The HevCaLP Protein Mediates Binding Specificity of the Cry1A Class of *Bacillus thuringiensis* Toxins in *Heliothis virescens*

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ABSTRACT: Retrotransposon-mediated disruption of the *BtR-4* gene encoding the *Heliothis virescens* cadherin-like protein (HevCaLP) is linked to high levels of resistance in the YHD2 strain to Cry1Ac toxin from *Bacillus thuringiensis*. This suggests that HevCaLP functions as a Cry1Ac toxin receptor on the surface of midgut cells in susceptible larvae and that the *BtR-4* gene disruption eliminates this protein in resistant larvae. However, Cry1Ac toxin binding to HevCaLP is yet to be reported. We used the polymerase chain reaction and immunoblotting as tools to discriminate between individual *H. virescens* larval midguts from susceptible (YDK) and resistant (CXC, KCBhyb, and YHD2-B) strains according to their *BtR-4* gene disruption genotype and phenotype. This approach allowed us to test the correlation between *BtR-4* gene disruption, lack of HevCaLP, and altered Cry1A toxin binding. Toxin-binding assays using brush border membrane vesicles revealed that a wild-type *BtR-4* allele is necessary for HevCaLP production and Cry1Aa toxin binding, while most of Cry1Ab and Cry1Ac binding was independent of the *BtR-4* genotype. Moreover, toxin competition experiments show that KCBhyb midguts lacking HevCaLP are more similar to midguts of the original YHD2 strain than to the current YHD2-B strain. This resolves discrepancies in published studies of Cry1A binding in YHD2 and supports our earlier suggestion that a separate genetic change occurred in YHD2 after appearance of the cadherin disruption, conferring even higher resistance in the resulting YHD2-B strain as well as a large reduction in Cry1Ab and Cry1Ac binding.

Effectiveness of transgenic plants producing Cry proteins from the bacterium *Bacillus thuringiensis* (Bt)[1] has led to an increase in their use for insect pest control (*1*). To maintain utility of this technology, the potential for evolution of pest resistance needs consideration. Although resistance to Bt spray formulations has appeared in open-field populations of the diamondback moth (*2*), no episodes of insect resistance to Bt crops in the field have been reported. However, results from insect selection in the laboratory demonstrate that the genetic potential for resistance to Cry toxins exists in many lepidopteran pests (*3*), including the tobacco budworm (*Heliothis virescens*). It is estimated that cotton plants expressing Cry1Ac toxin (Bt cotton) to control *H. virescens* infestation account for up to 60% of the cotton grown in the southern U.S. Laboratory selection experiments are useful to understand what mechanisms insects may evolve to cope with Cry toxins. This information can then be used to design and implement specific measures to delay or detect evolution of insect resistance to Cry toxins in the field.

After ingestion by the insect, crystalline Cry proteins are solubilized and processed by the midgut juice to an active toxin core. Active toxins bind to receptors on the brush border membrane and oligomerize to form pores that result in cell lysis, gut paralysis, and insect death (*4*). Although any variation in a step of the Cry toxin mode of action can potentially result in decreased susceptibility, alteration of toxin−receptor interactions is the most reported resistance mechanism (*3*).

Current "toxin-binding models" to explain these interactions are based on binding competition studies using radiolabeled toxins and insect midgut brush border membrane vesicles (BBMVs). On the basis of their toxin binding specificity, three populations of Cry1 toxin-binding sites (A, B, and C) were described in BBMVs from Bt-susceptible *H. virescens* larvae (*5*). One binding site (receptor A) was shared by Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja toxins (*6*). Primarily on the basis of ligand blot results, receptor A was proposed to comprise *N*-aminopeptidases (APNs) of 170, 130, and 110 kDa (*7*−*9*) and an unidentified

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[1] Abbreviations: Bt, *Bacillus thuringiensis*; BBMV, brush border membrane vesicle; HevCaLP, *Heliothis virescens* cadherin-like protein; PCR, polymerase chain reaction; APN, *N*-aminopeptidase; HvALP, *Heliothis virescens* alkaline phosphatase; dNTP deoxyribonucleoside triphosphate; BSA, bovine serum albumin; PVDF, polyvinylidiene difluoride Q; HRP, horseradish peroxidase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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210-kDa protein (*9*). Binding of Cry1Ac to the 110-kDa APN seems to be irrelevant for toxicity (*10*), while the 170-kDa APN has been shown to promote both high-affinity Cry1Ac binding and pore formation when reconstituted on phospholipid vesicles (*7*). A 130-kDa protein binding both Cry1Ab and Cry1Ac has been proposed to constitute receptor B, while several proteins of less than 100-kDa in size specific for Cry1Ac have been suggested to constitute receptor C (*6*).

In the Cry1Ac-selected YHD2 strain of *H. virescens* (*11*), resistance was associated with greatly decreased Cry1Aa binding, while Cry1Ab and Cry1Ac binding were unaffected (*12*). Disruption of a cadherin-superfamily gene (*BtR-4*) that resulted in the absence of a potential toxin-binding cadherin-like protein (HevCaLP) in midguts from YHD2 larvae was proposed as the main mechanism of resistance in this strain (*13*). After continuous selection with Cry1Ac, YHD2 larvae developed higher levels of resistance to Cry1Ac, which correlated with greatly decreased Cry1Ab, Cry1Ac, and Cry1Fa toxin binding (*14*) and reduced levels of a midgut alkaline phosphatase protein (*15*). Although Cry1A toxin binding to HevCaLP has not been demonstrated, cadherin-like proteins homologous to HevCaLP function as receptors for Cry toxins in other insects (*16*, *17*).

Another resistant *H. virescens* strain, KCBhyb, that was sequentially selected with Cry1Ac and Cry2A, developed a pattern of toxin binding (*18*) similar to the one previously observed in BBMVs from YHD2 larvae (*12*). A separate strain, CXC, selected with the same regime, did not develop any changes in toxin binding. These observations led to the hypothesis that a similar mechanism of resistance existed in larvae from both YHD2 and KCBhyb strains (*18*).

To test this hypothesis and the role of HevCaLP in toxin binding and toxicity, we have used discriminative polymerase chain reaction (PCR) and immunodetection to establish the *BtR-4* genotype and HevCaLP phenotype of midguts from susceptible (YDK) and resistant (YHD2-B, KCBhyb, and CXC) *H. virescens* larvae. Because *BtR-4* disruption resulted in the absence of HevCaLP from BBMVs, we investigated the correlation between the lack of HevCaLP, alteration of toxin binding, and resistance. Qualitative and quantitative binding assays demonstrated that the lack of HevCaLP results in greatly reduced Cry1Aa binding to BBMVs, while Cry1Ab and Cry1Ac binding were almost unaffected. These results are evidence for HevCaLP as a key component of receptor A and for the important role of this protein in Cry1A toxicity.

MATERIALS AND METHODS

Insect Strains. H. virescens strains used have been previously described. Strain YDK (*11*) is an unselected susceptible strain, while CXC (*19*), YHD2 (*11*), and KCBhyb (*18*) were generated after selection with Cry1Ac in the laboratory. Both CXC and KCBhyb originated from backcrossing moths from resistant strains (CP73-3 and KCB, respectively) to susceptible adults to counteract the negative effects of inbreeding, followed by reselection. Parental CP73-3 and KCB strains were selected with Cry1Ac and evolved cross-resistance to Cry2Aa (*20*, *21*). After backcrossing, resulting CXC and KCBhyb larvae were selected with Cry2Aa to increase resistance to this toxin. Both CXC and KCBhyb larvae were resistant to Cry1Ac (200−300-fold) and Cry2Aa (more than 250-fold) when compared to YDK larvae (*18*). In contrast, after selection with Cry1Ac, YHD2 larvae developed a 10 000-fold resistance to Cry1Ac but only low levels of cross-resistance to Cry2Aa (*11*). Subsequent selection with Cry1Ac resulted in even higher levels of resistance; here, we refer to this later descendant of YHD2 as YHD2-B. Our earlier binding studies were conducted on YHD2-B not YHD2 as reported (*14*). All insects were reared in the laboratory using an artificial diet as previously described (*11*).

DNA Isolation. Following dissection of the larval midgut, each carcass (i.e., larval body after the midgut was removed) was placed separately in a microfuge tube with DNA isolation buffer (50 mM Tris at pH 8.0, 10 mM EDTA, 0.2 M NaCl, and 2% SDS). Tissue was homogenized using a Retsch MM300 Mixer Mill. Homogenates were spun at 13000*g* for 5 min before adding 1 volume of phenol (pH 8.0). Samples were mixed by inversion and allowed to stand overnight. After centrifugation at 13000*g* for 5 min, the aqueous layer was removed to a clean microfuge tube and extracted with chloroform. Genomic DNA was precipitated from the aqueous layer using a $^1/_{10}$ volume of 2 M NaCl and 2 volumes of absolute ethanol. After a spin for 15 min at 13000*g*, the pellet was air-dried for 10 min before dissolving in TE buffer (10 mM Tris, and 1 mM EDTA at pH 8.0).

Detection of BtR-4 Disruption by PCR. PCR reactions contained 10 mM Tris (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM Rint SF1 primer (5′-ATAC-GAGCTGACGACACGCTGGGAGA-3′), 0.2 μM Rint SR2 primer (5′-TCTGAGCGTAGGAGGTGTGTTGTTGATGTC-3′), 0.2 μM Rint RR3 primer (5′-GCGCGATGTGACAGTC-CGGAACAG-3′), 0.5 units of AmpliTaq DNA polymerase (Applied Biosystems), and 100−200 ng of genomic DNA. PCR cycling conditions were 1 cycle at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s. Electrophoresis of PCR products was performed on 2.5% Metaphor agarose (BioWhittaker Molecular Applications) using Tris-borate-EDTA (TBE) buffer.

BBMV Purification. BBMVs were prepared from midguts isolated from fifth instar larvae as described elsewhere (*22*). Briefly, 6−8 individual midguts with the same *BtR-4* genotype were homogenized in SET buffer [250 mM sucrose, 17 mM Tris (pH 7.5), 5 mM EGTA], and BBMVs were purified by differential centrifugation in the presence of 12 mM $MgCl_2$ (final concentration). Measurements of aminopeptidase activity using L-leucine *p*-nitroanilide as the substrate were used to monitor BBMV enrichment. APN activity in the BBMV samples was 7−10-fold the activity detected in the initial midgut homogenates. After protein quantification (*23*) using bovine serum albumin (BSA) as the standard, BBMVs were kept at −80 °C until used.

Immunodetection of HevCaLP. A 367 amino acid region of HevCaLP, including ectodomain 7 of this protein, was cloned and expressed as a His-tagged peptide in *Escherichia coli* using pET30A (Novagen). Expressed peptide was purified using a nickel-affinity resin and used to produce rabbit antisera following previously described methods (*24*).

BBMV proteins (15 μg) were separated by electrophoresis and transferred to polyvinylidiene difluoride Q (PVDF) membrane filters (Millipore). After blocking in PBS buffer (135 mM NaCl, 2 mM KCl, and 10 mM Na_2HPO_4 at pH 7.5) containing 0.1% Tween-20 (PBST) and 3% BSA, filters

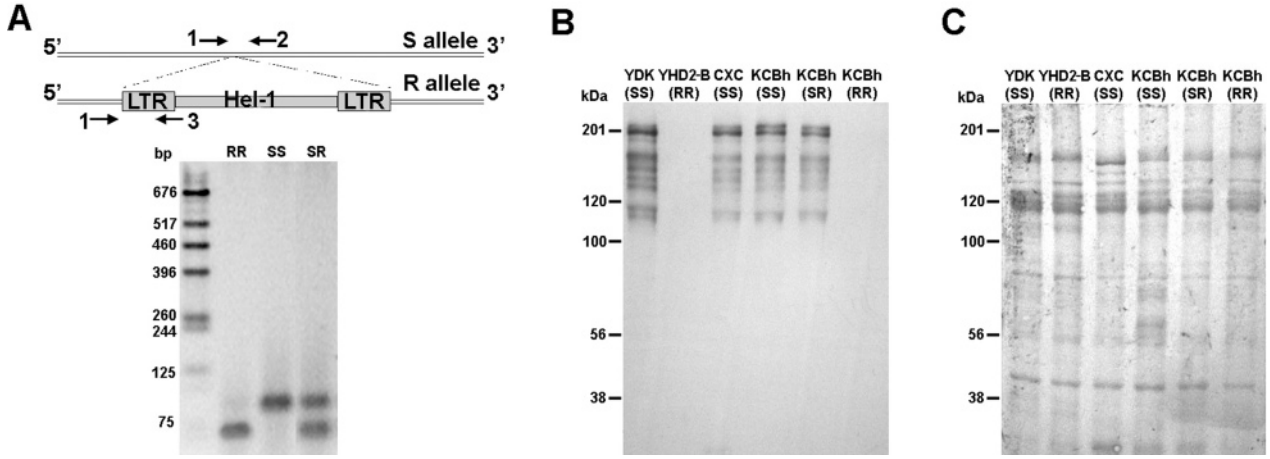

FIGURE 1: Detection of *BtR-4* gene disruption in DNA from individual larvae (A) and of HevCaLP in BBMVs from isolated midguts (B). Primers were designed to align with regions flanking the insertion point and internally to the left long terminal repeat of the Hel-1 retrotransposon (A) in a mutant allele. PCR products were separated by 2.5% agarose gel electrophoresis (B). Wild-type susceptible (S) alleles produced fragments of 99 bp, while alleles with the retrotransposon insertion (R) were detected as fragments of 71 bp. Both fragments were detected in heterozygotes. Immunodetection of HevCaLP in blots of BBMV proteins (B) was done using antiserum against HevCaLP. Blots were visualized by enhanced chemiluminescence and then stained with amido black to detect protein loads (C).

were probed with a 1:5000 dilution of polyclonal serum against HevCaLP for 1 h. After the blots were washed with PBST containing 0.1% BSA, they were probed with anti-rabbit serum (Sigma) conjugated to horseradish peroxidase (HRP) and developed using enhanced chemiluminescence (ECL, Amersham Biosciences). Controls with preimmune sera did not detect any BBMV protein (data not shown).

Toxin Purification and Labeling. Bacillus thuringiensis strains HD-37 and HD-73, producing Cry1Aa and Cry1Ac, respectively, were obtained from the *Bacillus* Genetic Stock Collection (Columbus, OH). Production and purification of Cry1Ab from inclusion bodies produced in an *E. coli* strain carrying the Bt NRD-12 *cry1Ab* toxin gene were the same as those described elsewhere (*25*). Cry1Aa and Cry1Ac toxin production and purification were the same as those previously described (*26*). Fractions containing pure toxin [a single band on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)] were pooled and stored at −80 °C until used. Protein concentrations were determined for BBMVs.

Purified Cry1A toxins (10 μg) were radiolabeled with 0.5 mCi ^{125}I using either iodo-beads (Pierce) following the instructions of the manufacturer (Cry1Aa and Cry1Ab) or the chloramine-T method (Cry1Ac labeling) as described elsewhere (*27*). We calculated bindability of labeled Cry1Aa and Cry1Ac samples (percentage of labeled Cry1A toxins that were able to specifically bind to BBMVs) according to the method of Schumacher and von Tscharner (*28*). Specific activities based on this method were 5 μCi/μg for Cry1Aa, 16 μCi/μg for Cry1Ab, and 18 μCi/μg for Cry1Ac.

^{125}I-Cry1A Toxin-Binding Assays. Qualitative binding of ^{125}I-Cry1A toxins to BBMVs from *H. virescens* strains was measured as previously described (*14*). BBMVs (20 μg of protein) were incubated with 1 nM ^{125}I-Cry1A toxins in 0.1 mL of binding buffer (PBS at pH 7.5 and 0.1% BSA) for 1 h at room temperature. Binding reactions were stopped by centrifugation for 10 min at 16100*g*, and pellets were washed once with 1 mL of ice-cold binding buffer before solubilization and electrophoresis in 10% SDS−PAGE gels. After the run, gels were dried and exposed to photographic film at −80 °C to detect the presence of BBMV-bound radiolabeled toxins.

For ^{125}I-Cry1A binding saturation assays, BBMVs (25 μg of protein/mL) were incubated with increasing amounts of ^{125}I-Cry1A toxin for 1 h at room temperature in binding buffer. Binding reactions were stopped by centrifugation, and pellets were washed as mentioned above. Activity of the resulting pellets was counted in a Beckman model 4000 γ detector. Nonspecific binding was determined by addition of homologous unlabeled toxin at a concentration 100-fold greater than the highest radioligand concentration tested. Binding affinity and concentration of receptors for each toxin were calculated through nonlinear regression using the KELL software (BIOSOFT, Cambridge, U.K.).

Competition of ^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding to 25 μg/mL of BBMV proteins by increasing concentrations of unlabeled homologous toxins or Cry1Aa was tested as described in Jurat-Fuentes and Adang (*6*).

RESULTS

Detection of BtR-4 Disruption Genotype and HevCaLP Phenotype. PCR reactions with specific primers to the regions flanking the Hel-1 retrotransposon insertion in the *BtR-4* gene (Figure 1A) allowed us to investigate the existence of the susceptible (not disrupted) and resistant (containing retrotransposon insert) *BtR-4* alleles in DNA from individual larvae. As previously reported for the YDK and YHD2 strains (*13*), PCR products from susceptible (S, wild-type) *BtR-4* alleles were 99 bp, while resistant alleles (R, disrupted gene) yielded a 71-bp PCR product (Figure 1A). Data obtained from the PCR reactions of larvae from four *H. virescens* strains with different susceptibility to Cry1A toxins are summarized in Table 1. It is important to note that only *BtR-4* gene alterations related to Hel-1 retrotransposon insertion could be detected by our PCR reaction and that the symbols R and S refer specifically to alleles of the *BtR-4* gene. All tested individuals from the YHD2-B strain were homozygous for the retrotransposon insertion in *BtR-4* (RR genotype). In larvae from the KCBhyb strain, similar numbers of homozygous susceptible (SS), homozygous resistant (RR), and heterozygous (SR) individuals for the *BtR-4* gene disruption were detected. No RR individuals were

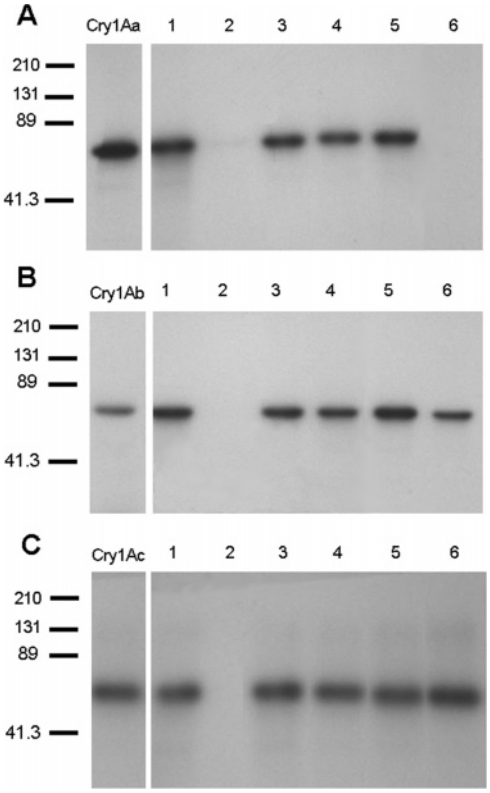

FIGURE 2: Qualitative analysis of ^{125}I-Cry1A binding to BBMVs from YDK-SS (lane 1), YHD2-B-RR (lane 2), CXC-SS (lane 3), KCBhyb-SS (lane 4), KCBhyb-SR (lane 5), and KCBhyb-RR (lane 6) strains. BBMVs were incubated with ^{125}I-Cry1Aa (A), ^{125}I-Cry1Ab (B), or ^{125}I-Cry1Ac (C) for 1 h. Reactions were stopped by centrifugation, and pellets were washed before 10% SDS−PAGE. Bound toxin was detected by autoradiography.

Table 1: Classification of Individual *H. virescens* Larvae from Different Strains According to Their *BtR-4* Genotype as Obtained from PCR Reactions

	BtR-4 genotype				
strain	SS	SR	RR	unknown	total
YDK	46	9	0	3	58
YHD2-B	0	0	40	0	40
CXC	55	4	0	3	62
KCBhyb	15	25	20	4	64

detected in larvae from the CXC or YDK strains. Most CXC and YDK individuals were SS, but we found a low proportion of RS individuals in both strains.

To investigate the HevCaLP phenotype of the different *BtR-4* genotypes, we performed immunodetection of HevCaLP in blots of BBMV proteins. Antiserum to HevCaLP detected seven proteins ranging in size from 110 to 200 kDa in BBMVs from larvae with SS or SR *BtR-4* genotypes (Figure 1B). Conversely, no proteins were detected by HevCaLP antiserum in BBMVs from RR larvae.

^{125}I-Cry1A Toxin-Binding Assays. Using qualitative binding assays, we detected Cry1Aa, Cry1Ab, and Cry1Ac toxin binding to BBMVs from larvae that were SS or SR for the *BtR-4* gene disruption (Figure 2). BBMVs from larvae that had both *BtR-4* alleles disrupted (RR), did not bind Cry1Aa. As previously reported (*14*), BBMVs from YHD2-B (all of which we found to be RR) did not bind Cry1Ab or Cry1Ac toxins. Unexpectedly, Cry1Ab and Cry1Ac bound to BBMVs from KCBhyb-RR comparably to the binding detected for

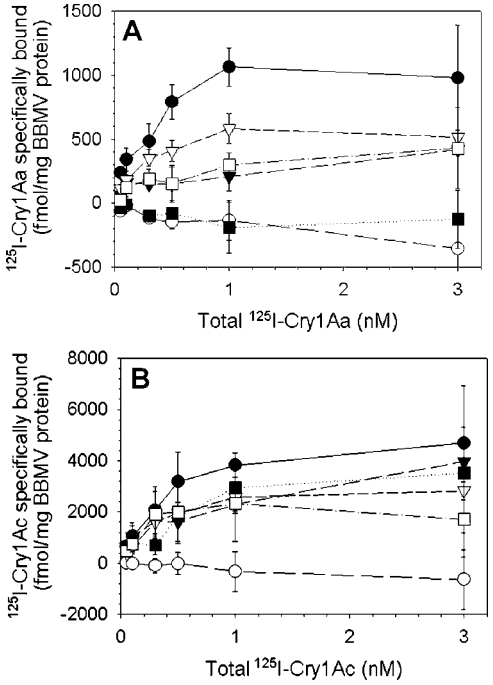

FIGURE 3: Saturation of ^{125}I-Cry1Aa (A) and ^{125}I-Cry1Ac (B) binding to BBMV proteins from different *H. virescens* strains. BBMV proteins from YDK-SS (●), YHD2-B-RR (○), KCBhyb-SS (▼), KCBhyb-SR (▽), KCBhyb-RR (■), or CXC-SS (□) were incubated with increasing amounts of labeled Cry1Aa or Cry1Ac. Binding reactions were stopped by centrifugation, and pellets were washed before counting their activity. Specific binding calculated as the difference between the total and nonspecific binding is shown. Bars denote the standard deviation from the mean calculated from at least two independent experiments done 3 times.

vesicles from larvae with SS or SR *BtR-4* genotypes (parts B and C of Figure 2). Therefore, binding of Cry1Aa to BBMVs was dependent on the existence of at least one wild-type *BtR-4* allele, while Cry1Ab and Cry1Ac binding seemed independent of the *BtR-4* genotype.

Quantitative parameters for Cry1A toxin binding were obtained from toxin-binding saturation assays with ^{125}I-Cry1Aa and ^{125}I-Cry1Ac (Figure 3). We did not perform ^{125}I-Cry1Ab saturation binding assays because of limited insect materials. Binding affinities (K_{com}) and concentration of receptors (B_{max}) calculated from the binding saturation curves (Table 2) were in agreement with previous reports of toxin binding to BBMVs from YDK, CXC, and KCBhyb larvae (*6*, *18*).

In agreement with qualitative binding assays, BBMVs with SS or SR *BtR-4* genotypes bound ^{125}I-Cry1Aa and ^{125}I-Cry1Ac toxins specifically and saturably (Figure 3). Cry1Aa-binding affinities were similar between KCBhyb-SS, KCBhyb-SR, and CXC-SS vesicles, while YDK-SS had a slightly lower binding affinity and higher concentration of the binding sites. All of these strains bound Cry1Ac with a similar affinity and concentration of the binding sites. As expected from the toxin-binding model, all of the strains binding Cry1A toxins had a higher concentration of Cry1Ac than Cry1Aa-binding sites.

BBMVs with the RR *BtR-4* genotype, whether from YHD2-B or KCBhyb, did not bind ^{125}I-Cry1Aa toxin, even at the highest ligand concentration tested. Additionally, BBMVs from the YHD2-B strain did not bind ^{125}I-Cry1Ac specifically, even at the highest ligand concentration tested

Table 2: Dissociation Constants (K_{com}, in nM Units) and Concentration of Receptors (B_{max}, in pmol/mg of BBMV Protein Units) Calculated from ^{125}I-Cry1A Toxin-Binding Saturation Assays with BBMVs from YDK, YHD2-B, CXC, and KCBhyb *H. virescens* strains with different *BtR-4* genotypes

		Cry1Aa		Cry1Ac	
H. virescens strain	*BtR-4* genotype	$K_{com} \pm$ error	$B_{max} \pm$ error	$K_{com} \pm$ error	$B_{max} \pm$ error
YDK	SS	0.56 ± 0.11	2.17 ± 0.23	0.56 ± 0.20	9.75 ± 2.98
YHD2-B	RR	NB[a]	NB	NB	NB
CXC	SS	0.26 ± 0.04	0.53 ± 0.05	0.57 ± 0.27	10.57 ± 4.16
KCBhyb	SS	0.24 ± 0.06	0.42 ± 0.06	0.44 ± 0.38	5.28 ± 2.34
KCBhyb	SR	0.29 ± 0.05	1.00 ± 0.09	0.49 ± 0.22	6.07 ± 2.72
KCBhyb	RR	NB	NB	0.96 ± 0.81	9.54 ± 6.56

[a] NB = no binding detected.

(10 nM). Binding of Cry1Ac to BBMVs from KCBhyb-RR suggested that disruption of *BtR-4* did not have a detectable effect on Cry1Ac binding. These results were evidence that *BtR-4* gene disruption resulted in reduced ^{125}I-Cry1Aa binding but not ^{125}I-Cry1Ac binding.

Heterologous Binding Competition with Cry1Aa. Our quantitative binding results with KCBhyb-RR were similar to those of Lee et al. (*12*), who reported that YHD2 showed a marked reduction of specific Cry1Aa binding but not Cry1Ab or Cry1Ac binding, in comparison to YDK. Moreover, they found a weaker competition of Cry1Ab and Cry1Ac binding by unlabeled Cry1Aa when comparing BBMVs from YHD2 and YDK larvae. The *BtR-4* gene and HevCaLP protein were unknown at the time of that study. To investigate whether this weaker competition effect was dependent on the *BtR-4* genotype, we performed heterologous competition of ^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding by Cry1Aa in BBMVs containing (KCBhyb-SS, KCBhyb-SR, and YDK-SS) or lacking (KCBhyb-RR) HevCaLP (Figure 4). From Lee et al. (*12*) and the toxin-binding model, we predicted that the absence of HevCaLP would affect the ability of Cry1Aa to compete ^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding.

Cry1Aa competed about 50% of ^{125}I-Cry1Ac binding and 60% of ^{125}I-Cry1Ab binding to BBMVs from YDK-SS, KCBhyb-SS, or KCBhyb-SR larvae. In contrast, only 20% of ^{125}I-Cry1Ac or ^{125}I-Cry1Ab binding to BBMVs from KCBhyb-RR larvae was competed by the highest Cry1Aa concentration tested. No differences in ^{125}I-Cry1Ab or ^{125}I-Cry1Ac homologous binding competition assays were found between BBMV populations (Figure 4).

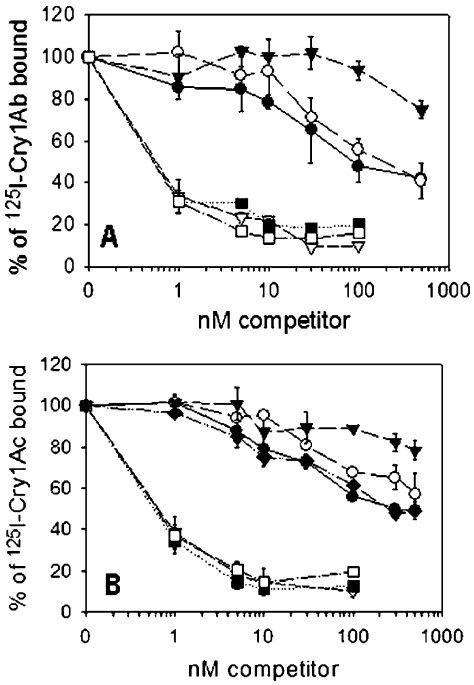

FIGURE 4: Homologous and heterologous Cry1Aa competition of ^{125}I-Cry1Ab (A) and ^{125}I-Cry1Ac (B) binding to BBMVs. BBMV proteins from YDK-SS (●), KCBhyb-SS (◆), KCBhyb-SR (○), or KCBhyb-RR (▼) were incubated with ^{125}I-Cry1Ab or ^{125}I-Cry1Ac in the presence of increasing concentrations of unlabeled competitor. Binding reactions were stopped by centrifugation, and pellets were washed before counting their activity. The amount of labeled toxin bound in the absence of the competitor was considered as 100% binding. Bars denote the standard deviation of the mean based on two independent experiments done 2 times. Also included are the homologous competition of Cry1Ab (A) and Cry1Ac (B) binding to BBMVs from YDK-SS (▽), KCBhyb-SR (■), and KCBhyb-RR (□) larvae.

DISCUSSION

Cadherin-like proteins function as receptors for Cry1A toxins in *Manduca sexta* (*29*, *30*) and *Bombyx mori* (*17*). Cadherin-like proteins from *Pectinophora gossypiella* (BT-R_2) and *H. virescens* (HevCaLP) have been hypothesized as Cry1A toxin receptors involved in resistance (*13*, *31*). In this paper, we investigated the effect of HevCaLP knockout for Cry1A toxin binding compared to known Cry1Ac and Cry2Aa toxicity. Using PCR with specific primers, we were able to detect and classify individual *H. virescens* midguts according to their *BtR-4* genotype. Immunodetection of HevCaLP confirmed the previous hypothesis (*13*) that at least one wild-type *BtR-4* allele is necessary for expression of full-length HevCaLP. We classified individual midguts as being SS, SR, or RR for the disruption of the *BtR-4* gene. This approach only detects the presence or absence of the retrotransposon insertion and not other alterations in the *BtR-4* gene that may prevent expression of a full-length protein. For example, cadherin-gene deletions have been reported in resistant strains of *P. gossypiella* (*31*). Nevertheless, along with immunodetection, this method of classification allowed us to correlate the *BtR-4* gene disruption, presence of HevCaLP, and Cry1A toxin binding and resistance in different *H. virescens* strains.

Antisera raised against HevCaLP fragments recognized seven proteins in BBMVs with at least one wild-type *BtR-4* allele. On the basis of the predicted size for HevCaLP (*13*), we believe the largest, 200-kDa protein detected in BBMVs is the full-length HevCaLP. Enzymatic degradation of HevCaLP, as previously reported for Bt-R1 cadherin from *M. sexta* (*32*), would explain detection of additional proteins.

As predicted by Gahan et al. (*13*), YHD2-B larvae were homozygous resistant for the *BtR-4* gene disruption and BBMVs from this strain did not contain detectable amounts of HevCaLP. No homozygous resistant individuals were detected in larvae from CXC or YDK strains, although a low percentage of heterozygosis was detected in both strains. This observation suggests that, as previously hypothesized (*18*), *BtR-4* gene disruption is not the mechanism of resistance in the CXC strain. We believe that the low frequencies of the *BtR-4* gene disruption in the YDK and CXC strains are due to accidental contamination with YHD2-B individuals during laboratory rearing.

Confirming our hypothesis (*18*), PCR reactions detected the existence of RR individuals for the *BtR-4* gene disruption in the KCBhyb strain. Larvae SS as well as SR were also detected in this strain, demonstrating high levels of heterogeneity. The presence of SS and SR midgut materials explains our previous binding results with BBMVs from KCBhyb larvae (*18*) in which only a small reduction but not an absence of Cry1Aa binding was detected.

HevCaLP immunodetection and ^{125}I-Cry1A-binding experiments demonstrated that lack of HevCaLP results in greatly decreased Cry1Aa binding. Because Cry1Aa shares its binding sites with Cry1Ab and Cry1Ac in BBMVs from *H. virescens* (*6*), one might anticipate an effect of HevCaLP knockout for both Cry1Ab and Cry1Ac binding. Unexpectedly, no significant differences in either Cry1Ac-binding affinity or concentration of the binding sites were detected between BBMVs from SS, SR, and RR KCBhyb midguts. In agreement with these results, mutations in the cadherin genes in resistant *P. gossypiella* larvae (*31*) did not affect Cry1Ac binding to BBMVs from these insects when compared to susceptible vesicles (*33*). Collectively, this evidence suggests either that cadherin from *P. gossypiella* and HevCaLP do not function as Cry1Ac-binding sites, that the binding methodology used was not sensitive enough to detect reduced Cry1Ac binding, or that these cadherin proteins may have a role in postbinding steps in the Cry1Ac mode of action.

Our finding that KCBhyb-RR midguts are still able to bind Cry1Ab and Cry1Ac, despite the loss of specific Cry1Aa-binding ability, is similar to that of Lee et al. (*12*), with BBMVs from the YHD2 strain. To explain the paradoxical result that high levels of Cry1Ac resistance could be achieved by YHD2 despite the retention of Cry1Ac-binding ability, they hypothesized that not all receptors of Cry1Ac were involved in toxic function (*12*). At the time of their study, the existence of *BtR-4* and HevCaLP was unknown, but it is likely that most or all YHD2 individuals in their study were homozygous RR for the *BtR-4* retrotransposon insertion, as later shown by Gahan et al. (*13*) and that most or all of the YDK individuals were homozygous SS. Our results with SS, SR, and RR genotypes from KCBhyb contribute to the resolution of this dilemma. The presence or absence of the HevCaLP protein appears to mediate binding properties that are not general but toxin-specific within the Cry1A class; i.e., Cry1Aa binding responds differently than Cry1Ab and Cry1Ac binding.

Interestingly, Lee et al. (*12*) detected a more subtle decrease in Cry1Ab and Cry1Ac binding to BBMVs from YHD2 larvae, using heterologous competition with Cry1Aa. Similarly, we detected decreased competition of Cry1Ab and Cry1Ac binding by Cry1Aa in BBMVs from KCBhyb-RR larvae when compared to YDK-SS, KCBhyb-SS, and KCBhyb-SR vesicles. Therefore, BBMVs from KCBhyb-RR lack a Cry1Aa-binding site also recognized by Cry1Ab and Cry1Ac. Because this binding site was present in YDK-SS, KCBhyb-SS, and KCBhyb-SR vesicles, we conclude that this missing site is located on HevCaLP and is part of receptor A in the binding site model (*6*). The fact that high concentrations of Cry1Aa competed low amounts of ^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding to KCBhyb-RR vesicles may suggest the presence of additional Cry1Aa-binding sites. Alternatively, this low level of competition may represent nonspecific interactions, because of the high Cry1Aa concentrations needed to detect it.

Our results with BBMVs from KCBhyb-RR are similar to the results of Lee et al. (*12*) with BBMVs from YHD2 in two key respects: (1) a loss of Cry1Aa but not Cry1Ab or Cry1Ac binding when measured directly by homologous competition with labeled and unlabeled toxin and (2) a more subtle reduction of Cry1Ab and Cry1Ac binding as measured indirectly by heterologous competition experiments with unlabeled Cry1Aa. These contrast with our results from BBMVs from YHD2-B, which have lost Cry1Aa-, Cry1Ab-, and Cry1Ac-binding ability in toxin-binding experiments in our earlier study (*14*) as well as this one. This difference cannot be attributed to properties of the HevCaLP protein, which is absent in both KCBhyb-RR and YHD2-B-RR vesicles.

We have previously hypothesized that an additional mutation occurred in YHD2, causing even higher levels of resistance after continued selection (*18*). Here, we extend this hypothesis by suggesting that (1) the second mutation directly or indirectly reduced the abundance or binding properties of receptor B, thus affecting Cry1Ab and Cry1Ac binding, (2) the mutation is genetically unlinked to BtR-4, and (3) the mutation is the main genetic difference between YHD2 and YHD2-B with respect to Bt toxin interactions. We are currently testing this hypothesis using genetic crosses with YHD2-B and related strains. Recently, we have found that reduction of Cry1Ac binding correlated with decreased amounts of an alkaline phosphatase (HvALP) in BBMVs from YHD2-B larvae (*15*).

From our results and previous reports, receptor A in BBMVs from susceptible *H. virescens* could be composed of the HevCaLP protein and the 130- (*8*) and 170-kDa (*7*) APNs. Neither of the APN genes mapped to a linkage group was responsible for Cry1Ac resistance in YHD2 (*13*). Therefore, only the HevCaLP can be considered as a putative Cry1A functional receptor. Other toxin-binding proteins from receptor A may be important to concentrate toxin molecules on the cell membrane. Alternatively, mutations in genes involved in APN expression with chromosomal location different to the APN genes may affect the toxin-receptor function of APNs.

Additional studies of HevCaLP should yield very useful information to understand the specificity of Cry1A-binding interactions and Bt resistance in *H. virescens*. Further experiments with HevCaLP expressed in heterologous systems are being currently conducted to study the toxin-binding specificities and potential role of HevCaLP as a functional Cry1A receptor.

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REFERENCES

1. Betz, F. S., Hammond, B. G., and Fuchs, R. L. (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests, *Regul. Toxicol. Pharmacol. 32*, 156−173.
2. Tabashnik, B. E., Cushing, N. L., Finson, N., and Johnson, M. W. (1990) Field development of resistance to *Bacillus thuringiensis* in diamondback moth (lepidoptera: plutellidae), *J. Econ. Entomol. 83*, 1671−1676.
3. Ferre, J., and van Rie, J. (2002) Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*, *Annu. Rev. Entomol. 47*, 501−533.
4. Aronson, A. I., and Shai, Y. (2001) Why *Bacillus thuringiensis* insecticidal toxins are so effective: Unique features of their mode of action, *FEMS Microbiol. Lett. 195*, 1−8.
5. van Rie, J., Jansens, S., Höfte, H., Degheele, D., and van Mellaert, H. (1989) Specificity of *Bacillus thuringiensis* δ-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects, *Eur. J. Biochem. 186*, 239−247.
6. Jurat-Fuentes, J. L., and Adang, M. J. (2001) Importance of Cry1 δ-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.), *Appl. Environ. Microbiol. 67*, 323−329.
7. Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R., and Adang, M. J. (1997) The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation, *Insect Biochem. Mol. Biol. 27*, 735−743.
8. Oltean, D. I., Pullikuth, A. K., Lee, H. K., and Gill, S. S. (1999) Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA, *Appl. Environ. Microbiol. 65*, 4760−4766.
9. Banks, D. J., Jurat-Fuentes, J. L., Dean, D. H., and Adang, M. J. (2001) *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ-endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis virescens* is not *N*-acetylgalactosamine mediated, *Insect Biochem. Mol. Biol. 31*, 909−918.
10. Banks, D. J., Hua, G., and Adang, M. J. (2003) Cloning of a *Heliothis virescens* 110 kDa aminopeptidase N and expression in *Drosophila* S2 cells, *Insect Biochem. Mol. Biol. 33*, 499−508.
11. Gould, F., Anderson, A., Reynolds, A., Bumgarner, L., and Moar, W. (1995) Selection and genetic analysis of a *Heliothis virescens* (lepidoptera: noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins, *J. Econ. Entomol. 88*, 1545−1559.
12. Lee, M. K., Rajamohan, F., Gould, F., and Dean, D. H. (1995) Resistance to *Bacillus thuringiensis* CryIA δ-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration, *Appl. Environ. Microbiol. 61*, 3836−3842.
13. Gahan, L. J., Gould, F., and Heckel, D. G. (2001) Identification of a gene associated with Bt resistance in *Heliothis virescens*, *Science 293*, 857−860.
14. Jurat-Fuentes, J. L., Gould, F. L., and Adang, M. J. (2002) Altered Glycosylation of 63- and 68-kDa microvillar proteins in *Heliothis virescens* correlates with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cry1 toxins, *Appl. Environ. Microbiol. 68*, 5711−5717.
15. Jurat-Fuentes, J. L., and Adang, M. J. (2004) Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae, *Eur. J. Biochem. 271*, 3127−3135.
16. Dorsch, J. A., Candas, M., Griko, N. B., Maaty, W. S., Midboe, E. G., Vadlamudi, R. K., and Bulla, L. A., Jr. (2002) Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R(1) in *Manduca sexta*: Involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*, *Insect Biochem. Mol. Biol. 32*, 1025−1036.
17. Hara, H., Atsumi, S., Yaoi, K., Nakanishi, K., Higurashi, S., Miura, N., Tabunoki, H., and Sato, R. (2003) A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins on midgut epithelial cells of *Bombyx mori* larvae, *FEBS Lett. 538*, 29−34.
18. Jurat-Fuentes, J. L., Gould, F. L., and Adang, M. J. (2003) Dual resistance to *Bacillus thuringiensis* Cry1Ac and Cry2Aa toxins in *Heliothis virescens* suggests multiple mechanisms of resistance, *Appl. Environ. Microbiol. 69*, 5898−5906.
19. Kota, M., Daniell, H., Varma, S., Garczynski, S. F., Gould, F., and Moar, W. J. (1999) Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects, *Proc. Natl. Acad. Sci. U.S.A. 96*, 1840−1845.
20. Gould, F., Martinez-Ramirez, A., Anderson, A., Ferre, J., Silva, F. J., and Moar, W. J. (1992) Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*, *Proc. Natl. Acad. Sci. U.S.A. 89*, 7986−7990.
21. Martinez-Ramirez, A. C., Gould, F., and Ferre, J. (1999) Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (lepidoptera: noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*, *Biocontrol Sci. 9*, 239−246.
22. Wolfersberger, M. G., Luthy, P., Maurer, A., Parenti, P., Sacchi, V. F., Giordana, B., and Hanozet, G. M. (1987) Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*), *Comp. Biochem. Physiol., A 86*, 301−308.
23. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
24. Luo, K., McLachlin, J., Brown, M. R., and Adang, M. J. (1999) Expression of *Manduca sexta* aminopeptidase in insect cells, *Protein Expression Purif. 17*, 113−122.
25. Masson, L., Prefontaine, G., Peloquin, L., Lau, P. C., and Brousseau, R. (1990) Comparative analysis of the individual protoxin components in P1 crystals of *Bacillus thuringiensis* subsp. *kurstaki* isolates NRD-12 and HD-1, *Biochem. J. 269*, 507−512.
26. Luo, K., Banks, D., and Adang, M. J. (1999) Toxicity, binding, and permeability analyses of four *Bacillus thuringiensis* Cry1 δ-endotoxins by use of brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*, *Appl. Environ. Microbiol. 65*, 457−464.
27. Garczynski, S. F., Crim, J. W., and Adang, M. J. (1991) Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis, *Appl. Environ. Microbiol. 57*, 2816−2820.
28. Schumacher, C., and von Tscharner, V. (1994) Practical instructions for radioactively labeled ligand receptor binding studies, *Anal. Biochem. 222*, 262−269.
29. Vadlamudi, R. K., Ji, T. H., and Bulla, L. A., Jr. (1993) A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner*, *J. Biol. Chem. 268*, 12334−12340.
30. Hua, G., Jurat-Fuentes, J. L., and Adang, M. J. (2004) Fluorescent-based assays establish *Manduca sexta* Bt-R_{1a} cadherin as a receptor for multiple *Bacillus thuringiensis* Cry1A toxins in *Drosophila* S2 cells, *Insect Biochem. Mol. Biol. 34*, 193−202.
31. Morin, S., Biggs, R. W., Sisterson, M. S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L. J., Heckel, D. G., Carriere, Y., Dennehy, T. J., Brown, J. K., and Tabashnik, B. E. (2003) Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm, *Proc. Natl. Acad. Sci. U.S.A. 100*, 5004−5009.
32. Candas, M., Francis, B. R., Griko, N. B., Midboe, E. G., and Bulla, J., L. A. (2002) Proteolytic cleavage of the cevelopmentally important cadherin BT-R_1 in the midgut epithelium of *Manduca sexta*, *Biochemistry 41*, 13717−13724.
33. Gonzalez-Cabrera, J., Escriche, B., Tabashnik, B. E., and Ferre, J. (2003) Binding of *Bacillus thuringiensis* toxins in resistant and susceptible strains of pink bollworm (*Pectinophora gossypiella*), *Insect Biochem. Mol. Biol. 33*, 929−935.

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