 91 (event MON810) and 65 kDa (event Bt11) (Koziel et al. 1993, Agbios 2002, Andow and Hilbeck 2004).

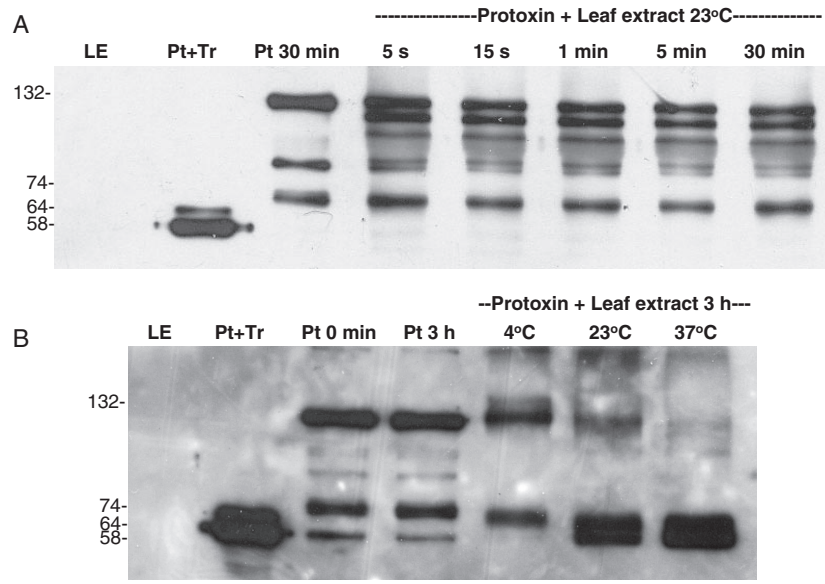

Fig. 4. Western blot analysis of Cry1Ab protoxin (0.3 μg/lane, Pt) incubated alone (for the time indicated) or with non-Bt corn leaf extract (2.3 μg/lane) at 23°C for 5 s to 30 min (A) or at various temperatures for 3 h (B). LE, leaf extract; Pt+Tr, protoxin and trypsin incubation.

Cry1Ab protoxin is converted to a proteinase-resistant fragment of 58 kDa when hydrolyzed by bovine trypsin or enzymes in midgut extracts from *O. nubilalis* larvae (Li et al. 2004b). The truncated Cry1Ab toxins expressed in events MON810 and Bt11 are considered functionally inactive and presumably still need to be hydrolyzed to be toxic, and proteinase-mediated resistant insects would be resistant to these inactive toxins. However, the results of this study indicate that the KS-SC Cry1Ab-protoxin resistant strain responded to the plant-derived toxin as though they were fed functionally activated toxin. These insects had only two-fold resistance to plant-derived Cry1Ab protein, >99% reduction in resistance based on the 254-fold resistance to Cry1Ab protoxin (Li et al. 2005a). This resistance was more similar to that of activated Cry1Ab (12-fold) than to protoxin Cry1Ab (254-fold) from bacterial expression. The reduction in resistance ratios (from 12- to 2-fold) may be caused by natural resistance factors in the plant (such as DIMBOA) expressed at specific stages in corn development (Sachs et al. 1996, Olsen and Daly 2000, Mao et al. 2007).

Hydrolysis of the Cry1Ab protoxin by plant enzymes into several peptide fragments may indicate that the mechanical damage by *O. nubilalis* during feeding releases plant proteinases that hydrolyze the transgenic Cry1Ab expressed in Bt corn. Therefore, *O. nubilalis* larvae may be exposed to an activated form of Cry1Ab when they feed on Bt corn plants. This partial or full activation of transgenic Cry1Ab also may have occurred during the preparation of Bt corn leaf extract for bioassays. A recent study indicates that the full-length Cry1F produced in transgenic cotton plants was sensitive to the host cell proteinases, resulting in a biologically active form (Gao et al. 2006).

Resistance to Cry1Ab protoxin in the KS-SC strain of *O. nubilalis* has been attributed to reduced proteinase activation of protoxin (Huang et al. 1999a; Li et al. 2004b, 2005a), and it was not associated with receptor binding (Li et al. 2004a). When these insects were exposed to either partially or fully activated Cry1Ab, the level of resistance was dramatically reduced. Therefore, the loss of proteinase activity in KS-SC insects may be more critical to earlier activation steps (i.e., hydrolysis from the protoxin to the 74-kDa intermediate) than to the later activation steps (i.e., hydrolysis from the 74-kDa intermediate to the fully active toxin).

Our results indicate that insects feeding on transgenic Bt corn plants are exposed (directly or indirectly) to functionally activated Cry1Ab. This suggests that proteinase-based resistance in corn borer populations does not threaten transgenic plants unless they express the protoxin form of Cry1Ab. If, however, protoxin solubilization and activation are no longer necessary for toxicity, the selectivity of Cry proteins may be compromised and the potential risk to nontarget insects could be increased. Because nontarget insects do not feed directly on plant tissues, they do not cause mechanical damage to plants, and therefore the likelihood of exposure to active Bt toxins would be low.

The KS-SC strain of *O. nubilalis* was selected from a field population collected in 1995 in south central Kansas. This form of resistance seemed to be relatively common in the field, because all five *O. nubilalis* colonies that were exposed to selection pressure responded and expressed a level of resistance within eight selection generations (Huang et al. 1997). Field populations seem to be predisposed to expressing al-

ternative digestive enzymes as a compensation response to feeding on plants with proteinase inhibitors (for review, see Oppert 2000). Other laboratories have developed moderately resistant *O. nubilalis* strains (Bolin et al. 1999, Chaufaux et al. 2001, Siqueira et al. 2004a). Siqueira et al. (2004b) reported that Cry1Ab-selected resistant strains (Europe R and RSTT R) of *O. nubilalis* had much higher resistance ratios to trypsin-activated Cry1Ab than to full-length Cry1Ab and concluded that proteinases were not responsible for resistance. Our more recent efforts to select for resistance using activated Cry1Ab failed to produce changes in susceptibility after eight generations of selection in newly established colonies of *O. nubilalis* from western Kansas (unpublished data). This observation suggests that in *O. nubilalis*, resistance to activated Cry1Ab toxins is not as common as resistance to protoxin Cry1Ab. However, resistance to activated toxins has been associated with receptor-mediated resistance and implies more serious consequences for resistance management.

A number of resistant and susceptible third instars of *O. nubilalis* larvae survived 5 d of exposure to Bt corn tissues. When these larvae were transferred to fresh Bt corn tissues, they were not able to survive. However, when these surviving larvae were transferred to non-Bt corn tissues, many were able to recover and developed to the pupal stage. This suggests that older *O. nubilalis* larvae can survive a short period of exposure to Bt corn if they can move to another non-Bt host plant. Early instars of *O. nubilalis* can develop on a number of alternate hosts that may be present in a Bt cornfield (Losey et al. 2002). Older *O. nubilalis* larvae that develop on such hosts could migrate to corn plants to complete development. Later instars are relatively tolerant to Bt toxins and therefore may survive exposure to Bt-transgenic corn (Huang et al. 1999b, Walker et al. 2000). These larvae may sample several plants to identify a non-Bt corn plant on which to complete development. If there were resistance alleles in such a population, those individuals could potentially survive on Bt corn tissues for longer periods, resulting in increased selection of resistance genes. This could be especially problematic if Bt and non-Bt corn are planted together. However, a mixture of Bt and non-Bt seeds is not recommended as a strategy for resistance management (Mallet and Porter 1992, Davis and Onstad 2000). The more likely scenario is that volunteer corn plants pose a threat to resistance management, because ≈25% of these plants can be non-Bt plants due to F2 segregation.

These results complement the results from previous physiological studies of the resistance mechanism in this KS-SC-resistant strain. Huang et al. (1999a) and Li et al. (2004b, 2005a) concluded that resistance in this strain was primarily due to a reduced protoxin activation because of reduced digestive proteinase activity. Li et al. (2004a) also found that DiPel-resistance was not associated with a loss of receptor binding for Cry1Ab or Cry1Ac proteins. Resistance to Bt toxin that is associated with reduced activity of digestive enzymes involved in the solubilization and activation of Bt protoxins also has been reported for *Plodia interpunctella* (Hübner) and *Heliothis virescens* (F.) (Oppert et al. 1994, 1996; Forcada et al. 1996; Forcada et al. 1999). Our studies demonstrate that Bt resistance mediated by reduced proteinase activation from Cry1Ab protoxin to toxin cannot protect *O. nubilalis* larvae against Bt corn expressing a truncated Cry1Ab toxin. These studies indicate that resistance mediated by reduced proteinase activity can be defeated by plant expression of a functionally active toxin, produced either by genetic engineering, or by plant or insect gut proteinases.

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