

Article

Knockout of Two Cry-Binding Aminopeptidase N Isoforms Does Not Change Susceptibility of *Aedes aegypti* Larvae to *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba and Cry11Aa Toxins

Junxiang Wang ¹, Xiaozhen Yang ^{1,2}, Huan He ^{1,2}, Jingru Chen ^{1,2}, Yuanyuan Liu ^{1,2}, Wanting Huang ¹, Luru Ou ^{1,3}, Zhaohui Yang ^{1,3}, Xiong Guan ¹, Lingling Zhang ^{1,3,*} and Songqing Wu ^{1,2,4,*}

- ¹ State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, College of Plant Protection, Fujian Agriculture and Forestry University (FAFU), Fuzhou 350002, China; junxiangwang1201@163.com (J.W.); yangxiaozhen0907@163.com (X.Y.); hehuanpike@163.com (H.H.); cjr35057788@163.com (J.C.); liuyuan2021@163.com (Y.L.); hwthuangwanting@163.com (W.H.); olr13395087102@163.com (L.O.); buibuiyang@163.com (Z.Y.); guanxfafu@126.com (X.G.)
- ² College of Forestry, Fujian Agriculture and Forestry University (FAFU), Fuzhou 350002, China
- ³ College of Life Science, Fujian Agriculture and Forestry University (FAFU), Fuzhou 350002, China
- ⁴ Postdoctoral Station of Forestry, College of Forestry, Fujian Agriculture and Forestry University (FAFU), Fuzhou 350002, China
- * Correspondence: lingling00264@163.com (L.Z.); dabinyang@126.com (S.W.)



Citation: Wang, J.; Yang, X.; He, H.; Chen, J.; Liu, Y.; Huang, W.; Ou, L.; Yang, Z.; Guan, X.; Zhang, L.; et al. Knockout of Two Cry-Binding Aminopeptidase N Isoforms Does Not Change Susceptibility of *Aedes aegypti* Larvae to *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba and Cry11Aa Toxins. *Insects* **2021**, *12*, 223. <https://doi.org/10.3390/insects12030223>

Academic Editor: Kevin M. Myles

Received: 10 February 2021

Accepted: 2 March 2021

Published: 5 March 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Simple Summary: The midgut aminopeptidase N (APN) isoforms have been identified as the binding receptor of insecticidal Cry toxins in numerous insects, including the major arbovirus vector *Aedes aegypti* (*Ae. aegypti*). However, whether the Cry-binding APN acts as an essential functional receptor to mediate *Bacillus thuringiensis* subsp. *israelensis* (Bti) toxicity in *Ae. aegypti* larvae remains to be determined. In this study, our results provide the direct molecular evidence demonstrating that two Cry-binding APN isoforms (*AeAPN1* and *AeAPN2*) did not play a key role in mediating Bti Cry4Ba and Cry11Aa toxicity in *Ae. aegypti* larvae.

Abstract: The insecticidal Cry4Ba and Cry11Aa crystal proteins from *Bacillus thuringiensis* subsp. *israelensis* (Bti) are highly toxic to *Ae. aegypti* larvae. The glycosylphosphatidylinositol (GPI)-anchored APN was identified as an important membrane-bound receptor for multiple Cry toxins in numerous Lepidoptera, Coleoptera, and Diptera insects. However, there is no direct molecular evidence to link APN of *Ae. aegypti* to Bti toxicity *in vivo*. In this study, two Cry4Ba/Cry11Aa-binding *Ae. aegypti* GPI-APN isoforms (*AeAPN1* and *AeAPN2*) were individually knocked-out using CRISPR/Cas9 mutagenesis, and the *AeAPN1/AeAPN2* double-mutant homozygous strain was generated using the reverse genetics approach. ELISA assays showed that the high binding affinity of Cry4Ba and Cry11Aa protoxins to the midgut brush border membrane vesicles (BBMVs) from these APN knockouts was similar to the background from the wild-type (WT) strain. Likewise, the bioassay results showed that neither the single knockout of *AeAPN1* or *AeAPN2*, nor the simultaneous disruption of *AeAPN1* and *AeAPN2* resulted in significant changes in susceptibility of *Ae. aegypti* larvae to Cry4Ba and Cry11Aa toxins. Accordingly, our results suggest that *AeAPN1* and *AeAPN2* may not mediate Bti Cry4Ba and Cry11Aa toxicity in *Ae. aegypti* larvae as their binding proteins.

Keywords: *Aedes aegypti*; Cry4Ba; Cry11Aa; aminopeptidase N; CRISPR/Cas9

1. Introduction

Aedes aegypti (*Ae. aegypti*) is a principal vector of several human diseases, including dengue fever, yellow fever, chikungunya, and Zika fever [1]. The annual dengue incidence was estimated to be 390 million infections worldwide by the World Health Organization

(WHO) [2]. Unfortunately, no effective vaccines against some vector-borne viral diseases (i.e., chikungunya and Zika) have been developed to date [3,4]. Hence, there is an urgent need to establish an effective and comprehensive control system for reducing the population density of vector mosquitoes.

The microbial agents *Bacillus thuringiensis* (Bt) have been used worldwide for pest control in agriculture, forestry, and sanitation [5]. Bt subsp. *israelensis* (Bti) is one of the Bt subspecies that specifically kills mosquito larvae by secreting a variety of crystalline toxins (Cry4A, Cry4B, Cry10A, and Cry11A) and cytolytic toxins (Cyt1 and Cyt2) [6]. Bti has entered the WHO-recommended insecticide list against pathogenic mosquitoes due to its environmental safety profile, target specificity, and lack of potential for resistance development [7,8]. Nonetheless, the insecticidal mechanism of Bt has not been fully understood and remains under investigation [9,10]. According to the known model of action of the three-domain Cry (3d-Cry) toxins, the Cry protoxins are first hydrolyzed at the N-terminus by gut proteases of target insects, and then bind to multiple receptors in the midgut brush border. The activated Cry toxins further accumulate in the apical membrane of the epithelial cells to oligomerize, induce pore formation, and destroy the cell osmotic balance; mechanisms which eventually lead to midgut ulceration and larval death [10]. Currently, four major midgut molecules have been identified as receptors for lepidoptera-specific Cry1 toxins, including the cadherin-like (CAD), the glycosylphosphatidylinositol (GPI)-anchored aminopeptidase N (APN), the GPI-anchored alkaline phosphatase (ALP), and the ATP-binding cassette transporter subfamily C (ABCC2/3) [11]. Several recent studies have revealed that Cry resistance mechanisms were primarily associated with the genetic alterations of these membrane receptors in several field- and laboratory-selected Bt-resistant insects [12]. Hence, functional analysis of Bt receptors is the key to an in-depth understanding of the underlying molecular basis of Bt toxicity and resistance mechanisms in target insects.

The APN family (EC 3.4.11.2) is a ubiquitous hydrolase enzyme mainly responsible for digesting protein through hydrolyzing neutral and basic amino acids at the N-terminus of polypeptides [13]. Since a 120-kDa APN of *Manduca sexta* (*M. sexta*) was first identified as the Cry-binding receptor, many more Lepidoptera GPI-APNs have been proposed to be involved in the process of Bt toxin binding [14,15]. Similarly, APNs of *Anopheles quadrimaculatus* and *Anopheles gambiae* were identified as the binding receptors for the Bt subsp. *Jegathesan* (Bti) Cry11Ba toxin [16,17]. Two *Ae. aegypti* GPI-APN isoforms, named *AeAPN1* (AAEL012778), *AeAPN2* (AAEL008155, the VectorBase ID has been updated to AAEL019828) have been identified as Cry11Aa-binding receptors by pull-down assays and receptor-binding studies [18,19]. The toxicity of Cry11Aa against *Ae. aegypti* larvae was enhanced by adding the full-length and partial fragments of *AeAPN2* proteins [19]. Thus, the above evidence suggested that *Ae. aegypti* GPI-APNs may be involved in the pathogenesis of different Bti Cry toxins as important membrane-bound receptors.

To investigate whether the Cry-binding APN is the essential functional receptor for Bti Cry toxins, CRISPR/Cas9-mediated mutagenesis was used for the functional analysis of *AeAPN1* and *AeAPN2* isoforms by loss-of-function approach in *Ae. aegypti*. Two *AeAPNs* are hypothesized to be the functional receptor molecules involved in the toxicity of Bti Cry4Ba and Cry11Aa, which in APN knockouts should be more tolerant to Cry4B and Cry11A toxins than the WT strain. However, our study showed that both the binding affinity and the larval susceptibility of the single *AeAPN1* or *AeAPN2* knockout strain, and the double *AeAPN1/AeAPN2* mutant strain to Cry4B and Cry11A toxins did not differ significantly compared to the wild-type (WT) strain. Our results provide direct molecular evidence demonstrating that *AeAPN1* and *AeAPN2* may not mediate Bti Cry4Ba and Cry11Aa toxicity in *Ae. aegypti*.

2. Materials and Methods

2.1. Mosquito and Bt Strains

The laboratory WT strain of *Ae. aegypti* (*Ae. aegypti* Haikou strain) was provided by the Fujian International Travel Health Care Center (Fujian, Fuzhou, China) and reared without exposure to Bt toxins for over 50 generations. All *Ae. aegypti* strains were maintained at 26 ± 1 °C and $83\% \pm 3\%$ relative humidity with a photoperiod of 14-h light/10-h dark cycles. The recombinant Bt strains individually producing Cry4Ba and Cry11Aa (pCG6-Cry4Ba and pCG6-Cry11Aa) were provided by Dr. Sarjeet R Gill's Laboratory, University of California Riverside, USA and stored at -80 °C.

2.2. Purification of Cry4Ba and Cry11Aa Protoxins

The pCG6-Cry4Ba and pCG6-Cry11Aa were grown in the 1/2 LB (Luria-Bertani) medium containing 12.5 µg/mL erythromycin at 30 °C. Subsequently, the crystal inclusions were completely released from the spores, the medium was removed by centrifugation at $9500 \times g$ and the pellet were washed three times with 1 M NaCl plus 0.03% Triton X-100 and three times with distilled water. The Cry4Ba crystal inclusion was purified by a repeated crystal solubilization method as previously described [20]. The Cry11Aa crystal inclusion was purified by discontinuous sucrose gradients as previously described [21]. The Cry4Ba protoxin was solubilized in alkaline buffer (50 mM Na₂CO₃/NaHCO₃, pH 10.5), and Cry11Aa protoxin was solubilized in distilled water. The protein concentrations of Cry protoxins were determined using the Bradford Protein Assay Kit (Beyotime, Shanghai, China) with Bovine Serum Albumin (BSA) as the standard.

2.3. sgRNA Design and Synthesis

The CRISPR/Cas9 target sites were designed in the third exon of *AeAPN1* and *AeAPN2* genes using CRISPOR program (<http://crispor.tefor.net/> (accessed on 8 October 2019)), and potential off-target effects were evaluated by Cas-OFFinder (<http://www.rgenome.net/cas-offinder/> (accessed on 8 October 2019)) (Table S1). DNA templates for small-guide RNAs (sgRNAs) were prepared by polymerase chain reaction (PCR) with target specific primers (Table 1) using KOD Hot Start Polymerase (TOYOBO, Osaka, Japan), and purified using a Gel Extraction Kit (OMEGA Bio-Tek, Norcross, GA, USA). In vitro transcription of sgRNAs was performed using the MEGAscript Kit (Ambion, Austin, MA, USA), and purified by phenol: chloroform extraction and isopropanol precipitation as per the manufacturer's instructions. The purified sgRNA concentration was determined using the ultra-micro spectrophotometer Q5000 (Quawell, San Jose, CA, USA) and stored at -80 °C until use.

Table 1. List of DNA oligo primers used in this study.

Primer Name	Sequence 5'-3'
sgRNA-R	ATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
APN1-sgRNA-1-F	GAAATTAATACGACTCACTATA GGTCTACAGTCGGCCATCCAGTT TTAGAGCTAGAAATAGC
APN1-sgRNA-2-F	GAAATTAATACGACTCACTATA GGTTCGTGCGCACTCAGCAGTT TTAGAGCTAGAAATAGC
APN2-sgRNA-1-F	GAAATTAATACGACTCACTATA GGATTGGAGCTAGCGGTAACGTT TTAGAGCTAGAAATAGC
APN2-sgRNA-2-F	GAAATTAATACGACTCACTATA GTGCGTCGAGACTACAAGACGTT TTAGAGCTAGAAATAGC
<i>AeAPN1</i> -F	GGAATGCCGATACTCCAAGATCAAT
<i>AeAPN1</i> -R	TGAAAATAATCCACTCATTGGCCGG
<i>AeAPN2</i> -F	AGTGTCTGAACATGTTCCGTGT
<i>AeAPN2</i> -R	TATGCGTCGTTGATCAGCTGAGC

2.4. Embryo Microinjection and Generation of *Ae. aegypti* Knockout Strains

The collection of mosquito embryos and microinjections were performed as described previously [22]. About 1 nL sgRNA/Cas9 mixtures of 300 ng/ μ L *spCas9* protein (NEB, USA), and 100 ng/ μ L each of sgRNAs and 1x Cas9 nuclease reaction buffer was injected into the fresh embryos using the Nanoject IIITM Microinjection System (Drummond, PA, USA). The hatched G₀ embryos were reared to pupation, and each pupa was transferred individually in cups for eclosion. The genomic DNA (gDNA) was extracted from one hindleg of each G₀ mosquito adult using 50 μ L of chelex-100 buffer containing 5% chelex-100 beads (BioRad, Berkeley, CA, USA) and 1 mg/ μ L Proteinase K (TaKaRa, Dalian, China), hatching at 55 °C overnight and 5 μ L supernatant for PCR template. G₀ mutations were identified by PCR amplification using Premix Taq Version 2.0 (TaKaRa, Dalian, China) with corresponding target specific primers (Table 1) followed by Sanger sequencing. The validated G₀ mutations were single outcrossed with the WT strain to generate F₁ families, and the F₁ individuals were validated by PCR and sequencing as described above. F₁ individuals with similar mutation sequences were pooled to generate F₂ families. The homozygous mutants were screened from F₂ individuals and pooled to establish the homozygous knockout strains.

2.5. Preparation of Brush Border Membrane Vesicles from *Ae. aegypti* Larvae

The alimentary tract tissues were dissected from approximately 500 early four-instar mosquito larvae, soaked in MET buffer (0.3 M mannitol, 5 mM EDTA, and 17 mM Tris-Cl, pH 7.5) and stored at –80 °C. The brush border membrane vesicles (BBMVs) were prepared using the differential magnesium precipitation method, as described previously [23]. The protein concentrations of the BBMVs were determined as described above.

2.6. Proteomic Identification of Midgut BBMVs from *Ae. aegypti* Larvae

To confirm the absence of *AeAPN1* and *AeAPN1* isoforms at the protein level in midgut brush border membrane of *AeAPN* knockouts, proteomic analysis of midgut BBMV proteins were performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Then, 15 μ g BBMV proteins were separated on 10% SDS-PAGE, and the target regions (~70 kDa–170 kDa) of Coomassie blue stained gels including *AeAPNs* were excised and stored at –20 °C. The gel pieces were in-gel digestion and LC-MS/MS analysis using an Orbitrap-Fusion-Tribrid mass spectrometer (Thermo Fisher Scientific, MA, USA) at the Basic Forestry and Proteomics Research Center, FAFU. The resulting MS/MS data were processed using Proteome Discoverer 1.3 were matched against the annotated protein databases of *Ae. aegypti* (https://vectorbase.org/vectorbase/app/downloads/Current_Release/AegyptiLVP_AGWG/fasta/data/ (accessed on 1 February 2021)).

2.7. ELISA Binding Assays

ELISA binding assay was performed as reported previously [24]. The ELISA plates were coated with 0.3 μ g *Ae. aegypti* BBMVs in a final volume of 100 μ L PBS/well overnight at 4 °C. Then, the plates were washed three times with 200 μ L PBS, and blocked with 200 μ L/well of blocking buffer (PBS with 2% low fat milk powder) for 2 h at 37 °C. The plates were washed three times with 200 μ L PBST and 100 μ L/well of different concentrations of Cry protoxin/PBST were added and the cultures were incubated at 37 °C for 1 h. After washing three times with PBST and PBS to remove unbound protoxins, the wells were incubated with 100 μ L blocking buffer containing anti-Cry4Ba or anti-Cry11Aa polyclonal antibody (1:10,000 dilution) for 1 h at 37 °C. After the required washing steps, 100 μ L/well of PBST containing HRP-labeled goat anti-rabbit IgG (H + L) antibodies (1:5000 dilution) (Beyotime, Shanghai, China) was added and allowed to incubate for 1 h at 37 °C. The wells were washed again and 100 μ L/well of TMB chromogenic solution (Beyotime, Shanghai, China) was added and allowed to react for 8–15 min at 37 °C, protected from light. Finally, 50 μ L/well of 2 M H₂SO₄ was added to terminate the reaction, and the optical density (OD) value of each well was read at 450 nm with an absorbance microplate

reader (BioTek, Winooski, VT, USA). The K_d values were calculated using Curve Expert 1.4 (Hyams DG Software) and the ELISA-binding plots were generated in GraphPad prism 8.0 (GraphPad Software).

2.8. Bioassay of Bti Cry Toxins

The susceptibility to Bti Cry4Ba and Cry11Aa toxins was determined for different *Ae. aegypti* strains as described [25]. At least 5 gradient concentrations of purified Cry4Ba and Cry11Aa protoxins were added to 20 mL filtered water containing 25 early fourth instar larvae and mixed fully. Each larvae bioassay was repeated with three biological replications. The larval mortality was recorded after 24 h, and the medium lethal concentration (LC_{50}) and the 95% confidence intervals (CI) of the LC_{50} were calculated by probit analysis using PoloPlus (LeOra Software).

3. Results

3.1. Generation of Individual *AeAPN1* and *AeAPN2* Knockout *Ae. aegypti* Strains by CRISPR/Cas9

To increase G_0 mutagenesis efficiency and to induce sequence fragment deletions for *AeAPN1* and *AeAPN2* gene regions, we designed two CRISPR/Cas9 target sites within 100 bp region of the corresponding third exons to disrupt the downstream GPI-anchored sites (Figure 1A, B). After 400 and 800 *Ae. aegypti* eggs were injected with sgRNA/Cas9 mixtures to knockout *AeAPN1* and *AeAPN2* genes, 26 and 88, respectively, they hatched into larvae within one month (Table 2). DNA sequencing of 560 bp gDNA fragments spanning the gRNA targeted region from *AeAPN1*- and *AeAPN2*-knockout G_0 individuals indicated that 4 and 36 of the larvae, respectively, presented undefined peaks around the CRISPR/Cas9 target sites (Table 2). The homozygous knockout (KO) *Ae. aegypti* strain for *AeAPN1* (named *AeAPN1*-KO) and *APN2* (named *AeAPN2*-KO) were generated using the reverse genetics approach. The *AeAPN1*-KO presented a 107 bp deletion and 9 bp insertion between 2 of the CRISPR/Cas9 target sites, causing an amino acid mismatch and protein translation to terminate prematurely upstream of the GPI-anchored site (Figure 1C and Figure S1). The *AeAPN2*-KO had a 14 bp deletion and 99 bp deletion in the 2 of CRISPR/Cas9 target sites respectively, also resulting in the deletion of the GPI-anchored site and a loss of function as the membrane receptor (Figure 1D and Figure S2). The complementary DNA (cDNA) PCR sequencing of the two homozygous knockouts showed the genotypes of the coding region were consistent with the gDNA (Figure 1C,D).

Table 2. Transformation data of G_0 embryos injected with CRISPR/Cas9 constructs.

Injected Component	Injected G_0 Embryos	G_0 Survivors	G_0 Mutants
Cas9 protein, APN1-sgRNA-1 and APN1-sgRNA-2	400	26	4
Cas9 protein, APN2-sgRNA-1 and APN2-sgRNA-2	800	88	36

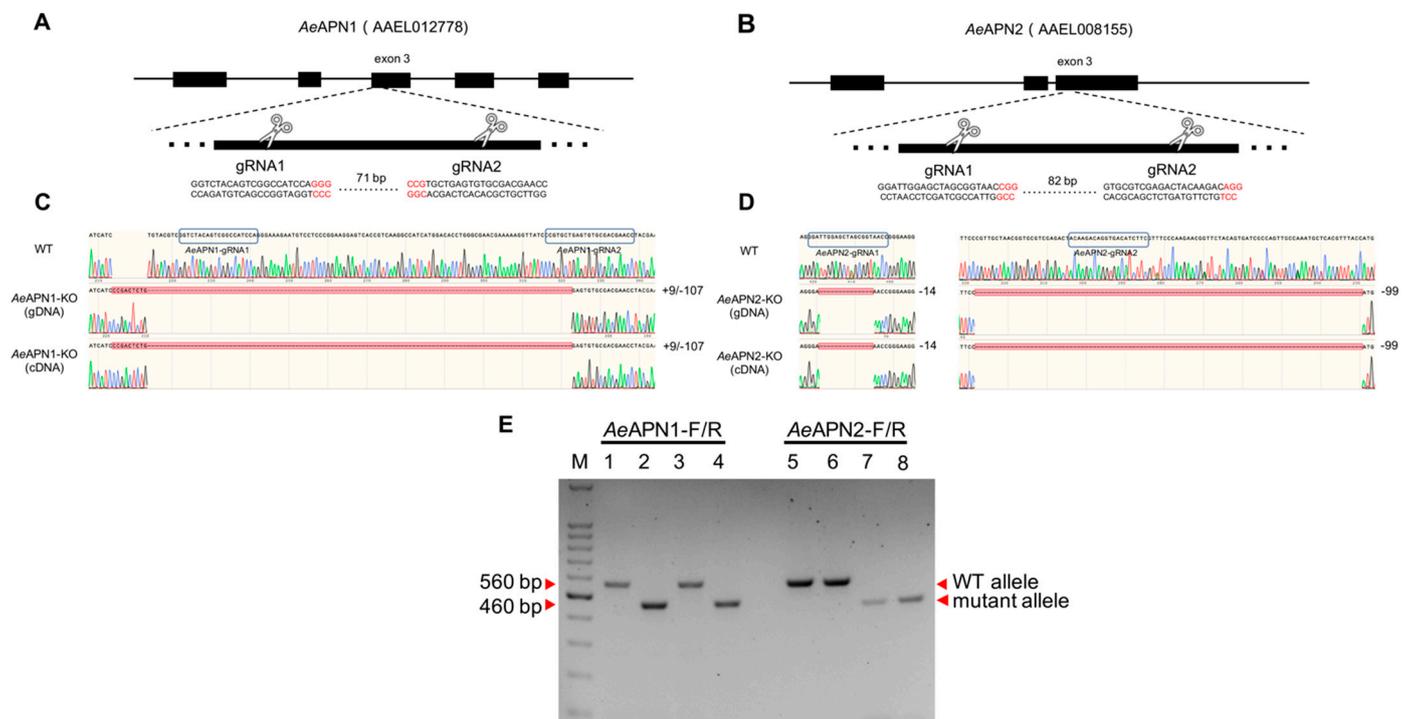


Figure 1. Generation of *AeAPN* knockout *Ae. aegypti* strains. Schematic representations of the *AeAPN1* (A) and *AeAPN2* (B) locus, where enlarged is the third exon contain 20-nucleotide sgRNA target sequences, and the PAM sequence shown in red. Aligned Sanger-sequencing trace of PCR-amplified using gDNA and cDNA from WT, *AeAPN1*-KO and *AeAPN2*-KO strains with specific primers (Table 1) spanning the gRNA targeted region (C,D). Electrophoresis of genomic-PCR products spanning target region from WT, *AeAPN1*-KO, *AeAPN2*-KO, and *AeAPN1/AeAPN2*-KO strains with specific primers *AeAPN1*-F/R and *AeAPN2*-F/R (E), Lane M: 100 bp DNA Ladder (TaKaRa, Dalian, China), Lane 1 and 5: the fragments were amplified from gDNA of the WT strain, Lane 2 and 6: the fragments were amplified from gDNA of the *AeAPN1*-KO strain, Lane 3 and 7: the fragments were amplified from gDNA of the *AeAPN2*-KO strain, Lane 4 and 8: the fragments were amplified from gDNA of the *AeAPN1/AeAPN2*-KO strain.

3.2. Generation of *AeAPN1/AeAPN2* Double-Mutant *Ae. aegypti* Strain

To generate a homozygous *Ae. aegypti* strain with double *AeAPN1/AeAPN2* knockout, *AeAPN1*-KO males were crossed with *AeAPN2*-KO females, and the homozygous mutants (named *AeAPN1/AeAPN2*-KO) were screened by the reverse genetics approach described above. The PCR products of *AeAPN1* and *AeAPN2* mutant allele were significantly smaller than the WT allele, which were easily separated by gel electrophoresis (Figure 1E). The proteomic analysis of target midgut BBMVs (~70–170 kDa) from WT and *AeAPN1/AeAPN2*-KO strains by LC-MS/MS, showed that 1382 proteins were identified from WT sample including 43 unique peptides of *AeAPN1* and 36 peptides (34 unique peptides) of *AeAPN2*, while no *AeAPN1* and *AeAPN2* peptides were identified in 1245 proteins of *AeAPN1/AeAPN2*-KO sample, suggesting the loss of GPI-anchoring signal of *AeAPN1* and *AeAPN2* cannot anchor to the midgut apical microvilli of *Ae. aegypti* (Figure S3 and Table S2).

3.3. *Cry4Ba* and *Cry11Aa* Protoxins Binding to BBMVs of the APN Knockouts and the WT Strains

The ELISA binding assays using the *Cry4Ba* protoxin to BBMVs of *AeAPN1*-KO, *AeAPN2*-KO, *AeAPN1/AeAPN2*-KO, and WT strains showed that the *Cry4Ba* bound to BBMVs of APN knockouts with high affinity (K_d *AeAPN1*-KO = 7.82 nM, K_d *AeAPN2*-KO = 8.31 nM and K_d *AeAPN1/AeAPN2*-KO = 8.94 nM), which was not significantly different than the binding of the WT strain (K_d WT = 8.07 nM) (Figure 2A). The *Cry11Aa* protoxin also had a high affinity to BBMVs of APN knockouts (K_d *AeAPN1*-KO = 9.9 nM, K_d *AeAPN2*-KO = 11.01 nM,

K_d *AeAPN1/AeAPN2-KO* = 10.35 nM, and K_d *WT* = 7.88 nM) (Figure 2B). These data indicated that losing of *AeAPN1* and *AeAPN2* cannot affect the high affinity of Cry4Ba and Cry11Aa toxins binding the midgut epithelial cells of *Ae. aegypti* larvae.

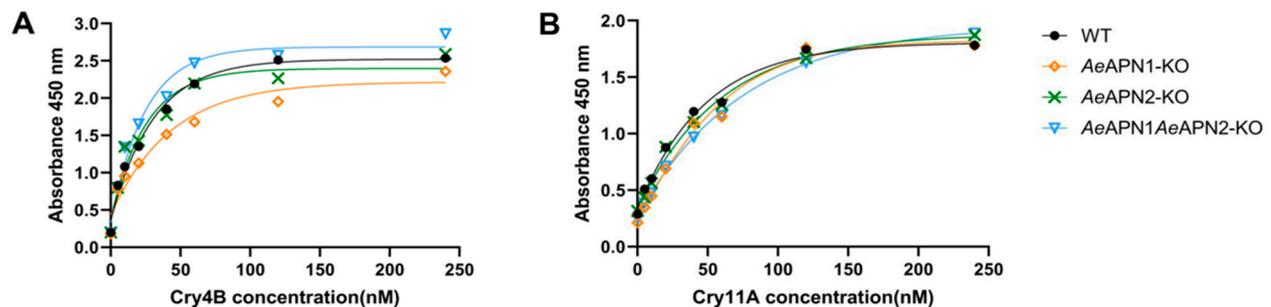


Figure 2. Binding of Cry4Ba and Cry11Aa toxins to *Ae. aegypti* BBMVs by ELISA. Different concentrations (0 nM, 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 120 nM, 240 nM) of Cry4Ba (A) and Cry11Aa (B) protoxins bound to 0.3 μ g midgut BBMVs from APN mutant strains and WT strain in the ELISA plates.

3.4. Response of the *Ae. aegypti* Larvae to Cry4Ba and Cry11Aa Protoxins

Bioassays of the *AeAPN1-KO*, *AeAPN2-KO*, and *AeAPN1/AeAPN2-KO* strains with Cry4Ba and Cry11Aa showed that the LC_{50} of Cry4Ba and Cry11Aa for these three APN mutant strains were similar to that of the WT strain, which indicated that neither the single knockout of *AeAPN1* or *AeAPN2*, nor simultaneous disruption of *AeAPN1* and *AeAPN2* resulted in a significant reduction of *Ae. aegypti* larvae susceptibility to Cry4Ba and Cry11Aa toxins (Tables 3 and 4).

Table 3. Susceptibility of *Ae. aegypti* strains to Cry4Ba toxin.

<i>Ae. aegypti</i> Strain	n	Slope (SE)	LC_{50} (μ g/mL) (95% CI)	RR ^a
WT	1125	2.947 (0.098)	1.771 (1.663–1.888)	1
<i>AeAPN1-KO</i>	1125	3.774 (0.181)	1.504 (1.421–1.591)	0.849
<i>AeAPN2-KO</i>	1125	3.664 (0.171)	1.863 (1.771–1.958)	1.052
<i>AeAPN1/AeAPN2-KO</i>	1125	3.079 (0.135)	2.221 (2.092–2.367)	1.254

^a RR (Relative Resistance) = LC_{50} of the knockout strain/ LC_{50} of the WT strain.

Table 4. Susceptibility of *Ae. aegypti* strains to Cry11Aa toxin.

<i>Ae. aegypti</i> Strain	n	Slope (SE)	LC_{50} (μ g/mL) (95% CI)	RR ^a
WT	1125	1.747 (0.110)	0.602 (0.526–0.685)	1
<i>AeAPN1-KO</i>	1125	3.192 (0.224)	0.556 (0.496–0.619)	0.924
<i>AeAPN2-KO</i>	1125	1.989 (0.124)	0.599 (0.546–0.653)	0.995
<i>AeAPN1/AeAPN2-KO</i>	1125	2.770 (0.163)	0.826 (0.769–0.897)	1.372

^a RR (Relative Resistance) = LC_{50} of the knockout strain/ LC_{50} of the WT strain.

4. Discussion

In recent decades, the identification of midgut molecules as Bt receptors in target insects has been a research objective to define Bt activity. The sequential binding model proposes that the activated Cry toxin forms a Pre-pore oligomer after interacting with the transmembrane receptor CAD, and further binding to the GPI-anchored membrane receptors (APN or ALP) leads to membrane insertion, pore formation, and cell lysis [14]. The GPI-APN was the first to be described as a Cry-binding receptor in the midgut of Lepidopteran insects [15]. However, whether the Cry-binding APN acts as an essential functional receptor to mediate Bti toxicity in *Ae. aegypti* larvae remains to be determined. In this study, our results demonstrated that deficiency in *AeAPN1* and *AeAPN2* isoforms do not affect either the binding affinity or susceptibility of *Ae. aegypti* larvae to Cry4Ba and Cry11Aa toxins.

Early functional analyses of APN as Cry receptors showed that the GPI-APNs of *M. sexta* and *Heliothis virescens* (*H. virescens*) were reconstituted into phospholipid vesicles and planar lipid bilayers to increase the binding affinity of Cry1 toxins and catalyze channel formation [26–28]. The *in vitro* cytotoxicity analysis indicated that heterologous expression of different APNs in insect cell lines (S2, Sf9, and Sf21), which are not susceptible to Cry toxin, decrease the cell viability or facilitate cell swelling under activated Cry toxins infection, such as following *Helicoverpa armigera* (*H. armigera*) APN1 [29], *H. virescens* APN1 [30] and two of *Ae. aegypti* APNs [31] infections. Whereas, the expression of *M. sexta* APN1 in S2 cells did not lead to host cell sensitivity to activated Cry1 toxins [32]. Furthermore, the down-regulated APN expression using *in vivo* RNAi was associated with tolerance to Cry toxins in several insects, including *Spodoptera exigua* APN1 [33], *M. sexta* APN1 [34], three *Chilo suppressalis* APNs [35], three *Diatraea saccharalis* APNs [36] and three *Ae. aegypti* APNs [37]. Another important source of APN involvement in Bt toxicity is suggested by deletion mutation studies of *HaAPN1* and the down-regulation of *Trichoplusia ni* (*T. ni*) APN1 transcription, which have been shown to be genetically linked with Cry1Ac resistance [38,39]. The transcriptome analysis of a laboratory-selected Cry11Aa resistant *Ae. aegypti* strain indicated that the transcript levels of two APNs (AAEL008158 and AAEL008162) were significantly down-regulated compared to the WT strain. Nonetheless, no changes in expression and non-synonymous mutations have been observed in *AeAPN1* and *AeAPN2* [40]. Thus, further functional analysis of APN applying genome-editing strategies are required to confirm the role of APN as a Bt functional receptor rather than as a Cry-binding protein.

Recently, CRISPR/Cas9-mediated genomic editing technology has provided a powerful tool to generate KO/knock-in models, and has been applied to the identification of Bt receptors in some lepidopteran insects. For example, the ABCA2 mutant generated by CRISPR/Cas9 in *T. ni* and *H. armigera* resulting in high-level resistance to Cry2A toxins [41,42]. CRISPR/Cas9-mediated double knockout of ABCC2 and ABCC3 in *H. armigera* and *Plutella xylostella* (*P. xylostella*) resulted in more than 1000-fold resistance to Cry1Ac [43,44]. KO of *T. ni* CAD and *Spodoptera frugiperda* (*S. frugiperda*) CAD did not affect the larvae susceptibility to Cry1 and Cry2 toxins [45,46]. Conversely, knockout of *H. armigera* CAD by CRISPR/Cas9 in a Bt-susceptible strain could increase resistance to Cry1Ac toxin by more than 500-fold [47]. Moreover, none of the 3 *H. armigera* APNs (*HaAPN1*, *HaAPN2*, and *HaAPN5*) individually knocked out using CRISPR/Cas9 resulted in any change in susceptibility of the larvae to Cry1A and Cry2A toxins [48].

Genetic and molecular studies have indicated that the entomopathogenicity of Bt is complex and may involve multiple membrane-bound receptors and intracellular pathways [9,49]. Generating single-receptor knockout insects by genome editing often does not achieve significantly different results in larvae susceptibility to Bt toxins. For example, neither *PxABCC2* nor *PxABCC3* knockout in *P. xylostella* strains produced any significant resistance to Cry1Ac, while simultaneous mutations of the two genes exhibited high-level resistance (>8000-fold) to Cry1Ac, revealing the functional redundancy between ABCC2 and ABCC3 as receptors in the activity of Cry1Ac toxins [44]. In this study, the double mutant *AeAPN1/AeAPN2-KO* strain did not show increased tolerance to Cry4Ba and Cry11Aa toxins, suggesting *AeAPN1* and *AeAPN2* did not exhibit synergistic effects on mediating the entomopathogenicity of Cry4Ba and Cry11Aa (Tables 2 and 3). The genome-wide analysis of the APN gene family showed that 29 APN isoforms were identified in *Ae. aegypti* genome, 11 of them were predicted to carry the GPI-anchoring signal. Moreover, a previous pulldown assay and our Co-immunoprecipitation assay (unpublished data) showed that *AeAPN3* (AAEL012774) could bind to Cry4Ba and Cry11Aa toxins in the midgut BBMV of *Ae. aegypti* [18]. Therefore, the potential complementary roles of other *AeAPNs* as Cry-binding receptors in determining susceptibility to Cry4Ba/Cry11Aa toxins should not be ruled out. Comprehensive determination of the role of APN in the action mechanism of Bt requires further functional analysis of multiple APNs in a variety of

insects. Overall, our study revealed that two Cry-binding APNs (*AeAPN1* and *AeAPN2*) may not play a key role in mediating Bti Cry4Ba and Cry11Aa toxicity in *Ae. aegypti*.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2075-4450/12/3/223/s1>, Figure S1: Amino acid sequence alignment of *AeAPN1* isoform from WT and *AeAPN1*-KO strains, Figure S2: Amino acid sequence alignment of *AeAPN2* isoform from WT and *AeAPN1*-KO strains, Figure S3: SDS-PAGE profile of midgut BBMV protein from the WT strain and the *AeAPN1/AeAPN2*-KO strain, Table S1: In silico analysis of off-target activity of sgRNAs used in this study, Table S2: *AeAPN1* and *AeAPN2* peptides identified from midgut BBMV of the WT strain.

Author Contributions: Conceptualization, J.W., S.W., L.Z., and X.G.; methodology, J.W., X.Y., and H.H.; software, J.W., X.Y., H.H.; investigation, J.W., X.Y., H.H., J.C., Y.L., W.H., L.O. and Z.Y.; formal analysis, J.W., X.Y., H.H.; writing—original draft preparation, J.W.; writing—review and editing, J.W., S.W., L.Z., and X.G.; supervision, S.W., L.Z., and X.G.; funding acquisition, S.W., L.Z., and X.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Program of China (grant number 2017YFD0600105); the National Natural Science Foundation of China (grant numbers U1905201 and 31601905); the Natural Science Foundation of Fujian Province (2020J01550); the United Fujian Provincial Health and Education Project for Tackling Key Research (Grant No. 2019-WJ-29); Open Project Funds of State Key Laboratory of Pathogen and Biosecurity (SKLPBS1838); China Postdoctoral Science Foundation (grant number 2017M612107); Forestry Programs of Science and Technology in Fujian Province (grant number Mincaizhi [2020] 601).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Exclude this statement.

Acknowledgments: We thank Yajie Guo provided constructive suggestions in ELISA binding assay. We also thank the Basic Forestry and Proteomics Research Center, FAFU for providing proteomic analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Kyle, J.L.; Harris, E. Global spread and persistence of dengue. *Annu. Rev. Microbiol.* **2008**, *62*, 71–92. [CrossRef]
- World Health Organization. Global Strategy for Dengue Prevention and Control, 2012–2020. World Health Organization. 2012. Available online: <https://apps.who.int/iris/handle/10665/75303> (accessed on 1 March 2021).
- Wilder-Smith, A. Dengue vaccine development: Status and future. *Bundesgesundheitsblatt Gesundh. Gesundh.* **2020**, *63*, 40–44. [CrossRef]
- Zanotto, P.M.d.A.; Leite, L.C.d.C. The Challenges Imposed by Dengue, Zika, and Chikungunya to Brazil. *Front. Immunol.* **2018**, *9*, 1964. [CrossRef] [PubMed]
- Crickmore, N.; Zeigler, D.R.; Feitelson, J.; Schnepf, E.; Van Rie, J.; Lereclus, D.; Baum, J.; Dean, D.H. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 807–813. [CrossRef] [PubMed]
- Berry, C.; O’Neil, S.; Ben-Dov, E.; Jones, A.F.; Murphy, L.; Quail, M.A.; Holden, M.T.; Harris, D.; Zaritsky, A.; Parkhill, J. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Appl. Environ. Microbiol.* **2002**, *68*, 5082–5095. [CrossRef] [PubMed]
- World Health Organization. Pesticides and Their Application: For the Control of Vectors and Pests of Public Health Importance. World Health Organization. 2006. Available online: <https://apps.who.int/iris/handle/10665/69223> (accessed on 1 March 2021).
- Ben-Dov, E. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins (Basel)* **2014**, *6*, 1222–1243. [CrossRef] [PubMed]
- Vachon, V.; Laprade, R.; Schwartz, J.-L. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. *J. Invertebr. Pathol.* **2012**, *111*, 1–12. [CrossRef]
- Bravo, A.; Gómez, I.; Mendoza Almanza, G.; Gaytán, M.; Soberón, M. *Different Models of the Mode of Action of Bt 3d-Cry Toxins*; CABI: Oxford, UK, 2015; pp. 56–68.
- Pardo-López, L.; Soberón, M.; Bravo, A. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: Mode of action, insect resistance and consequences for crop protection. *FEMS Microbiol. Rev.* **2013**, *37*, 3–22. [CrossRef]
- Peterson, B.; Bezuidenhout, C.C.; Van den Berg, J. An Overview of Mechanisms of Cry Toxin Resistance in Lepidopteran Insects. *J. Econ. Entomol.* **2017**, *110*, 362–377. [CrossRef] [PubMed]
- Nocek, B.; Mulligan, R.; Bargassa, M.; Collart, F.; Joachimiak, A. Crystal structure of aminopeptidase N from human pathogen *Neisseria meningitidis*. *Proteins* **2008**, *70*, 273–279. [CrossRef] [PubMed]

14. Pigott, C.R.; Ellar, D.J. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 255–281. [[CrossRef](#)]
15. Knight, P.J.K.; Knowles, B.H.; Ellar, D.J. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. *J. Biol. Chem.* **1995**, *270*, 17765–17770. [[CrossRef](#)] [[PubMed](#)]
16. Abdullah, M.A.; Valaitis, A.P.; Dean, D.H. Identification of a *Bacillus thuringiensis* Cry11Ba toxin-binding aminopeptidase from the mosquito, *Anopheles quadrimaculatus*. *BMC Biochem.* **2006**, *7*, 16. [[CrossRef](#)]
17. Zhang, R.; Hua, G.; Urbauer, J.L.; Adang, M.J. Synergistic and inhibitory effects of aminopeptidase peptides on *Bacillus thuringiensis* Cry11Ba toxicity in the mosquito *Anopheles gambiae*. *Biochemistry* **2010**, *49*, 8512–8519. [[CrossRef](#)]
18. Chen, J.; Aimanova, K.G.; Pan, S.; Gill, S.S. Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem. Mol. Biol.* **2009**, *39*, 688–696. [[CrossRef](#)]
19. Chen, J.; Likitvivatanavong, S.; Aimanova, K.G.; Gill, S.S. A 104 kDa *Aedes aegypti* aminopeptidase N is a putative receptor for the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Insect Biochem. Mol. Biol.* **2013**, *43*, 1201–1208. [[CrossRef](#)]
20. Zhou, Z.-s.; Yang, S.-j.; Shu, C.-l.; Song, F.-p.; Zhou, X.-p.; Zhang, J. Comparison and optimization of the method for Cry1Ac protoxin preparation in HD73 strain. *J. Integr. Agric.* **2015**, *14*, 1598–1603. [[CrossRef](#)]
21. Thomas, W.E.; Ellar, D.J. *Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: Effects on insect and mammalian cells in vitro and in vivo. *J. Cell Sci.* **1983**, *60*, 181–197. [[PubMed](#)]
22. Kistler, K.E.; Voshall, L.B.; Matthews, B.J. Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Rep.* **2015**, *11*, 51–60. [[CrossRef](#)] [[PubMed](#)]
23. Wolfersberger, M.; Luethy, P.; Maurer, A.; Parenti, P.; Sacchi, F.V.; Giordana, B.; Hanozet, G.M. 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. Part A Physiol.* **1987**, *86*, 301–308. [[CrossRef](#)]
24. Peña-Cardena, A.; Grande, R.; Sánchez, J.; Tabashnik, B.E.; Bravo, A.; Soberón, M.; Gómez, I. The C-terminal protoxin domain of *Bacillus thuringiensis* Cry1Ab toxin has a functional role in binding to GPI-anchored receptors in the insect midgut. *J. Biol. Chem.* **2018**, *293*. [[CrossRef](#)] [[PubMed](#)]
25. Batool, K.; Alam, I.; Zhao, G.; Wang, J.; Xu, J.; Yu, X.; Huang, E.; Guan, X.; Zhang, L. C-Type Lectin-20 Interacts with ALP1 Receptor to Reduce Cry Toxicity in *Aedes aegypti*. *Toxins* **2018**, *10*, 390. [[CrossRef](#)]
26. Sangadala, S.; Walters, F.S.; English, L.H.; Adang, M.J. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and 86Rb(+)-K⁺ efflux in vitro. *J. Biol. Chem.* **1994**, *269*, 10088–10092. [[CrossRef](#)] [[PubMed](#)]
27. Schwartz, J.L.; Lu, Y.J.; Söhnlein, P.; Brousseau, R.; Laprade, R.; Masson, L.; Adang, M.J. Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors. *FEBS Lett.* **1997**, *412*, 270–276. [[CrossRef](#)]
28. Luo, K.; Sangadala, S.; Masson, L.; Mazza, A.; Brousseau, R.; Adang, M.J. The *Heliothis virescens* 170kDa aminopeptidase functions as “Receptor A” by mediating specific *Bacillus thuringiensis* Cry1A δ -endotoxin binding and pore formation. *Insect Biochem. Mol. Biol.* **1997**, *27*, 735–743. [[CrossRef](#)]
29. Sivakumar, S.; Rajagopal, R.; Venkatesh, G.R.; Srivastava, A.; Bhatnagar, R.K. Knockdown of Aminopeptidase-N from *Helicoverpa armigera* Larvae and in Transfected Sf21 Cells by RNA Interference Reveals Its Functional Interaction with *Bacillus thuringiensis* Insecticidal Protein Cry1Ac. *J. Biol. Chem.* **2007**, *282*, 7312–7319. [[CrossRef](#)] [[PubMed](#)]
30. Wei, J.; Zhang, M.; Liang, G.; Wu, K.; Guo, Y.; Ni, X.; Li, X. APN1 is a functional receptor of Cry1Ac but not Cry2Ab in *Helicoverpa zea*. *Sci. Rep.* **2016**, *6*, 19179. [[CrossRef](#)] [[PubMed](#)]
31. Aroonkesorn, A.; Pootanakit, K.; Katzenmeier, G.; Angsuthanasombat, C. Two specific membrane-bound aminopeptidase N isoforms from *Aedes aegypti* larvae serve as functional receptors for the *Bacillus thuringiensis* Cry4Ba toxin implicating counterpart specificity. *Biochem. Biophys. Res. Commun.* **2015**, *461*, 300–306. [[CrossRef](#)]
32. Banks, D.J.; Hua, G.; Adang, M.J. Cloning of a *Heliothis virescens* 110 kDa aminopeptidase N and expression in Drosophila S2 cells. *Insect Biochem. Mol. Biol.* **2003**, *33*, 499–508. [[CrossRef](#)]
33. Qiu, L.; Cui, S.; Liu, L.; Zhang, B.; Ma, W.; Wang, X.; Lei, C.; Chen, L. Aminopeptidase N1 is involved in *Bacillus thuringiensis* Cry1Ac toxicity in the beet armyworm, *Spodoptera exigua*. *Sci. Rep.* **2017**, *7*, 45007. [[CrossRef](#)]
34. Flores-Escobar, B.; Rodríguez-Magadan, H.; Bravo, A.; Soberón, M.; Gómez, I. Differential role of *Manduca sexta* aminopeptidase-N and alkaline phosphatase in the mode of action of Cry1Aa, Cry1Ab, and Cry1Ac toxins from *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **2013**, *79*, 4543–4550. [[CrossRef](#)]
35. Wang, X.Y.; Du, L.X.; Liu, C.X.; Gong, L.; Han, L.Z.; Peng, Y.F. RNAi in the striped stem borer, *Chilo suppressalis*, establishes a functional role for aminopeptidase N in Cry1Ab intoxication. *J. Invertebr. Pathol.* **2017**, *143*, 1–10. [[CrossRef](#)]
36. Yang, Y.; Zhu, Y.C.; Ottea, J.; Husseneder, C.; Rogers Leonard, B.; Abel, C.; Huang, F. Molecular characterization and RNA interference of three midgut aminopeptidase N isozymes from *Bacillus thuringiensis*-susceptible and -resistant strains of sugarcane borer, *Diatraea saccharalis*. *Insect Biochem. Mol. Biol.* **2010**, *40*, 592–603. [[CrossRef](#)]
37. Saengwiman, S.; Aroonkesorn, A.; Dedvisitsakul, P.; Sakdee, S.; Leetachewa, S.; Angsuthanasombat, C.; Pootanakit, K. In vivo identification of *Bacillus thuringiensis* Cry4Ba toxin receptors by RNA interference knockdown of glycosylphosphatidylinositol-linked aminopeptidase N transcripts in *Aedes aegypti* larvae. *Biochem. Biophys. Res. Commun.* **2011**, *407*, 708–713. [[CrossRef](#)]
38. Tiewsirir, K.; Wang, P. Differential alteration of two aminopeptidases N associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in cabbage looper. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 14037–14042. [[CrossRef](#)] [[PubMed](#)]

39. Zhang, S.; Cheng, H.; Gao, Y.; Wang, G.; Liang, G.; Wu, K. Mutation of an aminopeptidase N gene is associated with *Helicoverpa armigera* resistance to *Bacillus thuringiensis* Cry1Ac toxin. *Insect Biochem. Mol. Biol.* **2009**, *39*, 421–429. [[CrossRef](#)] [[PubMed](#)]
40. Lee, S.B.; Aimanova, K.G.; Gill, S.S. Alkaline phosphatases and aminopeptidases are altered in a Cry11Aa resistant strain of *Aedes aegypti*. *Insect Biochem. Mol. Biol.* **2014**, *54*, 112–121. [[CrossRef](#)] [[PubMed](#)]
41. Yang, X.; Chen, W.; Song, X.; Ma, X.; Cotto-Rivera, R.O.; Kain, W.; Chu, H.; Chen, Y.R.; Fei, Z.; Wang, P. Mutation of ABC transporter ABCA2 confers resistance to Bt toxin Cry2Ab in *Trichoplusia ni*. *Insect Biochem. Mol. Biol.* **2019**, *112*, 103209. [[CrossRef](#)]
42. Wang, J.; Wang, H.; Liu, S.; Liu, L.; Tay, W.T.; Walsh, T.K.; Yang, Y.; Wu, Y. CRISPR/Cas9 mediated genome editing of *Helicoverpa armigera* with mutations of an ABC transporter gene HaABCA2 confers resistance to *Bacillus thuringiensis* Cry2A toxins. *Insect Biochem. Mol. Biol.* **2017**, *87*, 147–153. [[CrossRef](#)]
43. Wang, J.; Ma, H.; Zhao, S.; Huang, J.; Yang, Y.; Tabashnik, B.E.; Wu, Y. Functional redundancy of two ABC transporter proteins in mediating toxicity of *Bacillus thuringiensis* to cotton bollworm. *PLoS Pathog.* **2020**, *16*, e1008427. [[CrossRef](#)]
44. Liu, Z.; Fu, S.; Ma, X.; Baxter, S.W.; Vasseur, L.; Xiong, L.; Huang, Y.; Yang, G.; You, S.; You, M. Resistance to *Bacillus thuringiensis* Cry1Ac toxin requires mutations in two *Plutella xylostella* ATP-binding cassette transporter paralogs. *PLoS Pathog.* **2020**, *16*, e1008697. [[CrossRef](#)] [[PubMed](#)]
45. Wang, S.; Kain, W.; Wang, P. *Bacillus thuringiensis* Cry1A toxins exert toxicity by multiple pathways in insects. *Insect Biochem. Mol. Biol.* **2018**, *102*, 59–66. [[CrossRef](#)] [[PubMed](#)]
46. Zhang, J.; Jin, M.; Yang, Y.; Liu, L.; Yang, Y.; Gómez, I.; Bravo, A.; Soberón, M.; Xiao, Y.; Liu, K. The Cadherin Protein Is Not Involved in Susceptibility to *Bacillus thuringiensis* Cry1Ab or Cry1Fa Toxins in *Spodoptera frugiperda*. *Toxins* **2020**, *12*, 375. [[CrossRef](#)] [[PubMed](#)]
47. Wang, J.; Zhang, H.; Wang, H.; Zhao, S.; Zuo, Y.; Yang, Y.; Wu, Y. Functional validation of cadherin as a receptor of Bt toxin Cry1Ac in *Helicoverpa armigera* utilizing the CRISPR/Cas9 system. *Insect Biochem. Mol. Biol.* **2016**, *76*, 11–17. [[CrossRef](#)]
48. Wang, J.; Zuo, Y.Y.; Li, L.L.; Wang, H.; Liu, S.Y.; Yang, Y.H.; Wu, Y.D. Knockout of three aminopeptidase N genes does not affect susceptibility of *Helicoverpa armigera* larvae to *Bacillus thuringiensis* Cry1A and Cry2A toxins. *Insect Sci.* **2020**, *27*, 440–448. [[CrossRef](#)]
49. Zhang, X.; Candas, M.; Griko, N.B.; Taussig, R.; Bulla, L.A., Jr. A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 9897–9902. [[CrossRef](#)] [[PubMed](#)]