Identification of Bt toxin receptors in diamondback moth Plutella xylostella

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Abstract

Bt toxins kill insects by binding to various receptors at insect midgut and creating pores on the midgut membranes. However, the application of Bt toxins in agriculture is threatened by evolved resistance of insects. Our understanding of molecular basis in insects involved in Bt toxin binding is incomplete, especially in diamondback moth (DBM, Plutella xylostella), a major agricultural pest. Identifying Bt toxin receptors has remained challenging due to lacking sufficiently sensitive methods. Here, we report a novel technique, on-membrane capture, that identified numbers of previously unknown proteins, in addition to known receptors like cadherin and APN2, from the DBM midgut as binding partners of three Bt toxins Cry1Ac, Cry1Ab, and Cry1Bd. Among them, glucosinolate sulfatases GSS1 and GSS2 are receptors specifically interacted with Cry1Bd. Reduction in GSSs expression increased tolerance of DBM to Bt toxins. Expressing DBM GSSs in silkworms dramatically decreased the tolerance of the transgenic silkworms to Cry1Bd. Therefore, the on-membrane capture provides a new solution to identify Bt toxin receptors in insects.

1. Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, soil-dwelling bacterium that produces δ-endotoxin proteins known as Bt toxins or Cry toxins (crystalline toxins). Bt toxins efficiently kill lepidopteran, dipteran, and coleopteran pests, but are harmless to humans and other vertebrate animals (Bravo et al 2011). The Bt toxins belong to a class of bacterial pore-forming toxins. Once ingested by insects, Bt protoxins are solubilized in the insect midgut, and are then cleaved by proteases to produce activated toxins (Bravo et al 2007). These activated toxins penetrate the insect midgut protrophic membrane and bind to specific target sites, called primary receptors (such as cadherin), of the brush border membrane vesicles (BBMV) (Bravo et al 2008; Bravo et al 2011). Interactions between Bt toxins and cadherin facilitate protease cleavage of the helix α-1 of the toxins, promoting toxin oligomerization (Soberon et al 2007). These toxin oligomers are thought to have increased binding affinity to secondary receptors including glycosylphosphatidylinositol (GPI)-anchored proteins, aminopeptidase N (APN) (Bravo et al 2004), and alkaline phosphatases (ALP) (Jurat-Fuentes et al 2004). Binding of the toxin oligomers to these secondary receptors creates pores in the midgut membranes, thus causing osmotic shock, breakdown of the midgut cells, and insect death (Bravo et al 2004, Soberon et al 2007, Jurat-Fuentes et al 2004,). However, others have proposed that binding of the activated Bt toxin monomers to cadherin initiates a magnesium-dependent signalling pathway, causing cell disruption (Zhang et al 2006). In either model, the binding of Bt toxins to various midgut receptors is essential for disrupting the midgut membranes, which leads to cell lysis and insect death.

The application of Bt toxins in agriculture is threatened by evolved resistance of insects (Tabashnik and Carriere 2017). The diamondback moth (DBM) *Plutella xylostella* (Lepidoptera: Plutellidae), caused US $4–5 billion in management costs annually (Furlong et al 2013), is the first insect reported in the fields to have evolved resistance to Bt toxins (Tabashnik 1994). The DBM resistance phenotype involves the reduced binding of toxins to the brush border membranes, a trait that is inherited in a recessive manner but which achieves high resistance levels (Tabashnik et al 1996, Tabashnik et al 1997). Worriedly, our understanding of the molecular basis in DBM midguts involved in Bt toxin binding is scant. So far, all known Bt receptors identified from other lepidopteran insects have been conclusively eliminated as factors conferring resistance to Cry1A in DBM (Heckel et al 2007). Although, the resistance mechanisms in DBM are reported to involve alterations in the expression levels of Bt toxin receptor genes like adenosine triphosphate (ATP)-binding cassette transporter subfamily C (ABCC) gene, no obvious mutations in sequences of these genes between susceptible and resistant DBM strains, casting doubt on the role of these genes in Cry1Ac resistance phenotype of DBM (Guo et al 2015). It is an urgent need in identifying Bt toxin receptors in insects regarding the long-term and sustainable use of Bt and their Cry toxins as insecticides.

Here, we report a novel technique, on-membrane capture, that identified numbers of previously unknown candidates, in addition to well-known receptors cadherin and APN2, from DBM midgut as binding partners of Bt toxins. Among these candidates, we discovered new molecular components, glucosinolate sulfatases, that contribute to the action of Bt toxin Cry1Bd in the DBM midgut. Reduction in *GSSs* expression remarkably increased tolerance of DBM Bt toxins. Expressing DBM GSSs in silkworms dramatically decreased the tolerance of the transgenic silkworms to Cry1Bd. Therefore, the on-membrane capture provides a new solution to identify Bt toxin receptors in insects.

2. Material and Methods

2.1 DBM strain

The DBM strain 'Fuzhou lab' were reared on radish seedlings without exposure to insecticides for 5 years, spanning at least 100 generations (You et al. 2013).

2.2 Preparation of brush border membrane vesicles (BBMV)

Midgut BBMVs were prepared as described previously (Wolfersberger et al. 1987). Fifth-instar larvae were immobilized on ice and dissected in cold dissection buffer (17 mM Tris-HCl, pH 7.5, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 300 mM mannitol, 1 mM phenylmethane sulfonyl fluoride (PMSF)) to isolate the midgut epithelium. Midgut epithelial tissue was homogenized in an equal volume of ice-cold 24 mM $MgCl_2$, then incubated on ice for 15 min, followed by centrifugation at 25,006 *g* at 4°C for 15 min to collect the supernatant. The centrifuged pellet was resuspended in ice-cold dissection buffer in 0.5 volume of the initial homogenate and then the BBMV extraction procedure was repeated as described above. The supernatants collected from the two extractions were combined and BBMVs were precipitated by centrifugation at 30,000 *g* at 4°C for 1 h and stored at -80degC. Protein concentration was measured using the BCA Protein Assay Kit (Rockford, USA) according to the manufacturer's instructions.

2.3 On-membrane capture

Ten micrograms of each Bt protein was separated in a 10% gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In parallel, same amount of Bt proteins was separated by another 10% gel. Then, proteins were transferred to nitrocellulose membrane using an Amersham Semi-Dry Transfer Unit (Freiburg, Germany). Proteins one NC membrane were denatured by incubating the membrane in denaturing and renaturing buffer (100 mM NaCl, 20 mM Tris (pH 7.6), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.1% Tween-20, and 1 mM dithiothreitol (DTT)) containing 6 M guanidine-HCl for 30 min at room temperature (Wu et al. 2007). The membrane was then washed with denaturing and renaturing buffer containing 3 M guanidine-HCl for 30 min at room temperature, then washed with denaturing and renaturing buffer containing 0.1 M and no guanidine-HCl for 1 h at 4degC. The membrane was blocked with Pierce protein-free buffer (Rockford, USA) for 1 h at room temperature. Membranes were

incubated with 30 μg total BBMV proteins (final concentration 10 μg/mL) in protein-binding buffer (100 mM NaCl, 20 mM Tris (pH 7.6), 0.5 mM EDTA, 10% glycerol, 0.1% Tween-20, and 1 mM DTT) overnight at 4°C. The Bt proteins on the NC membrane were positioned by aligning to Bt proteins on the other NC membrane that visualized with Ponceau S, then were cut for trypsin digestion.

On-membrane digestion was carried out as described by Luque-Garcia et al. (Luque-Garcia et al. 2008). Nitrocellulose bands were washed at least six times with Milli-Q water (Merck Millipore, Shanghai, China), then incubated in trypsin solution (12.5 ng/μl prepared in 50 mM NH_4HCO_3 buffer (pH 8)) at 37°C overnight. After digestion, samples were dried in a vacuum, dissolved in acetone (90 μl acetone/4 mm^2 nitrocellulose), vortexed, and incubated for 30 min at room temperature. Acetone containing dissolved nitrocellulose was carefully removed and precipitated peptides were air-dried. Peptides were resuspended by adding 20 μl of 2% acetonitrile in 0.1% formic acid. All solutions were sonicated for 10 min before mass spectrometry analysis.

2.4 Q Exactive LC MS/MS analysis

In the analysis of complex mixtures, peptides of similar mass often co-elute; therefore, resolution is key in mass spectrometry (Michalski et al. 2011). Shotgun proteomics using the Q-Exactive instruments (Thermo Fisher Scientific, USA) is usually performed at 17,500 resolution at m/z 200 with a transient length of 60 ms. The higher resolution in MS/MS spectra helps to assign fragments of large precursors. Data were analyzed in MaxQuant using the integrated Mascot search engine (Michalski et al. 2011). The total number of MS scans exceeded 5000, and the total number of MS/MS scans exceeded 16 000. The average number of isotope patterns detected was close to 35,000, a very high number considering that the gradient was not particularly long, presumably because of the short MS and MS/MS cycle time of 1 s.

2.5 Far western blot

Far western blot was performed as previously described (Wu et al. 2007). Briefly, Bt proteins (Cry1Ac, Cry1Ab, and Cry1Bd) were separated on a 12% SDS-PAGE gel, then transferred to polyvinylidene difluoride (PVDF) membrane. Protein denaturing and renaturing on the membrane was performed exactly as per the protein co-blotting procedure described above. The membrane was then blocked with 5% milk in phosphate-buffered saline (PBS) for 1 h at room temperature. The membrane was then incubated with 5 μg purified His-GSS1 or His-GSS2 proteins (final concentration 1 μg/mL) in protein-binding buffer overnight at 4°C. Membranes were probed with anti-His primary antibodies, then washed with PBST (1% Tween-20 in PBS buffer), incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, and exposed to X-ray films after reacting with electrochemiluminescence (ECL) substrates.

2.6 Purification of GSS1and GSS2 proteins

Primers used for cloning in this study are listed in**Supplemental Table 1** . *GSS1* and *GSS2* were cloned into the pET28A vector using BamHI and HindIII sites, and over-expressed in the BL21 (DE3) *Escherichia coli* strain.

Bacterial cells were harvested by centrifuging at 3000 *g* for 15 min at 4°C. Cells were washed with bacterial cell lysis buffer to remove residual culture medium. Washed cells were harvested by repeating centrifugation at 3000 *g* for 15 min at 4°C. After decanting the supernatant, the wet pellet was weighed, and *E. coli* cells resuspended in about 3 mL of lysis buffer per gram of cell pellet. The suspension was stirred for 30 min at 4°C, lysozyme was added to a concentration of 0.1% (*w/v*), then the mixture was incubated for 30 min at 4°C while shaking gently. The suspension was centrifuged at 23,000 *g* for 30 min at 4°C and the supernatant discarded. The pellet was dissolved in inclusion body binding buffer (20 mM Tris-HCL (pH 7.9), 5 mM imidazole, 0.5 M NaCl, 8 M urea). The cleared inclusion bodies solution was applied to a column containing Ni-Agarose beads equilibrated with 3 × 5 mL ultrapure water and 3 × 5 mL protein binding buffer. The column was washed with 15 bed volumes of inclusion body binding buffer before eluting polyhistidine-tagged proteins with 5–10 bed volumes of inclusion body elution buffer (20 mM Tris-HCL (pH 7.9), 500 mM imidazole, 0.5 M NaCl, 8 M urea). Protein purity was typically >90% as determined by SDS-PAGE and Coomassie blue staining. Protein concentrations were measured using the BCA Protein Assay Kit according

to the manufacturer's instructions.

2.7 His-tag pull-down

The Pierce His Tag Protein Interaction Pull-Down Kit (catalog number 21277) was used to detect the binding of GSS1 and GSS2 with Bt proteins. Solubilization of proteins (His-GSS1 and His-GSS2) from inclusion bodies (a requirement of this kit) was carried out according to the method developed by Simpson (55). Cells were lysed as described in "Cloning and purification of GSS1and GSS2". The cell lysate was centrifuged at 23,000 *g* for 30 min at 4°C before decanting the supernatant and measuring the wet mass of the pellet. The pellet was resuspended in 10 volumes of lysate washing buffer and the suspension stirred for 1 h at room temperature. The mixture was again centrifuged at 23,000 *g*for 30 min at 4°C, the supernatant decanted and the pellet recovered. Wash steps were repeated three more times. The pellet was then dissolved in 9 volumes of solubilization buffer C per gram wet weight of inclusion body pellet, and the mixture incubated for 1 h at room temperature. Nine volumes of renaturation buffer C were added slowly to the solubilized pellet and the mixture incubated for 2 - 4 h at 25°C. Two milliliters of Ni-Agarose beads were added to renaturation buffer C and incubated overnight on a magnetic stirrer at 4°C. Protein–bead complexes were collected by pressing renaturation buffer C through a column with a 0.25 nm filter. Polyhistidine-tagged proteins were eluted with 5–10 bed volumes of elution buffer (20 mM Tris-HCL (pH 7.9), 500 mM imidazole, 0.5 M NaCl). Imidazole was removed from purified His-GSS1, His-GSS2 and Bt proteins (Cry1Ac, Cry1Ab and Cry1Bd) by dialysis against Tris-buffered saline buffer (25 mM Tris-HCl, 0.15 M NaCl (pH 7.2)).

A His-tag protein was added to spin columns containing equilibrated HisPur Cobalt Resin (Thermo Scientific, Rockford, USA) and incubated at 4°C for at least 30 min on a rotating platform with a gentle rocking motion. Spin columns were centrifuged at 1250 *g* for 30 s to 1 min to remove solution. Beads were washed 5 times with 400 µL of wash solution. Up to 150 µg of prepared Bt proteins was added to columns, which were then incubated overnight at 4°C. Beads were washed 5 times with washing buffer to remove non-specifically bound proteins. Proteins were eluted by adding 250 µL of elution buffer (1 mL of 290 mM imidazole elution buffer made with 70 µL of 4 M imidazole stock solution to 930 µL of wash solution) to the spin column. Spin columns were incubated for 5 min on a rotating platform with gentle rocking motion, before centrifuging at 1250 *g* for 30 s to 1 min. Proteins were analyzed by SDS-PAGE and visualized by silver stain.

2.8 Plasmids and plant transformation

Plasmids for double-stranded RNA (dsRNA) expression were constructed as previously described (56). The pBSK intron vector was a pBluescript II SK vector (Stratagene) containing a 120-nucleotide intron of the*Arabidopsis thaliana RTM1* gene between the NotI and XbaI sites. Sense and antisense target fragments with restriction enzyme sites at both ends were obtained by PCR amplifying DBM cDNA clones with primer pairs (**Supplemental Table 1**). The two PCR fragments were inserted at inverted repeats into the corresponding sites of the pBSK intron vector. The dsRNA construct generated was then used to replace GUS in pBI121 to generate the *Pro* 35S::dsRNA construct. The final RNAi vector was introduced into *Agrobacterium tumefaciens* strain GV3101. Transgenic Arabidopsis plants were generated using the floral dip method, screened on half-strength Murashige and Skoog (MS) agar medium containing 30 µg/mL kanamycin.

Analysis of dsRNA expression levels were analyzed using T2 homozygous plants. Freshly hatched DBM larvae were fed with leaves of the*dsGFP* and *dsGSS1* lines for 7 d, respectively. Expressions of *GSS1* and *GSS2* were detected by real-time PCR (primers listed in **Supplemental Table 1**).

2.9 Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was performed using an Eppendorf Mastercycler ep realplex, using gene-specific and allele-specific primers to detect expression patterns. Each reaction was performed in a 20 µL volume containing 10 µL SYBR Green (Fermentas), 0.4 µL Rox Reference Dye II, 1 µL of each primer (10 mM), 1 µL of sample cDNA, and 7.6 µL UltraPure distilled water (Invitrogen). The PCR program used was: 95°C for 10 s, 40 cycles at 95°C for 20 s, 60°C for 30 s. Relative quantification was calculated using the comparative 2^{-T} method

(Schmittgen and Livak 2008). All data were normalized to the level of *RP49* from the same sample.

2.10 Silkworm transformation

Transformation plasmids were constructed based on the initial piggyBac vectors pBac[3xp3-DsRed, IE1-EGFP]. The 3xp3 promoter was removed by cutting with NotI and AgeI, and was replaced with the HR5 enhancer followed by the IE1 promoter to generate the pBac[HR5IE1-DsRed, IE1-EGFP] plasmid based on homologous recombination using the ClonExpressTM II One Step Cloning Kit (Vazyme Biotech Co., Ltd.). The open reading frames (ORFs) of *GSS1* and *GSS2* were replaced with *EGFP* to generate the silkworm transformation plasmids, pBac[IE1-DsRed, IE1-GSS1] and pBac[IE1-DsRed, IE1-GSS2]. Primers are listed in **Supplemental Table 1** .

Silkworm microinjection was performed as described previously (Tan et al. 2013). The transformation plasmid was microinjected into preblastoderm G0 embryos (Nistari strain), which were then incubated at 25degC in a humidified chamber for 10–12 d until larval hatching. Larvae were reared on fresh mulberry leaves or an artificial diet (Nihonnosanko) under standard conditions. Putative transgenic adult G0 were mated with each other, and G1 progeny were scored for the presence of red fluorescence using fluorescence microscopy (Nikon AZ100). Positive G1 progeny were mated with wild-type moths to generate hybrid silkworms expressing one copy of dsRed and a target gene. Hybrid silkworms with red fluorescence were fed with Cry1Bd-treated leaves as described below.

2.11 Bt toxin treatment

To test the tolerance of RNAi-silenced DBM larvae, two leaves from 4-week-old *A. thaliana* plants with genotypes *dsGFP* and*dsGSS1* were laid on a moistened filter paper in a 15 mm petri dish. Freshly hatched DBM larvae were placed on leaves of each genotype to feed for 7 d. On the eighth day, larvae were transferred to fresh leaves coated with either a diluted suspension of Bt toxin in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0), or HEPES buffer as a control. Mortality was recorded after 24 h and LC50 values were calculated by probit analysis based on the dose determined to be high enough to kill 100% of larvae.

To test the tolerance of transgenic silkworms, four squares of mulberry leaf (4 x 4 cm), coated with 50 μL of a diluted suspension of Cry1Bd in HEPES buffer (pH 8.0) were fed to 10 second instar larvae for 24 h. The dose high enough to kill 100% of susceptible larvae was determined. Probit analysis was carried out using SPSS software (version 12.0) to determine LC50.

3. Results

3.1 Novel candidate receptors of Bt toxins in the DBM midgut identified by on-membrane capture

To investigate proteins at DBM midgut targeted by Bt toxins, we developed on-membrane capture method that is modified from far-western blotting (Wu et al. 2007, **Figure 1**). Briefly, three Bt proteins with different degree of toxicity to DBM (low activity Cry1Ac, middle activity Cry1Ab, and high activity Cry1Bd) were separated by an SDS-PAGE gel. In parallel, same amount of Bt proteins was separated by another SDS-PAGE gel. Then, proteins were transferred to two nitrocellulose membranes, respectively. Proteins on one NC membrane were denatured and renatured by gradually reducing the guanidine-HCl concentration (Wu et al. 2007). The membrane was blocked with protein-free buffer, then was incubated with total proteins isolated from DBM midgut epithelium. Bt protein on the membrane captures BBMV proteins if they form a complex. The Bt proteins on the NC membrane were positioned by aligning to Bt proteins on the other NC membrane that visualized with Ponceau S, then were cut for trypsin digestion. Digested peptides were precipitated in acetone that dissolves the NC membrane. Peptides were dried and were analyzed by nano liquid chromatography-tandem mass spectroscopy (nano LC-MS/MS) coupled with the mass spectrometer (**Figure 1**).

MS/MS spectra were queried against a combined protein database including DBM protein sequences and amino acid sequences of Cry1Ac, Cry1Ab, and Cry1Bd. A total of 520 peptides were detected in three bands.

In each band, the peptides of corresponding Bt toxin were most abundant (Figure 2A). 38.2% of peptides were identified as Cry1Ac in the band of Cry1Ac, 26.5% as Cry1Ab in the band of Cry1Ab, and 27.6% as Cry1Bd in Cry1Bd band (**Figure 2A**). The rest of the peptides corresponded to 81 unique proteins. Of these, 35 proteins were identified in the Cry1Ac band, 40 were in the Cry1Ab band, and 39 were in the Cry1Bd band (**Figure 2A**). Molecular weights of these proteins were dominantly at 50 kd and 200 kd, isoelectric points of proteins were approximately 5.5 (**Figure 2B**). These suggested that Bt toxins interacted with specific proteins at DBM midgut.

17 proteins were identified in all three bands (**Figure 2C**), indicating these proteins are unlikely associated with toxicity of Bt to DBM. Expectedly, two known receptors cadherin and APN2 previously excluded from DBM resistance to Bt toxins were found to interact with all three Bt toxins (Nakanishi et al. 2002, Chang et al. 2012, Guo et al. 2015). Some DBM proteins were identified uniquely from one of the bands, and these proteins are more likely to be associated with different degree of Bt toxicity (**Figure 2C, Supplemental Table 2**).

3.2 DBM glucosinolate sulfatases (GSSs) are receptors of Bt toxin Cry1Bd

We paid closer attention to the proteins uniquely interacted with Cry1Bd. The field population of DBM still remains highly susceptible to Cry1Bd but has evolved cross-resistance to other four Bt toxins including Cry1Ac and Cry1Ab (Ferre et al. 1991, Tabashnik et al. 1993, Tabashnik et al. 1994, Tabashnik et al. 1996, Kuo et al 2000, Liu et al. 2001, Zhao et al. 2007). Therefore, if Cry1Bd is to be used to control this pest, it is important to investigate potential Cry1Bd receptors.

13 DBM proteins were candidate receptors of Cry1Bd. We analyzed these candidates to identify the transmembrane helices and the GPI-anchor sequences which are known characteristics of Bt receptors, like the primary receptor cadherin and the secondary receptors ALP and APN. It resulted in identification of four proteins with GPI-anchor sites ATP synthase F0 subunit 8, β-1,3-glycosyltransferase 5, and two glucosinolate sulfatases. ATP synthase F0 subunit 8 is a mitochondrial protein that has been eliminated as a Bt toxin target, likewise, β-1,3-glycosyltransferase 5 has been ruled out as contributing to Bt resistance in *Plutella* (Baxter et al. 2008). Two glucosinolate sulfatases (GSS), GSS1 and GSS2, matched the peptide^{244}RIFAAMVK^{252} (**Supplemental Figure 1**), have predicted GPI-anchor sites at the position C485 (**Supplemental Figure 2**). In addition, GSS2 contains an N-terminal transmembrane helix. Likely, GSS2 is a membrane anchor protein with a C-terminal extending outside of the cell membrane (**Supplemental Figure 3**). These characteristics suggested GSSs are candidate receptors of Cry1Bd in the DBM.

To further investigate GSSs are the receptors of Cry1Bd, we tested whether GSSs directly interact with Cry1Bd. We carried out pull-down experiments and found that both GSS1 and GSS2 pulled down Cry1Bd, suggesting direct interactions between Cry1Bd and GSSs (**Figure 3A**). In contrast, either Cry1Ac or Cry1Ab were pulled down by GSSs, is consistent with the results that GSSs are absence in proteins captured by Cry1Ac or Cry1Ab (**Figure 3A** , **Supplemental Table 1**). The interactions of GSSs with Bt proteins were further validated by far-western blot. Similar to on-membrane capture, His-tagged GSS1 or His-tagged GSS2 were incubated with NC membrane containing Bt proteins. Consistently, both GSSs interact with Cry1Bd not Cry1Ac or Cry1Ab (**Figure 3B**). Altogether these results suggested GSSs bind with Cry1Bd directly and specifically, serving as receptors in the DBM to Cry1Bd toxin.

3.3 Reduction in *GSSs* expression increases tolerance of DBM Bt toxins

To investigate roles of GSSs in the toxicity of Bt to DBM, we generated transgenic lines expressing dsRNA corresponding to the *GSSs*sequence for plant-mediated RNAi of *GSSs* in DBM. The transgenic*A. thaliana* lines successfully knocked down *GSSs*expression compared to lines producing dsRNA corresponding to GFP (*dsGFP*) in DBM (**Figure 4A and 4B**). As results of reduction in expression of *GSSs* gene, *GSSs* -silenced larvae became more tolerant to Cry1Bd compared with controls. The LC50 of Cry1Bd to larvae were increased 69-fold from 0.05 mg/L of control larvae to 3.16 mg/L of *GSSs* -silenced larvae (**Figure 4C**). These results demonstrated that GSSs are susceptible factors that are critical for Cry1Bd toxicity to DBM. Interestingly, *GSSs* -silenced larvae also became more tolerant to Cry1Ac and Cry1Ab. Therefore, knock

down of the expression of *GSSs* is correlated with increased tolerance of DBM to Bt toxins.

3.4 GSSs are susceptible factors to Cry1Bd

To confirm that DBM GSSs are causative agents of susceptibility to Cry1Bd, we introduced DBM *GSS1* and *GSS2* into a Cry1Bd-resistant silkworm strain Nistari (Chen et al. 2014). Two transgenic silkworm lines expressing dsRed marker and one of the DBM*GSSs* were established (**Figure 5A**). Inverse PCR of genomic DNA confirmed the presence of transgenes, that *GSS1* lines had one copy of the transgene on chromosome 23 and GSS2 lines had one copy of *GSS2* on chromosome 11 (**Supplemental Figure 4**). As *GSSs* are more likely to be susceptible factors to Cry1Bd, a single allele of the gene would convert the resistance of transgenic Nistari. Therefore, the individuals with dsRed fluorescent were selected from the offspring of transgenic and wildtype Nistari, then were tested for tolerance to Cry1Bd. Remarkably, the silkworm larvae with one of GSSs became more sensitive to Cry1Bd compared to controls, as LC50 of Cry1Bd dropped to 1.86 mg/L of *GSS1* larvae and 1.30 mg/L of*GSS2* larvae from 33.90 mg/L of wildtype ones (**Figure 5B**). These results demonstrated DBM GSSs are susceptible factors of Cry1Bd toxin.

4. Discussion

Identifying Bt toxin receptors in insects has been challenging. Firstly, it is due to the complexity of Bt toxicology in insects. The action of a Bt toxin often requires binding to multiple insect proteins that unequally contributed to Bt toxicity. Besides, receptors to a Bt toxin in an insect species often are irrelevant to the toxicity of the same Bt toxin in other insect species. Secondly, there are no suitable methods that are efficient enough, regarding sensitivity and convenience, to detect Bt-receptor interaction in a wide range of insect species. So far, genetics-based methods like QTL analysis have demonstrated the strength in the identification of *Heliothis virescens* cadherin genes of and *Bombxy mori* ABBC2 genes in the resistance of those insects to Bt Cry1A toxins (Atsumi et al. 2012, Gahan et al. 2001, Gahan et al. 2010). Such approaches involve years of experiments in hybridizing of insects with different Bt resistance traits, which are often labour-intensity and time-consuming. Alternatively, affinity-abased methods, like widely used ligand dot (Wei et al. 2016, Qiu et al. 2017), require previous knowledge of Bt toxin binding partners often lacked for a novel Bt toxin and unclear to numbers of insect species. Furthermore, these methods are unlikely to identify low abundance membrane proteins, that potentially contribute to Bt toxin function (Candas et al. 2003, Krishnamoorthy et al 2007). Here, we presented the on-membrane capture, a reliable technique provided a new solution to identify Bt toxin receptors in insects. This technique allows us to rapidly and unbiasedly analyze the targets of a given Bt toxin in any insects without a presumption of the targets. Given that application of Bt toxins is consistently threatened by evolved resistance of insects, the on-membrane capture can evaluate the potential targets of in-service and new designed Bt toxins that will greatly facilitate assessing the risk of insect evolution resistance and choosing appropriate sets of toxins for delaying resistance.

Taking advantage of the on-membrane capture, we identified numbers of proteins in the DBM midgut as binding sites of Bt toxins. Among these proteins, some well-characterized proteins like cadherin and APN2 are captured by all three Bt toxins regardless of toxicity, which is consistent with the previous conclusion that cadherin and APN2 not associated with DBM resistance to Bt toxins (Nakanishi et al. 2002, Chang et al. 2012, Guo et al. 2015). Moreover, previously undescribed binding proteins of Bt toxins identified here shed new light on the mechanisms of Bt toxicity in DBM. Four Bt toxin-binding sites have been proposed to explain the action of Bt toxins in DBM (Ferré and van Rie 2002). Site 1 binds Cry1Aa only, site 2 binds Cry1Ab, Cry1Ac, and Cry1F (Ferré and van Rie 2002), site 3 specifically binds Cry1B, and site 4 binds Cry1C. Modification in these binding sites leads to DBM become cross-resistance to multiple Bt toxins (Ferré and van Rie 2002). For instance, modification of site 2 abolishes the binding to Cry1Aa, Cry1Ab, Cry1Ac and causes DBM cross-resistance to these Bt toxins (Ferré and van Rie 2002). We found DBM arylphorin subunit alpha proteins are likely belonging to site 2. Arylphorin is a mitogenic agent in the midgut stem cells of Lepidopteran insects (Blackburn et al. 2004, Micchelli and Perrimon 2006). Increased expression of arylphorin in*Spodoptera exigua* was found to be correlated with *B. thuringiensis* resistance (Hernandez-Martinez et al. 2010). Likelihood of arylphorin subunit alpha proteins involved in DBM resistance to Cry1Ab and Cry1Ac

toxins needs further investigations.

We also found that 13 previously unknown proteins specifically bind to Cry1Bd. DBM in the field have developed high resistance to Cry1Ac and become cross-resistance to Cry1Ab, and it remains highly susceptible to Cry1Bd. These 13 proteins are likely to be candidates to study the mechanisms of Cry1Bd toxin in DBM. Among them, GSSs are enzymes used by DBM to protect itself against the accumulation of toxic compounds from Brassicaceae (Ratzka et al 2002). When these plants are damaged by herbivory, a myrosinase processes glucosinolates into compounds that are toxic to the insect (Halkier and Gershenzon 2006). DBM counters this process by using GSSs to convert glucosinolates into non-toxic compounds (Ratzka et al 2002). Here, we found that GSS1 and GSS2 are receptors interacted specifically with Cry1Bd, suggesting the detoxification system of DBM is targeted by Cry1Bd. DBM GSSs are predicted extracellular proteins as they have predicted N-terminal secretory signal peptides. GSSs are likely to be selectively included in lipid rafts where the interaction with Cry1Bd happens, similar to many pore-forming toxins (Bravo et al. 2007).

Insect enzymes participated in detoxification of plant secondary compounds are frequently associated with insecticide resistance (Wang et al. 2018). GSSs may have roles in DBM develop resistance to Cry1A, because GSSs do not interact with Cry1Ac or Cry1Ab and *GSS* -silenced larvae showed increased resistance against Cry1Ac and Cry1Ab. GSSs have been found in higher levels in a Cry1Ac-resistant DBM strain NO-QA (McNall 2014). But GSSs of a resistant strain (NO-QA) and a susceptible strain (Geneva 88) showed no interaction with Cry1Ac, implying that GSSs might be indirectly involved in the action of Cry1A toxins. In eukaryotes, sulfatases are extensively glycosylated before being transported to their destinations (Hanson et al. 2004). GSSs may be likely involved in Cry1A toxicity through their terminal GalNAc residues. Indeed, GalNAc has been shown to bind with the carbohydrate-binding sites of domain III of Cry1Ac (Derbyshire et al. 2001). Alternatively, GSSs participate in Cry1A toxicity via a signalling pathway. Sulfatases have been attributed to pivotal roles in Wnt (Dhoot et al. 2001) and pheromone signalling (Ragsdale et al. 2013). The MAPK signalling pathway has found to manipulate the expression of multiple receptors relating DBM resistance to Cry1Ac (Guo et al. 2015). It will be important for future studies to investigate whether MAPK signalling pathways are involved in regulating the functions of GSSs, and thus whether they influence the development of DBM resistance.

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Data Accessibility

Data associated with the on-membrane capture can be found in Supplemental Figure 1 and Supplemental Table 2.

Conflicts of interest

The authors declare no competing financial interests.

Author contributions

Y.C., Yong.P.H., A.T. contributed to research design; Y.C. conducted modified far-western bloting, far-western bloting, and pull-down; Y.C. and Yu.P.H. generated transgenic Arabidopsis lines; Y.C., Q.L., M.C., J.X. generated transgenic silkworm lines; Y.C., Yu.P.H., M.C., and Q.L. conducted bioassay. Y.C. analyzed data; Y.C. and S.H. wrote the paper.

All authors discussed the results and commented on the manuscript.

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Figure legends

Figure 1 . **The workflow of on-membrane capture**

Figure 2 . **DBM midgut proteins revealed as binding candidates of Bt toxins** . (A) Peptide identification analysis on a Q-Exactive Orbitrap LC-MS system (60 min gradient). (B) Distribution of molecular weight and isoelectric point of DBM midgut proteins. (C) Venn diagram showing DBM midgut proteins captured by three Bt toxins Cry1Ac, Cry1Ab, and Cry1Bd.

Figure 3 . **DBM GSSs bind directly to Cry1Bd** . (A) Binding of GSS1 and GSS2 to Cry1Bd was detected by pull-down assay. His-GSS1 or His-GSS2 attached to the cobalt resin was incubated with one of Bt toxins Cry1Ac, Cry1Ab, and Cry1Bd. Cry1Ac or Cry1Ab was not eluted with either GSSs, and Cry1Bd was co-eluted with both GSS1 and GSS2. (B) Binding of GSS1 and GSS2 to Cry1Bd was detected by far-western blot. Bt proteins on nitrocellulose membranes were denatured and renatured by gradually reducing the guanidine-HCl concentration, then incubated with 5 μg His-GSS1 or His-GSS2 after the membrane was blocked. His-tagged GSSs was detected by anti-His antibody with Cry1Bd and not with Cry1A or Cry1Ab.

Figure 4. Reduction in *GSSs* expression increases tolerance of DBM Bt toxins . (A and B) Expression of *GSS1* and*GSS2* in DBM were knocked down. Freshly hatched DBM larvae were fed leaves of either *dsGFP* or *dsGSS1* lines for about 7 d until larvae reached the third instar, whereupon they were harvested for RNA analysis. Values (mean ± SD) were obtained from three independent experiments. ** above the columns indicates statistical significance between samples ($P < 0.01$). (C, D, E) LC_{50} of *GSS* -silenced larvae to three Bt toxins Cry1Ac, Cry1Ab, and Cry1Bd. Two leaves taken from 4-week-old *A. thaliana* plants of genotypes *dsGFP* or *dsGSS1* were laid on a moistened filter paper in a 15 mm petri dish. Freshly hatched DBM larvae were placed on leaves of each genotype and fed for 7 d. On the eighth day, larvae

were transferred to fresh leaves coated with a diluted suspension of Bt proteins in HEPES buffer (pH 8.0), or HEPES buffer as a control. Mortality was recorded after 24 h and LC50 was calculated by probit analysis based on the dose determined to be high enough to kill 100% of larvae. Values (mean ± SD) were obtained from three independent experiments. ** above the columns indicates statistical significance between samples ($p < 0.01$).

Figure 5. DBM GSSs are susceptible factors to Cry1Bd . (*A*) DsRed fluorescence phenotypes of hybrid offspring produced by transformed silkworms and wild type Nistari. Insects with DsRed fluorescence (indicated by arrow) were selected for Cry1Bd toxicity tests. (*B*) LC50 of hybrid silkworm against Cry1Bd. Four squares of mulberry leaf (4 × 4 cm), coated with 50 μL of a diluted suspension of Cry1Ab protoxin in HEPES buffer (pH 8.0), were fed to 10 second instar larvae for 24 h. The dose high enough to kill 100% of susceptible larvae was determined. Probit analysis was carried out using SPSS to determine the LC50 value. Values (mean ± SD) were obtained from three independent experiments. ** indicates statistical significance between samples ($p < 0.01$).

Supplemental Table 1 List of primers used in this study.

Supplemental Table 2 List of proteins captured by Cry1Ac, Cy1Ab, and Cry1Bd via the on-membrane capture.

Supplemental Figure 1 The peptide^{244}RIFAAMVK^{252}, identified by MS in the Cry1Bd sample, matched GSS1 and GSS2.

Supplemental Figure 2 GPI-anchor sites of GSS1 (A) and GSS2 (B) predicted by GPI Modification Site Prediction.

Supplemental Figure 3 Transmembrane helices of GSS1 (top) and GSS2 (bottom) analyzed by TMHMM. Both GSS1 and GSS2 have N-terminal secretory signal peptides, indicating that they are extracellular proteins. GSS2 also contains a transmembrane helix at its N-terminal, indicating that it is a membrane anchor protein with a C-terminal extending outside of the cell membrane.

Supplemental Figure 4 Genomic insertion of DBM *GSS1* (A) and DBM *GSS2* (B) in transgenic silkworm. Genomic insertion of*GSS1* and *GSS2* in transgenic silkworm lines, as revealed by inverse PCR and sequencing. The transgene integration site in*GSS1* lies in chromosome 23, between two genes KAIKOGA026485 and KAIKOGA026486. The transgene integration site in *GSS2* lies in chromosome 11, between BMgn01916 and BMgn011917. Chromosome localization and partial genomic DNA sequences between the Sau3AI site and the 3' or the 5' insert boundaries of the vector are shown. In all insertions, the TTAA insertion site found in canonical piggyBac insertions was found at the 3' and 5' insert boundary.

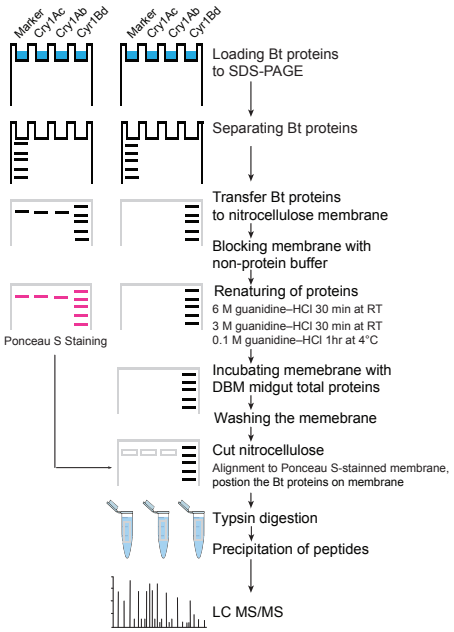

Figure 1

A

Bt proteins	MS Spectra	MSMS Spectra	Identified Peptides			Unique Peptides		Number of proteins
			Total	Bt	%	Total	Bt	
Cry1Ac	5946	16282	136	52	38.2%	60	17	35
Cry1Ab	5837	17088	189	49	25.9%	95	20	40
Cry1Bd	5820	16797	163	45	27.6%	64	11	40

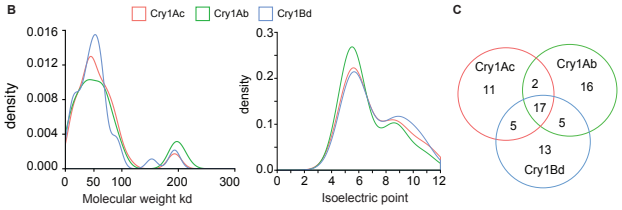

Figure 2

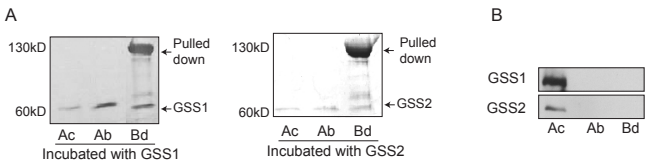

Figure 3

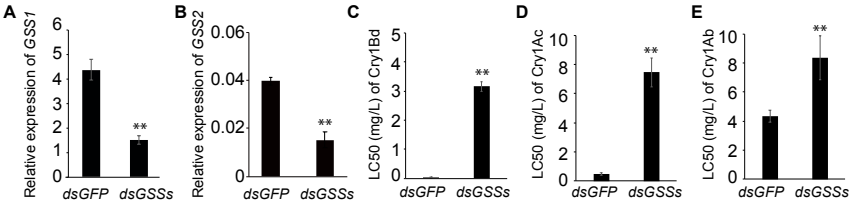

Figure 4

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`Figure 5.pdf` available at https://authorea.com/users/314508/articles/444850-identification-of-bt-toxin-receptors-in-diamondback-moth-plutella-xylostella