Peptide mediated, enhanced toxicity of a bacterial pesticidal protein against southern green stink bug

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Summary

The damage caused by stink bugs that feed on agricultural crops accounts for such significant losses that transgenic plant resistance to stink bugs would be highly desirable. As the level of toxicity of the *Bacillus thuringiensis*-derived, *ETX/Mtx2 pesticidal protein Mpp83Aa1 is insufficient for practical use against the southern green stink bug Nezara viridula*, we employed two disparate approaches to isolate peptides NvBP1 and ABP5 that bind to specific proteins (alpha amylase and aminopeptidase N respectively) on the surface of the *N. viridula* gut. Incorporation of these peptides into Mpp83Aa1 provided artificial anchors resulting in increased gut binding, and enhanced toxicity. These peptide-modified pesticidal proteins with increased toxicity provide a key advance for potential future use against *N. viridula* when delivered by transgenic plants to mitigate economic loss associated with this important pest.

Introduction

Stink bugs (Hemiptera; Pentatomidae) are among the most important pests of global agriculture. These piercing-sucking insects impact 12 major agricultural crops across the globe (Lavore *et al.*, 2018), including cotton, soybean and maize (McPherson and McPherson, 2000; Athey *et al.*, 2019). Southern green stink bug (*Nezara viridula*)

along with several other species in the complex constitute a considerable threat to agricultural productivity (Depieri and Panizzi, 2011; Lomate and Bonning, 2016). The polyphagous feeding behaviour of *N. viridula* (Depieri and Panizzi, 2011; Panizzi, 2015), makes management of this species particularly problematic. As current stink bug management relies heavily on the application of chemical insecticides that lack target specificity, the development of novel, environmentally friendly approaches has become a major goal in agrobiotechnology.

Bacillus thuringiensis (Bt)-derived pesticidal proteins offer the desired specificity for use against target pest species (van Frankenhuyzen, 2013) and have been widely used for the management of coleopteran and lepidopteran crop pests as well as mosquitoes (Bravo *et al.*, 2011). Toxic action mediated by binding of the pesticidal protein to the surface of the gut epithelium followed by pore formation results in disruption of gut function. Gut binding of these pesticidal proteins is mediated by a diverse set of molecules including cadherin-like proteins (Dorsch *et al.*, 2002; Chen *et al.*, 2009; Fabrick *et al.*, 2009), ABCC2 transporters (Atsumi *et al.*, 2012; Tanaka *et al.*, 2013; Banerjee *et al.*, 2017), aminopeptidase N (APN) (Knight *et al.*; 1994; Gill and Ellar, 2002; Rajagopal *et al.*, 2002), alkaline phosphatase (ALP) (Jurat-Fuentes and Adang, 2004; Arenas *et al.*, 2010), α- amylase (Fernandez-Luna *et al.*, 2010), ADAM metalloprotease (Ochoa-Campuzano *et al.*, 2007) and glycolipids (Griffitts *et al.*, 2005), although not all of these binding proteins have been shown to be functional receptors.

Few Bt-derived pesticidal proteins have sufficient activity against hemipteran pests for practical use (Porcar *et al.*, 2009; Chougule and Bonning, 2012). An exception to this are members of the β pore-forming group in the ETX/Mtx2 family, including Mpp51Aa2 (formerly Cry51Aa2 (Crickmore *et al.*, 2020)) employed for suppression of *Lygus* spp. and thrips (Baum *et al.*, 2012; Gowda *et al.*, 2016), and Mpp64Ba and Mpp64Ca (Liu *et al.*, 2018a,2018b) active against *Laodelphax striatellus* and *Sogatella furcifera*. Specific activity of an enhanced Mpp51Aa2 without harmful effects on non-target organisms has been demonstrated (Bachman *et al.*, 2017; Farmer *et al.*, 2017; Moar *et al.*, 2017). To address the lack of sufficiently active pesticidal proteins for management of hemipteran pest species, we previously demonstrated that modification of the Bt-derived Cyt2Aa by addition of a pea aphid gut-binding peptide

resulted in increased toxicity against the pea aphid, *Acyrthosiphon pisum* (Chougule *et al.*, 2013). Based on the same approach, enhanced toxicity of a three-domain toxin, Cry1Ab against *Nilaparvata lugens* has been reported (Shao *et al.*, 2016).

In this study we addressed the question of whether incorporation of a gut-binding peptide would enhance binding and insecticidal efficacy of an ETX/Mtx2 pesticidal protein, Mpp83Aa1 for use against stink bugs. In so doing, we also addressed whether isolation of peptides that bind specific gut surface proteins is more advantageous than screening for gut surface-binding peptides without knowledge of the binding partner, and whether information on relative abundance and location of gut surface proteins facilitates the peptide screening strategy. To this end, we isolated peptides that bound to *N. viridula* gut surface proteins and to recombinant APN. We used 2D ligand blot analysis to identify the binding partner of peptide NvBP1 selected in the former screen. Peptides NvBP1 and ABP5 selected on the basis of binding specificity were used to modify the pesticidal protein Mpp83Aa1 fused to maltose binding protein (MBP). Bioassays were then conducted to test the modified fusion proteins for enhanced efficacy against *N. viridula* nymphs.

Experimental procedures

Expression and purification of N. viridula APN

The appropriate APN contig from an *N. viridula* midgut transcript library (Liu *et al.*, 2018a,2018b) was identified based on the presence of conserved protein domains and a predicted GPI anchor (Eisenhaber *et al.*, 2000). Recombinant *N. viridula* APN was baculovirus expressed using pOET3 and Flashbac Ultra (Oxford Expression Technologies, Oxford, UK) in Sf9 and Sf21 cell monolayer cultures maintained in Sf900 SFMIII growth medium (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA, USA) at 27°C using standard procedures (Hitchman *et al.*, 2010; King and Possee, 1992). All protein concentrations reported herein were determined by Bradford assay (BioRad, Hercules, CA, USA) using bovine serum albumin as a standard. Recombinant protein samples were analysed with protein separation (10 μg per lane) in a 10% SDS PAGE gel and proteins transferred to a PVDF membrane (Amersham Life Science, Little Chalfont, UK). The membrane was blocked with $1\times$ PBS 0.2% Tween 20 and 5% non-fat dry milk. Recombinant APN was detected with a V5 epitope polyclonal antibody (Rockland Immunochemicals, Limerick, PA, USA; dilution 1:2,000) and an HRP-coupled secondary antibody (Thermo Fisher Scientific: dilution 1:5,000) followed by a chemiluminescent substrate (Pierce Thermo Scientific, Rockford, IL, USA).

Recombinant APN was purified using a Ni NTA agarose column (Sigma-Aldrich, St Louis, MO, USA) using standard procedures. Aminopeptidase N activity was assayed using L-leucine-p-nitroanilide (LpNA) as substrate as described previously (Lorence *et al.*, 1997). The specific activity was calculated with one unit of specific APN activity defined as the amount of enzyme catalysing the hydrolysis of 1 mol of LpNA min^{-1} mg of protein^{-1}.

Phage display library screens for peptides that bind N. viridula BBMV or Aminopeptidase N

The Ph.D.-C7C Phage Display Peptide Library (New England Biolabs, Ipswich, MA, USA) was screened either against *N. viridula* BBMV or recombinant APN (Fig. 1). *N. viridula* were maintained as described previously (Cantón and Bonning, 2019a, 2019b). For preparation of brush border membrane vesicles (BBMV), *N. viridula* were reared as described previously (Lomate and Bonning, 2016). For BBMV derived from newly molted adults, insects were immobilized for 30 min over ice and guts dissected. BBMV were prepared by use of the magnesium precipitation method (Wolfersberger, 1993) and stored at $-70°C$ until use. BBMV (60 μg ml^{-1} in TBS buffer with 0.1% Tween) or recombinant APN (100 μg ml^{-1} in TBS buffer with 0.1% Tween) were coated overnight on a rocking platform at 4°C on to polystyrene microtitre wells. Following incubation with the phage library and 10 washes, phages were eluted at pH 2.2 following the recommended screening procedures. Eluted phages were amplified for the next round of biopanning. After the third round of phage enrichment, the phage DNA from single plaque forming units was isolated according to the manufacturer's protocol and sequenced by Sanger sequencing at the Iowa State University DNA Facility. Sequences were analysed with the Clustal Omega server, with grand average of hydropathy (GRAVY) score estimated http://www.gravy-calculator.de/index.php and screened for unrelated target binding using the Scanner And Reporter Of Target-Unrelated Peptides (SAROTUP) server (http://i.uestc.edu.cn/sarotup3/cgi-bin/TUPScan.pl).

Two peptides from the BBMV library screen (NvBP1 and NvBP5) and five peptides from the APN library screen (ABP1-5) were selected. Peptide-linker-mCherry fusions were produced with the linker comprised of five alanine proline repeats (AP)5. Primer sequences are provided in Table S1. Peptide-(AP)5-mCherry constructs were cloned into pBAD/HisB (Invitrogen) and proteins expressed and purified as described by Chougule *et al.*, 2013 (Chougule *et al.*, 2013).

Identification of the gut protein bound by NvBP1

Two-dimensional ligand blot analysis using *N. viridula* gut-derived BBMV (50 μg treated with the 2D Clean-up kit; GE Healthcare, Chicago, IL, USA) was used for
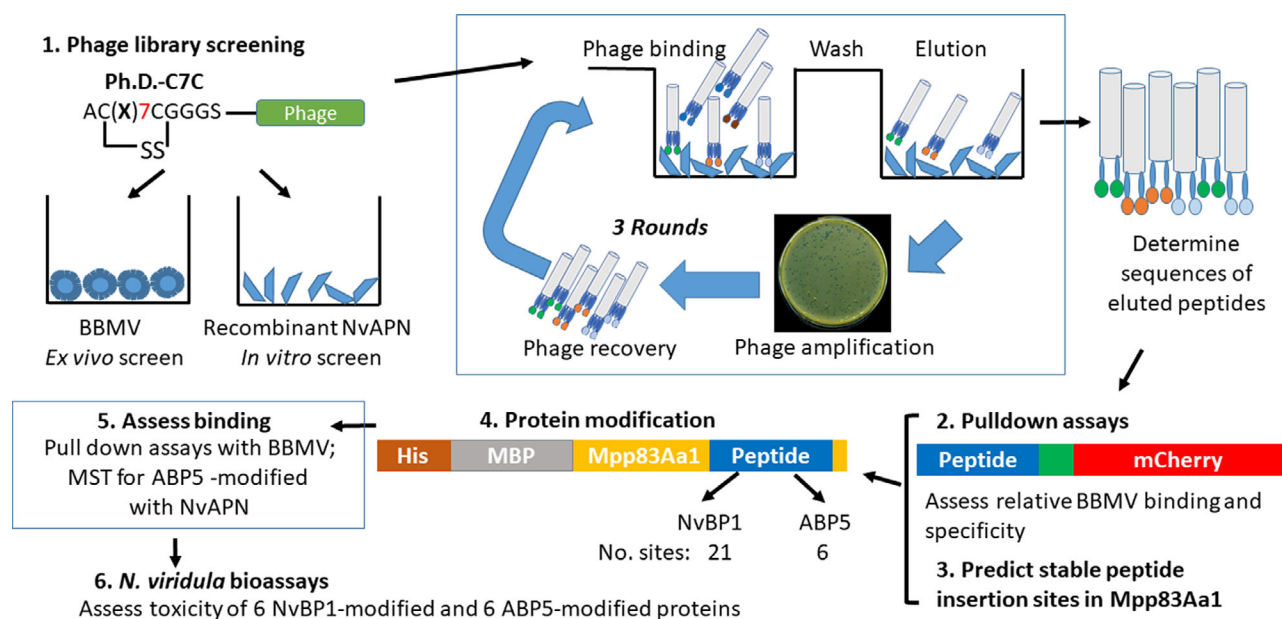
Fig. 1. Workflow for identification and use of gut-binding peptides for Mpp83Aa1 modification.

identification of the NvBP1 gut binding partner as described previously (Linz *et al.*, 2015). 2D-separated proteins were transferred from the gel to a PVDF membrane (Millipore, Burlington, MA, USA) for ligand blot, N-terminal sequencing, or LC MS/MS identification. For proteins transferred to PVDF membranes, the membrane was blocked 1 h with 1× PBS with 0.2% Tween 20 (PBST) and 5% fat free milk. Following five washes with (PBST), the blot was incubated 2 h with 10 mM of either NvBP1 peptide − mCherry fusion or (AP)5 linker − mCherry fusion in PBST with 0.1% fat free milk. The blots were rinsed and washed five times with PBST and incubated with the primary antibody, anti - mCherry (Novus Biologicals, Littleton, CO) at a 1:5000 dilution for 1 h. The membrane was washed as described, incubated with the secondary antibody anti-rabbit coupled to HRP (ratio 1:10000; Thermo Scientific) for 1 h and washed. A final wash with 1X PBS was performed. Antibody binding was detected with the West Pico Chemiluminescent kit (Pierce/Thermo) on exposure to film.

The NvBP1 binding protein was identified by LC MS/MS and N-terminal sequencing at the Iowa State University Protein Facility. For LC MS/MS protein identification, the protein spot was manually excised from the 2D gel, reduced, alkylated and digested with trypsin. The generated peptides were then separated by LC MS/MS with the Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific). The translated *N. viridula* transcriptome (Liu *et al.*, 2018a,2018b) was used as reference to identify *N. viridula* proteins. The TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/)

was used to predict the presence of transmembrane helices in candidate NvBP1-binding proteins.

N-terminal sequencing was performed after 2D gel electrophoresis and transfer of BBMV proteins to a PVDF membrane. N-terminal protein sequencing was carried out on proteins visualized by staining (Coomassie brilliant blue R250) by Edman degradation with a Perkin Elmer Applied Biosystems Model 494 Procise protein/peptide sequencer (Norwalk, CT) with an on-line Perkin Elmer Applied Biosystems Model 140C PTH Amino Acid Analyzer.

Confirmation of peptide binding to BBMV by pull-down assay

To confirm binding of selected peptides to *N. viridula* gut proteins, pull-down assays were conducted with purified *N. viridula*-binding peptide − linker-mCherry fusions and *N. viridula* BBMV. Negative controls for pull-down assays were linker-mCherry, mCherry alone, and BBMV alone. Fusion proteins (10 nM) were incubated with 10 μg of BBMV in binding solution (1XPBS 0.1% Tween 20 and 0.1% BSA) in a final volume of 100 μl. This solution was incubated for 2 h at room temperature and centrifuged at 20 800× *g* for 20 min at 4°C. The pellet was resuspended with 100 μl of binding solution and centrifugation repeated 3 times. Finally, the resulting pellet was resuspended in 10 μl of binding solution and analysed by western blot. Proteins were resolved in a 10% SDS PAGE gel and transferred to PVDF membrane (Amersham). The membrane was blocked with 1X× PBS 0.2%

Tween 20 and 5% non-fat dry milk. The peptide fusion was detected using an mCherry polyclonal antibody (dilution 1:5000) and a secondary HRP coupled antibody (Thermo Scientific, dilution 1:5000) followed by a chemiluminescent substrate (Pierce Thermo Scientific). For competition bioassays conducted with peptides ABP1-5, the peptide mCherry fusion proteins were incubated with or without excess peptide (biotinylated, 0.1 μM to 1000 μM) with BBMV derived from second instar nymphs, and the pull-down assay continued as described above.

Peptide modification of Mpp83Aa1

Mpp83Aa1 (NCBI Accession No. MZ005703) is an ETX/Mtx2 pesticidal protein that shares 24% amino acid identity to the β pore-forming E-toxin from *Clostridium perfringens*. The predicted structure of Mpp83Aa1 was modelled by I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Sites for introduction of peptides NvBP1 or ABP5 by addition to- or replacement of-existing sequence were selected on the basis of homology modelling, *in silico* protein stability and peptide exposure on the surface of the protein. Sequences for the modified Mpp83Aa1 constructs were synthesized by GenScript for expression as HisMBP fusion proteins in pMal-c2X (New England Biolabs). Native and peptide-modified Mpp83Aa1 proteins were expressed in *E. coli* BL21 cells as MBP fusion proteins and purified (pMal Protein Fusion and Purification System; New England Biolabs). The purified toxins were concentrated using the Amicon Ultra Centrifugal Filter 6 ml device (Millipore Sigma).

Assessment of peptide impact on toxin binding

Both pull-down assays and microscale thermophoresis (MST) were used to assess the relative binding of native and modified Mpp83Aa1 fused to MBP. For the pull-down assay, the protocol described above was followed using 50 nM of the respective MBP-Mpp83Aa1 along with 10 μg of *N. viridula* BBMV. Fusion proteins that bound BBMV were visualized by western blot with anti-Mpp83Aa1 antibody raised in rabbit.

For MST analysis, a Monolith NT 115 (NanoTemper, Cambridge, MA, USA) was used to determine the binding affinity of MBP fusion proteins between Mpp83Aa1 modified with ABP5 and recombinant *N. viridula* APN. The native and modified Mpp83Aa1 fusion proteins (~ 75 kDa) were labelled using the Monolith Protein Labeling Kit RED-NHS 2nd Generation (Amine Reactive) according to the manufacturer's directions and aliquoted in 20 μl or smaller volumes in Corning Costar low binding microcentrifuge tubes. To determine binding affinity,

50 nM of target (labelled MBP-Mpp83Aa1 diluted from a 1 μM of stock concentration with MST assay buffer; 1X PBS with 0.1% Tween-20) and 1 μM of ligand (recombinant APN) were used. A sixteen-fold serial dilution of the ligand was made in low binding tubes using ligand buffer (5% CHAPS buffer). Target (10 μl) was added to the serially diluted ligand (10 μl) and incubated in the dark at room temperature for 10 min. The Monolith NT115 series premium capillaries were dipped into the final mix, and arranged in the order of high to low ligand concentrations in the capillary stand, which was then inserted into the Monolith NT115. Readings were taken for the ΔF norm. The normalized fluorescence (Fnorm) for each data point represented by the interaction between fluorescent-labelled target molecule (MBP-Mpp83Aa1 constructs) at a particular concentration with a range of concentrations of unlabelled ligand (recombinant APN) was plotted into a sigmoid curve to obtain the Kd value, where half of the target molecules are in a bound state. Response amplitude and the signal to noise ratios were used as measures of quality control. Two to three biological replicates were performed at different times for selected modified toxins, using different aliquots from the same recombinant APN and fusion protein preparations.

Bioassays

Toxicity of native and modified MBP-Mpp83Aa1 to *N. viridula* was assessed using the bioassay method of Wellman-Desbiens and Côté ([2005](#)). Scintillation vials were set up with a layer of autoclaved sand, 100 μl of ddH$_2$0 followed by another layer of autoclaved sand and plugged with autoclaved cotton plugs. Four stink bugs were placed in each vial with five technical replicates per bioassay. The purified toxins (1 mg total protein in 1ml for the six selected constructs) were mixed with *Lygus hesperus* artificial diet (Frontier Scientific Services Inc, Newark, Delaware, US) supplemented with 150 μl of streptomycin (500 μg ml^{-1}) and 35 μl of Nystatin (50 mg ml^{-1}) poured into diet packets made with parafilm. The assay was conducted with three biological replicates of twenty, 2nd instar nymphs. Insect mortality was recorded daily for 7 days.

Statistical differences between the toxicity of native and modified MBP-Mpp83Aa1 fusion proteins on day 7 of bioassays were determined by one way ANOVA. Assumptions of normality (Shapiro–Wilk) and equal variance (Brown Forsythe) were met. Significant differences in mortality data between constructs modified with ABP5 or NvBP1 at the same site were also assessed by pairwise multiple comparison procedures by use of the Holm-Sidak method.

For the determination of LC$_{50}$ values, second instar nymphs were fed on five doses (30, 90, 270, 540,

1620 μg ml^{-1}) of test protein (MBP-Mpp83Aa1 native, 70-76 constructs and 172-178 constructs) or on buffer alone. Five vials with four insects per vial (n = 20 insects) were used for each treatment per replicate bioassay as described above. Mortality was scored daily for 8 days, and mortality on day 8 used for determination of LC$_{50}$ values. LC$_{50}$ values were determined using a PROC PROBIT model with Abbott`s correction to account for mortality in the control. The LC$_{50}$ was calculated in SAS program along with the 95% fiducial limits (see Methods S1 for SAS script). Goodness of fit tests were conducted to calculate the Pearson Chi-square values.

Results

Identification of peptides that bind BBMV or recombinant APN of N. viridula

Screening of the Ph.D.-C7C Phage Display Peptide Library for peptides that bound *N. viridula* BBMV (Fig. 1) resulted in identification of candidate peptides NAGHLSQ (NvBP1) and EVMSHKW (NvBP5) by Sanger sequencing. Of 60 phages sequenced after the third round of phage enrichment, 40 encoded NvBP1 and one expressed NvBP5. As SAROTUP analysis indicated that NvBP5 had polystyrene binder sequence, this peptide was omitted from further analysis (Table S2).

Aminopeptidase N (APN) was identified from Contig 9840 from the *N. viridula* transcriptome (Liu *et al.*, 2018a,2018b) (Fig. S1). Recombinant *N. viridula* APN was expressed in Sf21 cells using the baculovirus expression system, and affinity purified (Fig. S2). Five peptides (ABP1-5) were selected following phage display library screening for phage encoding peptides that bind recombinant *N. viridula* APN (Table S2). ABP5 was the most hydrophilic of the seven peptides assessed.

NvBP1 binds to N. viridula α-amylase N4

2D ligand blot analysis with NvBP1-(AP)5-mCherry showed that NvBP1-(AP)5-mCherry binds a ~ 50 kDa protein with a pI of 6 that was absent from the negative control blot with (AP)5-mCherry as ligand (Fig. 2A). While several candidate binding proteins were identified by LC-MS/MS with reference to the translated *N. viridula* gut transcriptome, the approximate pI (5.62 and 6.02) and molecular mass of proteins encoded by two contigs, 9247 (56 kDa) and 10931 (60 kDa), correlated with the protein observed in the ligand blot (Table S3). Alpha-amylase N4 on the surface of the *N. viridula* gut epithelium is the binding partner of NvBP1 based on localization (transmembrane helix from Y13 to A35; Table S3) and the N-terminal sequence of DTIXN. Ligand blot results (Fig. 2A) also indicate that the binding of NvBP1 is specific.

Peptide binding and specificity of binding to BBMV proteins

Pull-down assays conducted to confirm binding of peptides selected from the phage display library screens showed that NvBP1, but not NvBP5 bound to *N. viridula* BBMV proteins (Fig. 2B). This result is consistent with characterization of NvBP5 as a polystyrene-binding peptide (Table S2).

While binding of all five APN-binding peptide-(AP)5-mCherry fusions (ABP1-5) to BBMV was confirmed (Fig. S3), only ABP5-(AP)5-mCherry bound specifically to BBMV with binding outcompeted at ≥ 10 μM peptide in competition pull-down assays (Fig. 2B; Fig. S3). On the basis of these competition assays, peptide ABP5 was selected for modification of Mpp83Aa1, along with NvBP1.

Peptide addition to MBP-Mpp83Aa1

A homology model based on Mpp51Aa1 (Xu *et al.*, 2015) was used for selection of sites for peptide incorporation. As there was little information on domains of ETX/Mtx2 proteins that are important for toxicity, a wide range of sites including alpha helices, beta sheets and loop regions that are predicted to be on the exterior of Mpp83Aa1 were selected for modification. The sites and the mode of peptide addition (addition to- or substitution of- existing sequence), were selected on the basis of modelling with (1) the peptide predicted to be displayed on the surface of Mpp83Aa1 rather than folded in, and (2) the stability of the predicted modified structure.

NvBP1 was incorporated into eight sites in Mpp83Aa1 by addition- and into 13 sites by substitution- of existing amino acid sequences, resulting in a total of 21 constructs (Fig. 3). All 21 NvBP1-modified MBP-Mpp83Aa1 expressed stably in *E. coli* (Fig. S4). Based on data generated from initial bioassays with all 21 constructs, a subset of six NvBP1-modified constructs was selected for further analysis. These same six sites (one addition at AA43, five substitution; AA70-76, AA172-178, AA208-214, AA224-230 and AA269-275) were also used for Mpp83Aa1 modification with ABP5 (Fig. 3), and ABP5-modified MBP-Mpp83Aa1 were also stably expressed (Fig. S4).

Binding of MBP-modified Mpp83Aa1 to N. viridula gut proteins

The binding of the 12 MBP- modified Mpp83Aa1 fusion proteins to BBMV was first assessed by pull-down assay. While all ABP5-modified constructs showed increased binding relative to native (MBP-Mpp83Aa1), ABP5- modification at AA172-178 showed the greatest
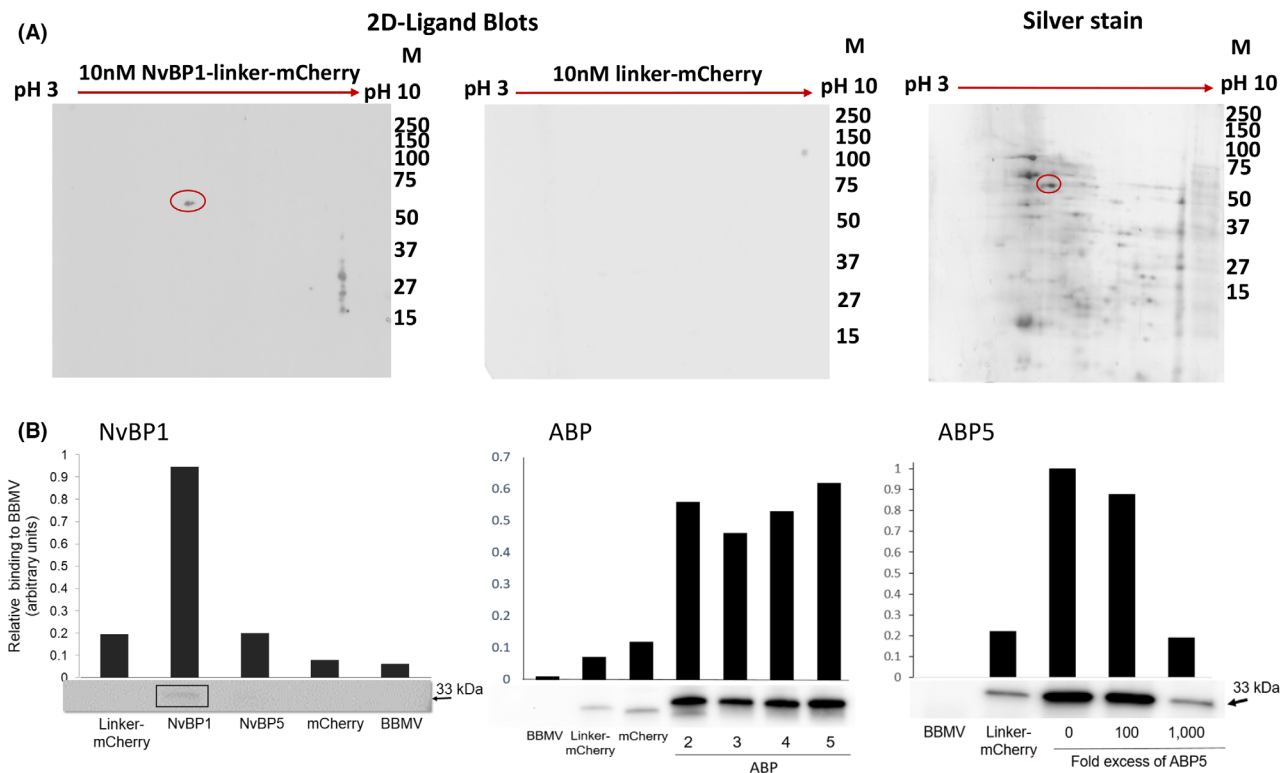
Fig. 2. Peptide binding (A) NvBP1 binds α-amylase N4. 2D gel electrophoresis of 50 μg BBMV, followed by ligand blot analysis with NvBP1-(AP)5-mCherry or (AP)5-mCherry (negative control) as ligand resulted in identification of a single protein spot. Ligand blots and a silver stained 2D gel are shown. M. molecular mass markers.
B. Assessment of peptide binding to *N. viridula* BBMV. Pull-down assays were conducted to assess the relative binding of peptide-(AP)5-mCherry (10 nM) to BBMV (10 μg). Western blots are shown beneath histograms of quantified band intensity for NvBP1 and NvBP5, and for ABP peptides. For ABP5, binding to BBMV was outcompeted by excess biotinylated synthetic peptide indicating that ABP5 binding is specific. Negative controls used in these assays were (AP)5-mCherry (linker-mCherry), mCherry and BBMV.

increase in binding relative to other modified constructs. NvBP1-modified constructs with modifications at AA172-178, 224-230 and 269-275 showed the greatest increase in binding relative to native, with the highest binding for NvBP1 substitution of AA224-230 (Fig. 4). NvBP1-modification at AA70-76 showed decreased binding relative to native in these pull-down assays. Taken together, sites AA172-178 resulted in increased binding for both peptides, while sites AA224-230 and AA269-275 resulted in increased binding for NvBP1 with a more moderate change relative to native on modification with ABP5.

The binding affinity (Kd) values for selected ABP5-modified Mpp83Aa1 proteins to recombinant *N. viridula* APN were determined by microscale thermophoresis assay (MST). The binding affinity of native MBP-Mpp83Aa1 to recombinant APN was 139 nM. The binding affinity of MBP-ABP5-modified Mpp83Aa1 to recombinant APN increased for AA172-178 (Kd 4.98 nM) and AA70-76 (123 nM) while the binding of AA 224-230 was (Kd 187 nM). These results are consistent with pull-down assay data (Fig. 4; Table S4).

Impact of MBP-peptide-modified Mpp83Aa1 on N. viridula

Bioassays were conducted to assess the impact of the 12 MBP-peptide-modified Mpp83Aa1 fusions on second instar *N. viridula* nymphs. Nymphs were fed at 1,000 μg of total protein per ml, containing ~442 μg of MBP-Mpp83Aa1 fusion protein per ml (Fig. S4) of which (based on molecular mass) 188 μg ml^{-1} was composed of Mpp83Aa1. Toxicity was significantly increased for four ABP5-modified constructs - AA43, 172-178, 224-230 and 269-275, and five NvBP1-modified constructs – AA70-76, 172-178, 208-214, 224-230 and 269-275 (Fig. 5, Table 1). Pairwise comparisons indicated that mortality resulting from modification with ABP5 or NvBP1 at the same site, was significantly different for sites AA172-178, 208-214, 224-230, but not for AA43, 70-76 or 269-275 ($p < 0.05$; Table 2).

Determination of LC$_{50}$ values for selected MBP-Mpp83Aa1 fusion constructs modified with ABP5 or NvBP1 at AA70-76 or AA172-178 showed a reduction in

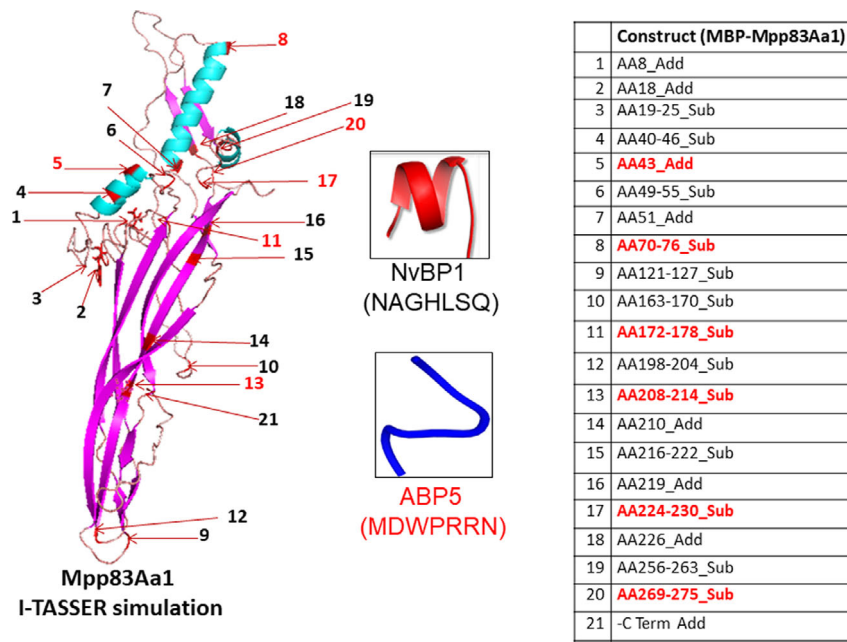|	Construct (MBP-Mpp83Aa1)
1	AA8_Add
2	AA18_Add
3	AA19-25_Sub
4	AA40-46_Sub
5	**AA43_Add**
6	AA49-55_Sub
7	AA51_Add
8	**AA70-76_Sub**
9	AA121-127_Sub
10	AA163-170_Sub
11	**AA172-178_Sub**
12	AA198-204_Sub
13	**AA208-214_Sub**
14	AA210_Add
15	AA216-222_Sub
16	AA219_Add
17	**AA224-230_Sub**
18	AA226_Add
19	AA256-263_Sub
20	**AA269-275_Sub**
21	-C Term Add

Fig. 3. Modification of Mpp83Aa1 with *Nezara viridula* gut binding peptides. The protein structure generated by PyMOL (The PyMOL Molecular Graphics System, Version 2.2 Schrödinger, LLC) with sites of modification indicated by arrows are shown. The protein was modified either by addition of peptide sequences to existing amino acid sequence, or by substitution of existing amino acids. Modifications made with NvBP1 or with ABP5 (a subset of sites modified with NvBP1, in red) are shown. Specific details of peptide addition to Mpp83Aa1 are provided in the table. The 21 sites in Mpp83Aa1 used for peptide addition are denoted in the construct name. A single amino acid (e.g. AA8) indicates the Mpp83Aa1 amino acid after which the 7 amino acid peptide was added. A peptide range (e.g. AA19-25) indicates the Mpp83Aa1 amino acids that were replaced.
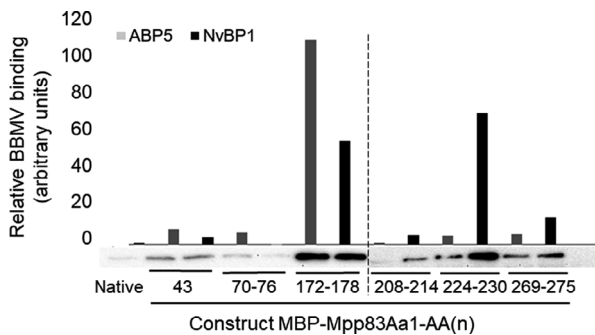

Fig. 4. Relative peptide binding. Relative binding of peptide-modified MBP-Mpp83Aa1 to *N. viridula* BBMV. Pull-down assays were performed with native and peptide modified MBP-Mpp83Aa1. Relative binding of MBP-Mpp83Aa1 modified with ABP5 or NvBP1 is indicated by quantification of western blot band intensities. Dashed line indicates two separate western blots with data normalized to the respective native bands. Data are representative of two biological replicate experiments.

LC_{50} in all cases apart from the fusion construct modified at AA70-76 with ABP5 (70-76A: Table S5). These results are consistent with mortality observed in single dose bioassays (Fig. 5). LC_{50} values could not be determined for MBP-Mpp83Aa1 native or the 70-76A as mortality levels were insufficient at the highest dose used in these bioassays.

Discussion

The aim of this study was to assess whether a gut-binding peptide could be used to enhance the efficacy of a bacteria-derived pesticidal protein for use against stink bugs. Achievement of this goal represents a significant advance for management of these difficult agricultural pests. From our results with the ETX/Mtx2 –type protein Mpp83Aa1, we conclude that: (i) peptides selected for binding to BBMV or to a specific gut surface protein were both effective for enhancing the binding of an ETX/Mtx2 pesticidal protein to the gut of *N. viridula*, (ii) as observed previously (Chougule *et al.*, 2013), the strength of binding of peptide-modified fusion proteins did not correlate directly with insecticidal activity, (iii) in terms of improving insecticidal efficacy, substitution of amino acids in a given ETX/Mtx2 pesticidal protein with gut-binding peptide sequences was more successful than addition of peptide sequence to existing sequence. The use of MBP-Mpp83Aa1 fusion proteins for this work facilitated both expression and stability of the Mpp83Aa1 native and modified toxins. Minimal impact of MBP on any of the parameters tested was observed. MBP would not be included on expression of modified Mpp83Aa1 in transgenic plants however, reflecting a very different environment for protein expression.
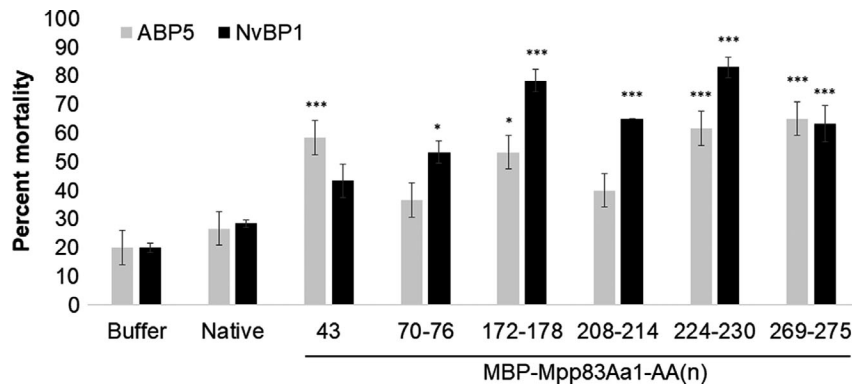
Fig. 5. Gut binding peptides ABP5 and NvBP1 increased MBP-Mpp83Aa1 toxicity against *N. viridula* nymphs. Membrane feeding assays with 1 mg ml^{-1} total protein containing ~442 μg ml^{-1} of MBP-Mpp83Aa1 (Fig. S4) in *Lygus hesperus* diet were conducted with 20 s instar *N. viridula* per biological replicate and three biological replicates. Mean percent mortality at day 7 and SE are shown. Statistically significant differences relative to MBP-Mpp83Aa1 (native) are indicated by * and ***, representing $P < 0.05$, or $P < 0.001$ respectively. See Table 1 for supporting data.

Table 1. *N. viridula* mortality resulting from ingestion of MBP-Mpp83Aa1 fusion proteins.

Treatment	Mean	STDEV	SEM	P-value
Buffer	20	6.45497224	3.2274861	NA
MBP fusions				
Mpp83Aa1 (Native)	26.66	6.2915287	3.1457643	NA
43A	58.33	9.12870929	4.5643546	<0.001
43N	43.33	15	7.5	0.176
70–76A	36.66	9.46484724	4.7324236	0.818
70–76N	53.33	9.12870929	4.5643546	0.002
172–178A	53.33	2.88675135	1.4433757	0.002
172–178N	78.33	11.0867789	5.5433895	<0.001
208–214A	40	5	2.5	0.497
208–214N	65	2.5	1.25	<0.001
224–230A	61.66	12.5	6.25	<0.001
224–230N	83	8.77021475	4.3851074	<0.001
269–275A	65	5	2.5	<0.001
269–275N	63.33	13.7689264	6.8844632	<0.001

Mean percentage mortality values, SD and SE for treatment of second instar *N. viridula* nymphs with native and modified MBP-Mpp83Aa1 are shown along with p values for comparison with mortality resulting from MBP-Mpp83Aa1 (Native) as determined by one-way ANOVA. Modifications are indicated by A, ABP5 and N, NvBP1.

Table 2. Comparison of mortality between Mpp83Aa1 constructs modified with ABP5 or NvBP1 at the same site. Pairwise Multiple Comparison (Holm-Sidak method).

Site for ABP5 vs. NvBP1 comparison	Difference of means	T	P	P < 0.050
AA43	15.000	2.745	0.315	No
AA70-76	16.667	3.050	0.184	No
AA172-178	25.000	4.575	0.005	Yes
AA208-214	25.000	4.575	0.005	Yes
AA224-230	21.333	3.904	0.027	Yes
AA269-275	1.667	0.305	0.997	No

Multiple methods have been adopted for screening phage libraries for peptides that bind to the surface of insect guts with the goal of blocking pathogen transmission (Ghosh *et al.*, 2001; Liu *et al.*, 2010) or enhancement of pesticidal proteins (Chougule *et al.*, 2013; Shao *et al.*, 2016). These methods include feeding the target insect on the phage library and eluting bound phage from the dissected gut (e.g. pea aphid, mosquito), the use of BBMV enriched for gut surface proteins, and the use of specific recombinant gut proteins as bait for the phage display library as described herein. For tractable insects, elution from the dissected gut provides the most direct approach for isolation of gut binding peptides without the potential for binding to intracellular gut proteins that are typically present in BBMV (Bayyareddy *et al.*, 2012). For *N. viridula*, BBMV were screened for gut-binding peptides followed by confirmation of binding to gut surface proteins to avoid the potential loss of phage on exposure to the diverse proteolytic enzymes present in the *N. viridula* gut and saliva (Lomate and Bonning, 2016; Cantón and Bonning, 2019a, 2019b).

In contrast to other insects, stink bugs rely on a biphasic digestive process, with serine proteases active at alkaline pH released in the saliva, and cathepsins active at acidic pH prevalent in the gut, to ensure complete digestion of ingested materials (Lomate and Bonning, 2016; Cantón and Bonning, 2020). This efficient digestive physiology suggests that pesticidal proteins that bind gut surface proteins abundant in the anterior midgut (M1 region) are more likely to exert an effect than proteins that would have to withstand prolonged exposure to proteases before binding gut proteins in the more distal M2 or M3 regions. Thus, knowledge of the relative abundance of surface proteins along the length of the stink bug gut (Cantón and Bonning, 2019a, 2019b) provides valuable information for selection of appropriate gut surface proteins to target for production of gut binding peptides. In this study, while APN activity predominates in M1, transcript levels are comparable along the

length of the gut, suggesting that inhibitors or post-translational mechanisms are involved in regulating APN activity (Cantón and Bonning, 2019a, 2019b). In contrast to APN, alpha amylase N4 transcripts are abundant in M1, but not in M2 and M3 (Fig. S5). Modification of Mpp83Aa1 with NvBP1, which binds alpha amylase resulted in overall greater toxicity than modification with ABP5, which binds APN, possibly due to increased contact with the gut epithelium in M1 and relatively short exposure to gut digestive enzymes.

While receptor proteins that mediate toxic action have been identified for a number of Bt-derived pesticidal proteins from different structural groups, none have been identified for ETX/Mtx2 proteins. Some of the three-domain Cry toxins bind the GPI anchored midgut proteins ALP and APN, resulting in pore formation (Bravo *et al.*, 2004; Pardo-Lopez *et al.*, 2006). APN is a functional receptor in several lepidopteran species (Gill *et al.*, 1995; Knight *et al.*, 1995) with mutation conferring resistance to three-domain Cry toxins in some pest species (Heckel, 2020). In this study, MBP-Mpp83Aa1 was shown to bind recombinant *N. viridula* APN with a Kd of 139 nM suggesting that APN is also a putative receptor for Mpp83Aa1.

Incorporation of peptides ABP5 and NvBP1 into Mpp83Aa1 provided artificial anchors for binding to APN and α-amylase N4 respectively. Alpha-amylase is a putative receptor for *B.t. israelensis* mosquitocidal proteins Cry4Ba and Cry11Aa in *Anopheles albimanus* with binding observed between these pesticidal proteins and recombinant *E. coli* –expressed alpha amylase (Fernandez-Luna *et al.*, 2010). The peptides ABP5 and NvBP1 therefore either increased MBP-Mpp83Aa1 binding to existing receptor proteins (i.e. putative receptor APN), or provided new binding sites (alpha amylase N4) to expedite toxic action. The extent of increased binding and toxic action varied at a given site according to peptide in some cases. At site AA43 for example, the only site for which peptide sequences were added to Mpp83Aa1 sequence (rather than replacing existing sequence), significant toxicity enhancement relative to native was seen with ABP5 but not with NvBP1. For sites 208–214, significantly increased mortality relative to native was seen with NvBP1 but not ABP5. Indeed, mortality data for Mpp83Aa1 modified with ABP5 at AA172-178, 208-214 and 224-230 were significantly different from constructs modified with NvBP1 at the same site (Table 2). In these cases, the impact of the peptide sequence on Mpp83Aa1 structure, or the orientation of Mpp83Aa1 on binding may drive these different outcomes.

Overall, higher mortality levels were seen for constructs modified with NvBP1 than ABP5, particularly for modifications at AA172-178, AA208-214 and AA224-230. Non mutually exclusive scenarios that may account for this observation include (i) NvBP1 mediates higher affinity binding to α-amylase than ABP5 binding to APN, (ii) α-amylase is more abundant in the gut resulting in higher toxicity, (iii) α-amylase expression is high in the anterior midgut (M1) relative to APN, such that toxicity occurs before significant enzymatic degradation of MBP-Mpp83Aa1, as discussed above, (iv) differences in stability of the modified constructs on exposure to salivary and gut enzymes. MBP-Mpp83Aa1 shows minimal degradation when treated with *N. viridula* saliva (Fig. S6).

The beta pore forming toxins have a highly variable head region that is hypothesized to interact with receptors in the host gut, and a highly conserved tail region proposed to function in pore formation and membrane integration (James *et al.*, 2012; Jerga *et al.*, 2016). An essential role has been proposed for the beta barrels in ETX/Mtx proteins in the formation of pores in their target insects (Popoff, 2011). The six sites employed for modification of Mpp83Aa1 were scattered throughout the head domain. It is notable that placement of gut-binding peptides at AA208–214 in the beta barrel domain did not interfere with toxicity.

Based on prior work with pea aphid gut-binding peptides (Liu *et al.*, 2010; Chougule *et al.*, 2013), we expect NvBP1 and ABP5 to bind to species closely related to *N. viridula* (i.e. other stink bugs), but to a lesser extent. The extent of peptide binding is expected to reflect the similarity of receptor proteins, particularly at the specific peptide-binding region. Importantly, none of the *N. viridula* gut binding peptides identified from this work were enriched from the same phage library screened *in vivo* for peptides that bind to the gut epithelium of the honey bee, *Apis mellifera* (Y. Guo, unpublished data; (Mishra *et al.*, 2021)). Although empirical tests are required to test for toxicity to nontarget organisms, the peptides used in this study are not expected to bind the honey bee gut.

From a practical standpoint, while the increase in toxicity of the peptide-modified MBP-Mpp83Aa1 fusion proteins (up to 78% mortality at ~442 μg of fusion protein per ml; ~188 μg of Mpp83Aa1 per ml) is modest relative to that of the native fusion protein (26%; Table 1), these may not reflect the toxicity levels of native and modified Mpp83Aa1 alone with sequences optimized for plant expression. Indeed, plant expression of the related pesticidal protein, Mpp51Aa2 in cotton was sufficiently high for effective use against plant bugs in the genus *Lygus* (Hemiptera; Miridae) with up to 1070 μg mg^{-1} dry weight in cotton squares and 500 μg mg^{-1} in leaf tissue (Gowda, *et al.*, 2016). This example provides a precedent for the deployment of peptide-modified Mpp-type proteins for stink bug management.

In conclusion, peptides ABP5 and NvBP1 were successfully used to enhance the toxicity of an ETX/Mtx2

pesticidal protein against *N. viridula* nymphs. This strategy is expected to facilitate use of modified bacteria-derived pesticidal proteins for the management of hemipteran pests, similar to the successful use of Bt proteins for suppression of lepidopteran and coleopteran pest species (Blanco *et al.*, 2009; Chougule and Bonning, 2012; Gassmann *et al.*, 2014).

Conflict of interest

N.P.C. is currently employed by BASF Corporation. R.D. was previously by BASF Corporation. R.D. and B.B. are authors on a patent submission associated with this work. R.B., B.F.E., P.E.C. do not have competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. *N. viridula* APN sequence (contig 9840) is predicted to have a 204 amino acid Endoplasmic Reticulum Associated Protein (ERAP) domain at the C terminus of the protein as predicted by the CATH software. This domain is present in 208 insect sequences of the aminopeptidase N family. *N. viridula* APN also has a predicted GPI anchor site at tyrosine 855.

Fig. S2. Purification of recombinant *N. viridula* APN. SDS PAGE gel (10%) showing baculovirus expressed recombinant APN (102 kDa; arrow) in eluted fractions from Ni-NTA affinity chromatography purification. FT, flow through; E1-5, Eluate fractions 1–5; M, molecular mass markers. In conjunction with APN expression, APN activity increased from 2.4 units in uninfected Sf21 cells to 6.3 units in Sf21 cells infected with the baculovirus expressing recombinant *N. viridula* APN.

Fig. S3. Assessment of ABP peptide-binding specificity. Competition pull-down assays were performed using 10 nM of gut- binding peptide-(AP)5-mCherry fusion proteins and 10 μg of BBMV prepared from fifth instar nymphs. Competing peptide was added at concentrations from 1 μM to 1000 μM. Fusion proteins (33KDa) bound to BBMV after a series of washes were detected with anti-mCherry antibodies (1:5000 dilution). Western blots are shown for ABP1-4. Binding of ABP2 was reduced at 100k-fold excess peptide (the highest concentration used), while the binding of ABP1, ABP3 and ABP4 was not. M, molecular mass markers.

Fig. S4. Expression of modified MBP-Mpp83Aa1. All modified MBP-Mpp83Aa1 proteins (75 kDa) were stably expressed. Fusion proteins modified with A) NvBP1 and B) ABP5 were run in SDS-PAGE gradient gels (4–20%; 20μg lane^{-1}) and the gel stained using Coomassie brilliant blue stain.

Fig. S5. Relative APN and alpha amylase N4 transcript abundance in the gut of *N. viridula*. A) Regions M1 to M3 of the *N. viridula* midgut, and M4, hindgut. B) Transcripts per million (mean TPM) for M1, M2 and M3. All transcripts had significant BLAST e-values compared to *N. viridula* alpha amylase N4, or *H. halys* aminopeptidase. The majority of alpha amylase N4 transcripts were located in M1, the anterior midgut, while APN transcripts were distributed across all three midgut regions.

Fig. S6. Stability of Mpp83Aa1 on exposure to *N. viridula* saliva. MBP-Mpp83Aa1 (0.5 μg) was incubated for 1 hr at 37^{0}C with or without 2 μl of saliva and the samples visualized by staining of the SDS-PAGE gel with Coomassie blue or by western blot with Mpp83Aa1 or MBP antisera. MBP (42.5 kDa) was used as a positive control. Some degradation of Mpp83Aa1 was apparent after 1 h

Table S1. Primers used for production of NvBP- and ABP-mCherry fusion protein constructs. Restriction sites are shown in bold, peptide sequences are underlined, the Alanine – Proline linker is shown in italics and mCherry sequence is highlighted.

Table S2. Analysis of peptides isolated from phage display screens. GRAVY scores and SAROTUP analysis data for peptides selected from *N. viridula* BBMV (NvBPn) or recombinant APN (ABPn) phage display screens. Negative GRAVY scores indicate hydrophilicity.

Table S3. Alpha amylase N4 is the most abundant NvBP1 binding proteins in *N. viridula* BBMV based on LC MS/MS analysis.

Table S4. Binding affinity of MBP-native Mpp83Aa1 and MBP- ABP5-modified Mpp83Aa1 for recombinant *N. viridula* APN. Data for up to 3 replicates are shown for selected constructs. -, not determined.

Table S5. LC$_{50}$ values for modified MBP-Mpp83Aa1 fusion proteins. NA, not applicable: LC$_{50}$ values could not be determined for native or for modified construct 70–76A as only 20% mortality was recorded at the highest dose of 1620 μg of total protein ml^{-1} in two biological replicates. These data reflect mortality seen in single dose bioassays (1000 μg of total protein ml^{-1} Figure 5).

Methods S1. Bioassays: LC$_{50}$ data analysis.