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Proteomic analysis of Cry2Aa-binding proteins and their receptor function in *Spodoptera exigua*

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The bacterium *Bacillus thuringiensis* produces Crystal (Cry) proteins that are toxic to a diverse range of insects. Transgenic crops that produce Bt Cry proteins are grown worldwide because of their improved resistance to insect pests. Although Bt "pyramid" cotton that produces both Cry1A and Cry2A is predicted to be more resistant to several lepidopteran pests, including *Spodoptera exigua,* than plants that produce Cry1Ac alone, the mechanisms responsible for the toxicity of Cry2Aa in *S. exigua* are not well understood. We identified several proteins that bind Cry2Aa (polycalin, V-ATPase subunits A and B, actin, 4-hydroxybutyrate CoA-transferase [4-HB-CoAT]), and a receptor for activated protein kinase C (Rack), in *S. exigua*. Recombinant, expressed versions of these proteins were able to bind the Cry2Aa toxin *in vitro* assays. RNA interference gene knockdown of the *Se*-V-ATPase subunit B significantly decreased the susceptibility of *S. exigua* larvae to Cry2Aa, whereas knockdown of the other putative binding proteins did not. Moreover, an *in vitro* homologous competition assay demonstrated that the *Se*-V-ATPase subunit B binds specifically to the Cry2Aa toxin, suggesting that this protein acts as a functional receptor of Cry2Aa in *S. exigua*. This the first Cry2Aa toxin receptor identified in *S. exigua* brush-border membrane vesicles.

e Crystal (Cry) toxins produced by *B* (Bt) are a diverse group of proteins that are used to control a broad range of insect pests[1]. Not only are Bt compounds used worldwide as pesticides, but *C* genes have been used to create transgenic crops with enhanced resistance to pest insects. Of the Cry2A subfamily, both Cry2Aa and Cry2Ab have been successfully incorporated into plants to produce transgenic insect-resistant crops[2,3].

In China, transgenic Bt cotton expressing the Cry2Ab toxin has not been commercialized. In contrast, transgenic Cry1Ac cotton, which was rst cultivated in 1997, is now grown on more than 3 million hectares in 2015[4]. Adoption of this Bt cotton variety has resulted in the decline of several important pest populations at the landscape level in China, as well as reductions in the application of broad-spectrum insecticides[5]. Nonetheless, the continued large-scale planting of Bt cotton has led to new problems, including the evolution of resistance among target pests[6,7] and rapid increases in non-target hemipteran[8] and lepidopteran pests[9–11]. Developing plants that express more than one Cry toxin could, however, both delay insect resistance to Bt crops and increase the target pest spectrum[12,13]. For example, transgenic plants that express both Cry1Ac and Cry2Ab toxin would be expected to be much more resistant to lepidopteran pests, especially the beet armyworm [3,14].

. (Hübner; Lepidoptera: Noctuidae) is a polyphagous insect that has not been a signi cant crop pest in China for some time[11]. However, because of the recent reduction in pesticide usage in cotton elds, and because it is insensitive to Cry1Ac, the beet armyworm has once again become a major economic pest of cotton in China[3,15–17]. Although some studies suggest that . is less sensitive to Cry2Aa/b than to Cry1B, Cry1C or other toxins[18,19], Bt crops producing both Cry1Ac and Cry2Aa/b (Cry2Ab in the case of cotton) are predicted to be more resistant to . , and several other lepidopteran pests, than those currently cultivated in China which produce only Cry1Ac[3,15,20–22]. However, except for cadherin[23], little is known about the receptor proteins that mediate the toxicity of the Cry2A subfamily of proteins in the Lepidoptera.

In this paper, we present the rst analysis of Cry2Aa receptor proteins in . brush-border membrane vesicles (BBMVs). Because the Cry2Aa protein has 87% sequence homology with Cry2Ab, and similar toxicity to both the Lepidoptera and Diptera, we chose Cry2Aa to represent the Cry2A subfamily[24,25]. In addition, and

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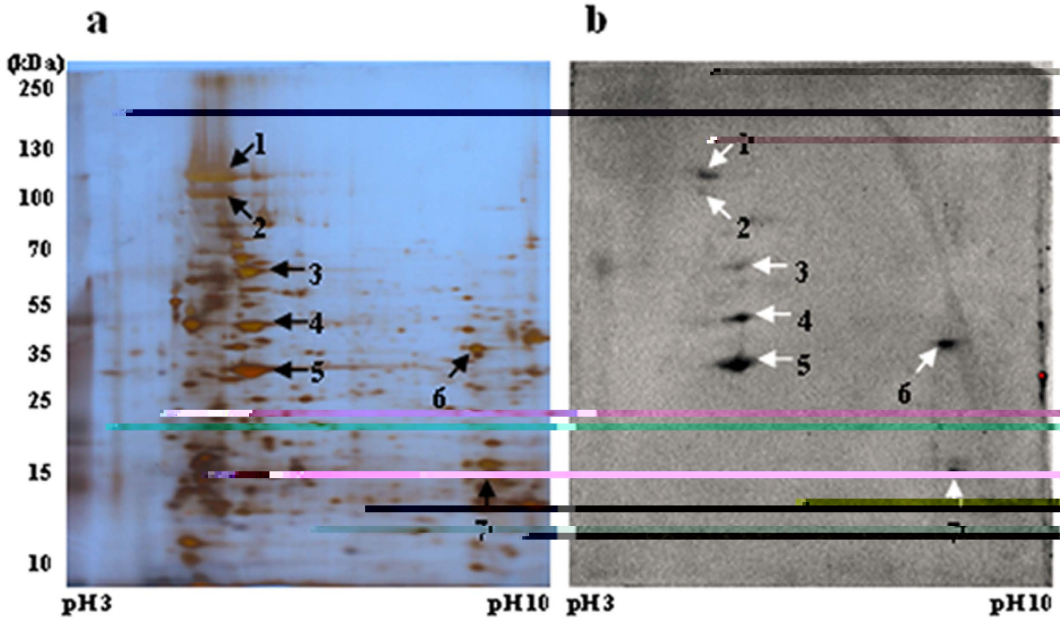


F 1. **2DE** ***S. exigua* BBM** **4w** **-C 2A** **.** ( ) . BBMV proteins (100 μg) separated by 2DE, marker positions are indicated on the le of the gel. e pH 3–10 IPG strip used for isoelectric focusing is shown at the bottom. ( ) . Cry2Aa-binding proteins are the spots numbered 1 to 7; spot positions correspond to those in Fig. 1a.

	A N	M (D)	PI	N	
1	gi|327082384	32.7	4.48	polycalin	
2	gi|327082384	32.7	4.48	polycalin	
3	gi|401323	68.46	5.14	V-type ATPase subunit A	
4	gi|401326	55.1	5.26	V-type ATPase subunit B	*H*
5	gi|157111829	41.9	5.29	Actin	*A*
6	gi|389613607	51.1	8.33	4-hydroxybutyrate CoA-transferase	
7	gi|328670883	36.4	7.64	Receptor for activated protein kinase C	*H*

1. **C 2A -** ***S. exigua* BBM** **NCBI** **M** **2.2** **w** **.** [a]Numbers correspond to those in Fig. 1. [b]Proteins in the NCBI database for which signi cant peptide mass matches or sequence similarity were available.

possibly more important, the puri ed toxin (purity > 98%) is only commercially available for Cry2Aa at present. e goal of this study was to identify Cry2Aa binding proteins in . BBMVs using two-dimension gel electrophoresis (2DE) and LC-MS (liquid chromatography-mass spectrometry)/MS techniques. e utility of using such a combination of protein binding assays and RNA interference to analyze the receptor function of binding proteins is also evaluated and discussed.

Results

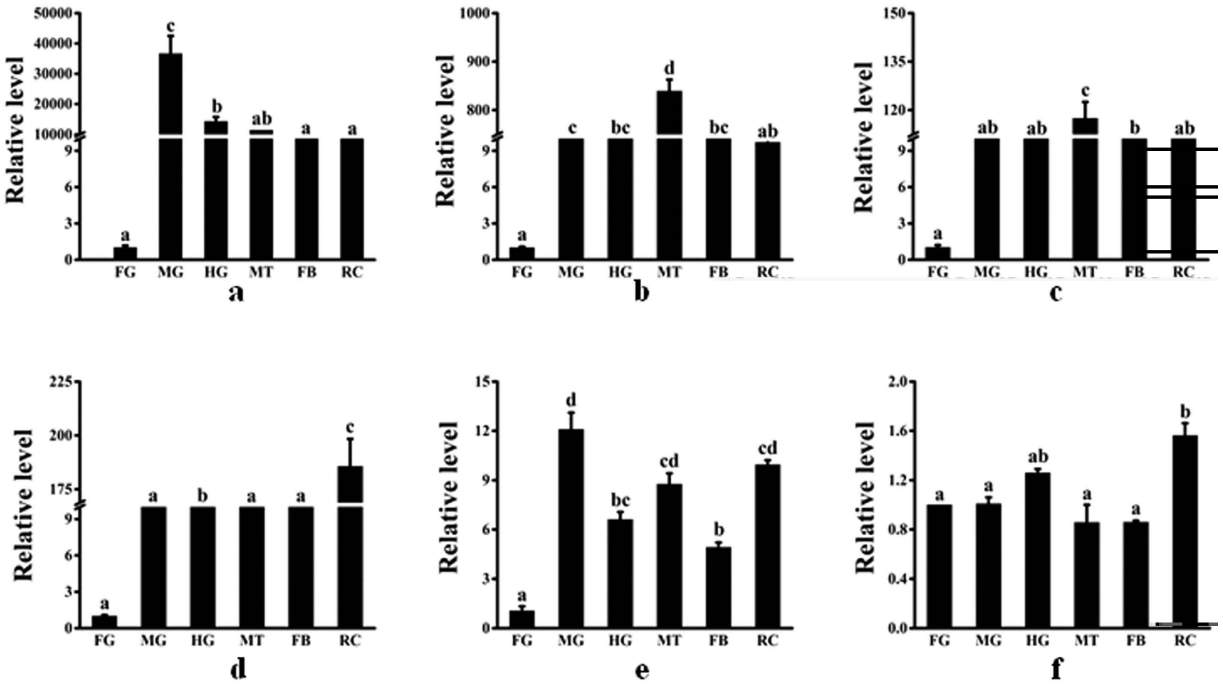
Binding of Cry2Aa to *S. exigua* BBMVs. Proteins of . BBMVs were separated by 2DE and silver stained (Fig. 1a). Proteins ranging in size from 10 kDa to 130 kDa were isolated using pH 3–10 IPG strips and 8% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels. Activated Cry2Aa toxin and a polyclonal antibody were used to identify speci c proteins binding to Cry2Aa. An antibody-speci city test was conducted before the binding assays to con rm that the Cry2Aa antibody recognizes Cry2Aa but not Cry1Ac (Supplementary Fig. S1).

Cry2Aa bound to seven proteins of approximately 100, 110, 65, 50, 30, 35 and 15 kDa (protein spots numbered 1 through 7 in Fig. 1b). To the best of our knowledge, this is the rst evidence that Cry2Aa binds to . BBMV proteins. Protein spots were excised from the silver-stained gel based on PVDF (polyvinylidene uoride) membrane signals and analyzed by LC-ESI (electrospray ionization)-MS/MS. A er searching protein databases, the protein spots in the silver-stained gel (Table 1) were identi ed as polycalin, V-type ATPase subunit A, V-type ATPase subunit B, actin, 4-hydroxybutyrate CoA-transferase (4-HB-CoAT), and a receptor for activated protein kinase C (Rack). Among these, 4-HB-CoAT and Rack were not previously known to bind to Cry toxin.

Cloning and sequence analysis of *S. exigua* genes encoding Cry2Aa-binding proteins. We cloned the full-length of - cDNA (GenBank accession no. KU234093) from the midguts of .

larvae. e 3,339-bp open reading frame (ORF) encodes a protein of 1,113 residues with a predicted mass of 122 kDa. e deduced protein sequence includes a signal peptide, glycosylphosphatidylinositol (GPI)-anchoring site, N-glycosylation sites and O-glycosylation sites (Supplementary Fig. S2). Phylogenetic analysis shows that -polycalin clusters with lepidopteran polycalin (Supplementary Fig. S3). Alignment using DNAMAN so ware indicates that -polycalin has highest homology with that of *M* and *H* (47.0% and 46.2%, respectively).

. -*A* *A* and *B* were also cloned, and their sequences submitted to GenBank (KX685519 and KX685520, respectively). eir respective cDNAs contained ORFs of 1,848 and 1,482 bp, encoding 616- and 494-amino acid proteins with estimated molecular weights of 68 kDa and 55 kDa. e conserved domains walker A motif/ATP-binding site, walker B motif, N-glycosylation sites, and O-glycosylation sites, are shown in Supplementary Figs S4 and S6. Phylogenetic analysis placed both proteins in the lepidopteran clade. High identity of V-ATPase subunits A and B among diverse insect species was detected; for example, . V-ATPase subunit A has 95.3% identity with that of



F 3. E ***S. exigua* C 2A -** . cDNA templates were derived from the foregut (FG), midgut (MG), hindgut (HG), fat body (FB), Malpighian tubules (MT), and the remainder (R), of 4th instar larvae. ree independent samples were examined for relative transcript levels using the $2^{-\Delta\Delta CT}$ method. a = -, b = - -*A* *A*, c = - -*A* *B*, d = -, e = *-4-HB-C A*, f = -. Expression levels were normalized to those of the reference genes - *L10* and -*GA DH*. Bars with di erent letters indicate values < 0.05 (ANOVA).

RNA interference knockdown of binding proteins. Compared to dsEGFP or H_2O, larval ingestion of dsRNAs speci c for the -V-ATPase subunit A, -V-ATPase subunit B, -actin, -4-HB-CoAT, -Rack, and -polycalin, signi cantly reduced transcript levels of these genes by 46.6%, 36.7%, 39.1%, 45.8%, 45.9% and 37.4%, respectively (Fig. 4a). Corrected mortalities following ingestion of Cry2Aa toxin for each of the above dsRNA treatment groups were 86.4%, 47.9%, 78.9%, 81.1%, 67.7% and 87.1%, respectively. e mortality of larvae fed dsRNA speci c for -V-ATPase subunit B was signi cantly lower than that of the water or dsEGFP control groups (Fig. 4b).

Production of recombinant peptides and binding assays. Expressed peptides were puri ed and separated by 8% SDS-PAGE gels (Fig. 5a). e results of an ELISA (enzyme-linked immunosorbent assay) indicate that the -V-ATPase subunit A, -V-ATPase subunit B, -actin, -4-HB-CoAT, -Rack and three partial fragments of -polycalin, all bound to Cry2Aa toxin (Fig. 5b).

Dot blot analysis of the Cry2Aa receptor in *S. exigua*. Based on the previous bioassays, we conducted homologous, competitive binding assays to test the speci city of binding between Cry2Aa and the recombinant -V-ATPase subunit B peptide. Binding between Cry2Aa and the -V-ATPase subunit B peptide was markedly reduced at higher concentrations of un-labelled Cry2Aa (Fig. 6).

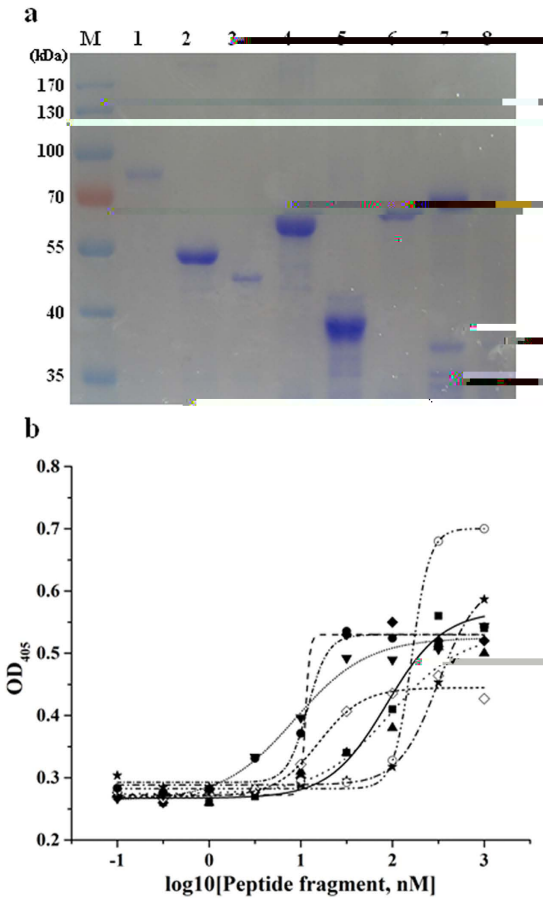
Discussion

Our results indicate that . V-ATPase subunit B is associated with Cry2Aa toxicity. Identifying this novel putative Cry2Aa receptor is potentially crucial to understanding how Cry2Aa is toxic to lepidopteran species. Since V-ATPase was rst reported in [26], a substantial amount of evidence has demonstrated that V-ATPases, which are located in the goblet cell apical membrane and rely on ATP hydrolysis to actively pump their substrates across membranes, are involved in energy production and conversion[27,28]. V-ATPase in the insect midgut mediates pH to create an alkaline environment and participates in ion-transport processes[29]. V-ATPase up-regulation has been found to be related to Cry1Ac resistance in - and . [30,31]. Although V-ATPase has been identi ed as a Cry toxin-binding protein in *H.* (Cry1Ac), *H.* (Cry1Ac) and . (Cry1Ab)[32–34], little is known about its function with regard to Cry toxins in other insects. Interestingly, RNA silencing of the *A.* ATP synthase subunit beta increased larval mortality to Cry toxins, which suggests that this protein is involved in Cry toxin resistance[35].

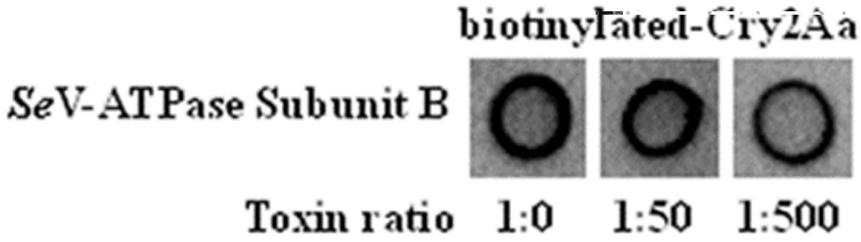
e model proposed by Jurat-Fuentes .[36] postulates that the Cry toxin binds to cadherin and is then inserted into the cell membrane, facilitating interaction of the toxin with other molecules such as V-ATPase. In fact, Cry1Ac binds to V-ATPase and disturbs H^+/K^+ transport, thereby destabilizing pH[37]. Moreover, Cry1Ac

has been found to inhibit (Na^+, K^+)-ATPase in mammals[38]. Two main classes of active transporters comprise the active transmembrane transport system. Sodium solute symporter (SSS) is driven by proton or sodium transmembrane gradients[39], and has been shown to act as a Cry toxin receptor[40]. Secondary active transporters include ATP-binding cassette (ABC) proteins and V-ATPase, both of which are ATP-dependent electrogenic proton pumps that actively move substrates across cell membranes. ABC proteins are also involved in Cry toxicity[41–43] and our results suggest that V-ATPase interacts with Cry2Aa toxin. However, further research is required to determine whether the V-ATPase subunit B interacts with Cry toxin in a manner similar to that of the ABC transporter.

Our results show that - was most highly expressed in the midgut of the 3rd to 5th larval instars of . , which may protect the larval midgut from viruses or oxidative damage[44]. - -*A* *B* was most highly expressed in 1st instar larvae and adults, and - -*A* *A*, - -*A* *B*, and -*4-HB-C A* , were most highly expressed in the gut and Malpighian tubules, which suggests that they may be involved in energy metabolism[27,28,45]. -*A* and - showed high transcript levels in other larval tissues, perhaps because their proteins comprise part of the cytoskeleton[46]. We also performed binding assays which showed that the polycalin peptides 2 and 3 had the highest binding a nities witDxpbarv;oin prhanrd 10.69s 14(b



F 5. B C 2A . ( ) Positions of puri ed, recombinant peptides a er staining with Coomassie Blue on SDS–PAGE gel. Peptides had been bacterially expressed and puri ed in a nickel-nitrilotriacetic acid (Ni-NTA) a nity column. Lanes 1 to 8 are: the -V-ATPase subunit A, -V-ATPase subunit B, -actin, -4-HB-CoAT, -Rack, and three truncated recombinant -polycalin peptides (6 = peptide1, 7 = peptide2, 8 = peptide3). ( ) Degree of binding, as indicated by optical density (OD), of Cry2Aa to di erent -peptide fragments. -V-ATPase subunit A (■), -V-ATPase subunit B ( ), -actin (▲), -4-HB-CoAT (★), -Rack (○), -polycalin peptide1 (◇), -polycalin peptide2 (▼) and -polycalin peptide3 (●).



F 6. B C 2A . Se- -A P B Weight ratios of 1:0, 1:50 and 1:500 of unlabeled Cry2Aa toxin were used to compete with the -V-ATPase subunit B in binding to biotinylated-Cry2Aa.

were overlaid on 8% SDS-PAGE gels for electrophoresis. e separated proteins were either stained or transferred to polyvinylidene di uoride (PVDF) membranes.

After proteins had been transferred to PVDF membranes, the membranes were blocked in PBST buffer (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 0.1% Tween-20, pH 7.5) containing 5% (w/v) skim milk for 2 h, then incubated with 0.3 μg/ml activated Cry2Aa (EnviroLogix Inc., Portland, ME, USA) in blocking bu er for 2 h at room temperature. e membranes were washed in PBST bu er three times, then incubated for 2 h with a polyclonal antibody against Cry2Aa (diluted 1:3,500; Genscript Biology Company, Nanjing, China). A er washing as above, the membranes were incubated with a goat anti-rabbit IgG horseradish

peroxidase (HRP)-linked antibody (diluted 1:5,000). A er nal washes, the membranes were developed with an ECL chemiluminescence detection kit (Fermentas/ ermo Fisher Scienti c, Waltham, MA, USA) following the manufacturer's recommendations.

Mass spectrometry. A er blotting, areas on the gel were excised according to the PVDF membrane signals and destained with destaining solution (30% acetonitrile/100 mM NH_4HCO_3). Each gel sample was then subject to a series of processes, including incubation with 100 mM DTT at 56 °C for 30 minutes, treatment with 200 mM indole-3-acetic acid (IAA) a er removal of the supernatant, and incubation with 100 mM NH_4HCO_3. e liquid was removed, and 100% acetonitrile was added for 5 minutes. e samples were freeze-dried before being subject to trypsin digestion for 24 hours at 37 °C, then analyzed using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) at the Shanghai Life Science Research Institute, Chinese Academy of Sciences, Shanghai, China. e amino acid sequence results were compared with those in the NCBI database using Mascot2.2 so ware.

Gene cloning and sequence analysis. Total RNA was isolated from the midguts of actively feeding 4th instar . larvae using the RNAiso reagent (TaKaRa, Dalian, China) a er contaminating genomic DNA had rst been eliminated with RNase-free DNase. e RNA preparation was subject to reverse transcription with the PrimeScript™ RT reagent Kit (TaKaRa, China), according to the manufacturer's instructions. Partial - , - -*A* *A*, - -*A* *B* and -4- *C* *A*- cDNA sequences were obtained from Prof. Fei Li (Zhejiang University). A smart RACE (rapid ampli cation of cDNA ends) cDNA ampli cation kit (Clontech, TaKaRa Bio Inc., Dalian, China) was used to amplify full-length target genes from . larvae for which pairs of gene-speci c primers were designed using Primer 5.0 so ware based on the partial sequences (Supplementary Tables S1–S5). e PCR products were subcloned into the PMD (18)-T vector (Takara, Dalian, China) and sequenced by the Nanjing Genscript Company, China. e resultant sequences were submitted to GenBank.

Full-length cDNAs were subjected to bioinformatic analysis using an ORF nder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence alignment was performed using DNAMAN so ware, and phylogenetic analysis was performed using MEGA4.0[51,52]. Amino acid sequences from other species were used to construct a phylogenetic tree (Supplementary Table S8). Deduced protein sequences were obtained using the ExPASy translate tool Translate (http://web.expasy.org/translate/) from the Swiss Institute of Bioinformatics. N-terminal signal peptides were predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). e GPI modi cation site prediction server (big-PI Predictor: http://mendel.imp.ac.at/sat/gpi/gpi_server.html was used to predict GPI-anchor signal sequences and GPI anchoring sites. e presence of N- and O-glycosylation sites in predicted protein sequences was assessed using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively.

Production of recombinant *S. exigua* proteins and microtiter plate binding assays. A method similar to a recently described protocol[23] was used to produce recombinant proteins. Brie y, PCR products were puri ed with Wizard PCR Preps DNA Puri cation System (Promega, Madison, WI, USA) and double digested with FastDigest restriction enzymes (Fermentas, ermo Fisher Scienti c, USA) for 10 min at 37 °C. e products were ligated into the previously digested pET-30a (+) vector to generate pET-30a/ -peptide plasmids. However, we failed to obtain a -polycalin fragment using this expression system, and the pGEX-6P-1 vector was therefore used to construct the recombinant plasmid pGEX-6P-1/ -polycalin containing a His tag (see primers in Supplementary Table S1). -polycalin was divided into three fragments corresponding to bases 1–1,113, 1,114–2,226 and 2,227–3,339 of the -polycalin coding sequence, which were named peptide1, peptide2 and peptide3. Insert sequence and orientation were con rmed by sequencing by the Genscript Biology Company, Nanjing, China.

For expression, 200 ng of each recombinant plasmid was transformed into *E* strain BL21 (DE3) (TransGen Biotech, Inc, Beijing, China) and positive clones were cultured overnight at 37 °C in LB medium containing 50 mg/ml kanamycin. When the absorbance was 0.5 to 0.8 at 600 nm, protein expression was induced with 0.1 mM isopropyl-D-thiogalactoside (IPTG) for 6 h at 37 °C, with horizontal shaking at 200 rpm. e *E.* cells were harvested by centrifugation at 10,000× g for 10 min at 4 °C, a er which pellets were resuspended in a solution of 20 mM sodium phosphate, 500 mM sodium chloride, 30 mM imidazole and 5 M urea (pH 7.4), containing 1 mM phenylmethanesulfonyl uoride (PMSF). Cells were lysed by sonication for 15 min on ice. e protein fragments with a His tag were puri ed using a nickel-nitrilotriacetic acid (Ni-NTA) a nity column (HisTrap HP column; GE Healthcare Life Sciences, Piscataway, NJ, USA) and eluted with eluting bu ers (20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole and 5 M urea, pH 7.4). e cleaved proteins were refolded by 0.006 rSmes

Competitive binding dot blot assay. Activated Cry2Aa protein was biotinylated using the EZ-Link sulpho-N-hydroxysuccinimide (NHS) liquid chromatography (LC) biotinylation kit (Pierce, FL, USA) according to the manufacturer's instructions. A homologous competitive binding assay for Cry2Aa by . recombinant peptides was then conducted. In total, 2 μg of puri ed peptides bound to a nitrocellulose membrane was blocked in PBST containing 3% BSA and incubated for 3 h with 0.2 μg/ml biotinylated Cry2Aa and unlabelled toxin at weight ratios of 1:0, 1:50 and 1:500. Following washing, streptavidin-HRP was used to detect biotinylated toxin using an ECL chemiluminescence detection kit (Fermentas/ ermo Fisher Scienti c, Waltham, MA USA), as described previously[23].

RNA interference knockdown of *S. exigua* target genes. A method adapted from Ren .[54] was used to produce a dsRNA-expressing vector. Target gene fragments were ampli ed from . midgut cDNA using PrimeSTAR HS DNA polymerase (TaKaRa Bio Inc., Dalian, China). e products were individually cloned into the plasmid pET-2P to generate recombinant pET2P/ -target-gene plasmids. pET2P/*EGF* recombinant plasmid production of EGFP dsRNA was used to generate the control EGFP dsRNA[23]. Recombinant plasmids were transferred into competent *E.* HT115 (DE3) cells. Individual colonies were cultured at 37 °C in 500 ml LB medium containing 50 μg/ml kanamycin. A er reaching an OD600 of 1.0, the production of dsRNA was induced by the addition of 0.4 mM IPTG, and the cultures were allowed to grow for an additional 5 h at 37 °C. e bacteria were precipitated by centrifugation at 5,000 rpm for 10 min, and dsRNA was extracted according to the method described by Timmons .[55] and Dong .[56]. Nucleic acids were analyzed for appropriate size by 1% agarose gel electrophoresis.

Newly hatched larvae were allowed to feed for 48 h at 27 °C on an arti cial diet to which either 50 μg/cm^2 -target genes, EGFP dsRNA, or water, had been added. e larvae were then transferred to the wells of a 6-well plate where they were allowed to continue feeding for 7 days at 27 °C. Each well contained 5 ml of arti cial diet plus either 2.6 μg/cm^2 activated Cry2Aa toxin (equivalent to the LC_{70} value determined by a pilot study), or water (the control). Five replicates were conducted with a total of 120 larvae in each treatment.

qPCR assay. Three groups of samples were prepared, including different tissues, developmental stages and dsRNA treatment groups (three replicates for each treatment). Quantitative real-time PCR (qPCR) was used to measure di erences in gene expression between tissues, developmental stages and dsRNA treatment groups. qPCR primers were designed using the NCBI profile server (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Supplementary Tables S1–S5). . *L10* and *GA DH* were used as internal reference genes (Supplementary Table S6)[54,57]. e following standard qPCR protocol was used: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 59 °C for 30 s. All qPCR samples were run in triplicate using SYBR Premix ExTaq™ (TaKaRa) and a Bio-Rad Detection iQ2 System. Melting curve analysis from 55 to 95 °C was performed to determine the speci city of the qPCR primers. To determine the e ciency of qRT-PCR primers, a 5-fold dilution series of second-instar larval cDNA corresponding to 1 μg total RNA was used to produce a standard curve (cDNA concentration vs. Ct) with e ciencies calculated from the slope using linear regression. e corresponding qRT-PCR e ciencies were calculated according to the equation: $E = (10^{[1/slope]} - 1)*100$[58,59] (Supplementary Table S7).

Data analysis. Quantitative expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method[58]. Corrected larval mortalities were calculated using Abbott's formula[60]. Means and variances of treatments were analyzed with one-way ANOVA implemented in SPSS for Windows (SPSS 18.0, Chicago, IL, USA).

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Author Contributions

L.Q., B.Z. and L.L. performed the experiments. W.M., C.L., X.W. and L.C. conceived and designed the experiments. L.Q. and L.C. analyzed the data and wrote the manuscript.

Additional Information

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