Research Article

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Increased toxicity of *Bacillus thuringiensis* Cry3Aa against *Crioceris quatuordecim-punctata*, *Phaedon brassicae* and *Colaphellus bowringi* by a *Tenebrio molitor* cadherin fragment

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Abstract

BACKGROUND: Biopesticides containing Cry insecticidal proteins from the bacterium *Bacillus thuringiensis* (*Bt*) are effective against many lepidopteran pests, but there is a lack of *Bt*-based pesticides for efficient control of important coleopteran pests. Based on the reported increase in *Bt* toxin oligomerization by a polypeptide from the Cry3Aa receptor cadherin in *Tenebrio molitor* (Coleoptera: Tenebrionidae), it was hypothesized that this cadherin peptide, rTmCad1p, would enhance Cry3Aa toxicity towards coleopteran larvae. To test this hypothesis, the relative toxicity of Cry3Aa, with or without rTmCad1p, against damaging chrysomelid vegetable pests of China was evaluated.

RESULTS: Cry3Aa toxicity was evaluated in the spotted asparagus beetle (*Crioceris quatuordecimpunctata*), cabbage leaf beetle (*Colaphellus bowringi*) and daikon leaf beetle (*Phaedon brassicae*). To assess the effect of rTmCad1p on Cry3Aa toxicity, neonate larvae were fed Cry3Aa toxin alone or in combination with increasing amounts of rTmCad1p. The data demonstrated that Cry3Aa toxicity was significantly increased in all three vegetable pests, resulting in as much as a 15.3-fold increase in larval mortality.

CONCLUSION: The application of rTmCad1p to enhance Cry3Aa insecticidal activity has potential for use in increasing range and activity levels against coleopteran pests displaying low susceptibility to *Bt*-based biopesticides.
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Keywords: *Bacillus thuringiensis*; Cry toxin; *Bt* synergist; cadherin; Coleoptera

1 INTRODUCTION

The spore-forming gram-positive bacterium *Bacillus thuringiensis* Berliner (*Bt*) is an environmentally safe biological insecticide.[1] During sporulation, *Bt* produces one or more crystal (Cry) proteins, which are stored as insoluble crystalline inclusions. *Bt*-based pesticides have been used worldwide for more than 60 years to control agricultural and forestry pests, and account for more than 90% of all biopesticides.[2] *Bt* toxins are also expressed in transgenic *Bt* crops to provide pest control with reduced chemical pesticide applications.[3–5]

In China, the production and application of *Bt* has developed quickly in the past 20 years, with the use of *Bt* formulations increasing from less than 0.3 million kg in 1985 to 30 million kg in 2005.[6] At present, the application of *Bt* has been extended to the production of vegetables. However, almost all *Bt* commercial formulations used in China have been based on *Bt kurstaki*, which contains five crystal proteins, Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry2B.[7] These *Bt* toxins are effective against lepidopteran pests on important cruciferous vegetable crops such as cabbage and cauliflower,[6] but are not effective in controlling coleopteran pests. In comparison, commercial Bt-based pesticide products targeting coleopteran pests are based on Bt san diego or tenebrionis expressing the Cry3Aa toxin.

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The mode of action of *Bt* insecticidal Cry toxins has been extensively studied in lepidopteran larvae.[8] A generally accepted mode of action for Cry toxins describes the sequential steps of protoxin activation, specific binding and cell toxicity.[9] Both the required activation and, more importantly, binding steps confer remarkable pest specificity to Cry proteins.[10] Ingested insecticidal crystal proteins are activated to a toxic form by proteinases from the digestive insect gut fluids. After crossing the peritrophic matrix, activated toxins bind to specific receptor proteins on the midgut microvilli. In lepidopterans, several insect midgut proteins have been proposed as Cry toxin receptors. Specifically, Cry1A receptor functionality has been demonstrated for cadherin proteins from *Bombyx mori*,[11] *Manduca sexta*,[12,13] *Ostrinia nubilalis*[14] and *Heliothis virescens*.[15] Alterations of toxin-binding motifs within lepidopteran cadherin genes are genetically linked to Cry1Ac resistance in *H. virescens*,[16,17] *Helicoverpa armigera*[18] and *Pectinophora gossypiella*.[19,20] More recently, Chen *et al*.[21] identified a putative cadherin *Bt* receptor in *Aedes aegypti*, and Fabrick *et al*.[22] identified the first functional Cry toxin receptor cadherin from a coleopteran insect, *Tenebrio molitor* (Coleoptera: Tenebrionidae), suggesting similarities in the mode of action of Cry toxins across insect taxonomic orders.

According to the model proposed by Bravo *et al*.,[23] Cry toxin binding to cadherin facilitates further proteolytic processing necessary for toxin oligomerization. Toxin oligomers display high affinity for aminopeptidase or alkaline phosphatase, secondary receptor proteins attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor.[23] Binding to these secondary receptors concentrates toxin oligomers in specific membrane regions called lipid rafts, where toxin oligomers insert and form pores that result in cell death by osmotic shock.[24] An alternative model suggests that Cry toxin binding to cadherin receptors activates intracellular pathways leading to cell death.[13] Notably, in both models, cadherin is a critical contact point for Cry toxins that is pivotal for intoxication.

In agreement with the crucial role of cadherin for Cry intoxication, some cadherin peptides fed with Cry toxins evidently compete for toxin binding and cause a reduction in toxicity in *H. armigera*[25] and *M. sexta*.[26] In contrast, different cadherin peptides have been demonstrated to enhance Cry toxicity against several insect pests. For example, a fragment of the BtR cadherin (CR12-MPED) from *M. sexta*, corresponding to cadherin ectodomain 12 and containing a critical toxin-binding region,[12] enhanced the activity of Cry1A toxins by promoting toxin oligomerization.[27] Another peptide containing the putative toxin-binding site of an *Anopheles gambiae* cadherin receptor for Cry4Ba potentiated Cry4Ba toxicity in *A. gambiae* larvae.[28] Similar Cry toxicity enhancement effects were discovered for fragments of cadherin from *H. armigera*[29] and *Diabrotica virgifera virgifera*.[30]

A protein fragment containing the predicted toxin-binding region from *T. molitor* cadherin, rTmCad1p, was demonstrated to bind Cry3Aa toxin specifically and promote Cry3Aa toxin oligomerization.[22] This cadherin fragment also increased Cry3Aa toxicity in *T. molitor* larvae.[31] To evaluate whether rTmCad1p can potentiate Cry3Aa toxicity in field pests, heterologously expressed rTmCad1p was used with Cry3Aa in bioassays of relevant coleopteran pests of vegetables in China, including the spotted asparagus beetle (SAB), *Crioceris quatuordecimpunctata*, the cabbage leaf beetle (CLB), *Colaphellus bowringi*, and the daikon leaf beetle (DLB), *Phaedon brassicae*.[32–34] The present data demonstrate that rTmCad1p can be used significantly to enhance Cry3Aa toxicity against several coleopteran field pests that currently have limited available control options. More importantly, the use of rTmCad1p as Cry3Aa enhancer may reduce the amount of toxin needed for effective control, lengthen residual activity and delay the onset of resistance in target insects.

2 MATERIALS AND METHODS

2.1 Source of insects

Adult SAB were obtained from the Changping Asparagus Experimental Station (Beijing). An SAB colony was maintained on asparagus plants within a controlled environmental chamber. Eggs of CLB and DLB were kindly provided by Jiangxi Academy of Agricultural Sciences (Jiangxi, China) and Huazhong Agricultural University (Wuhan, China) respectively. Eggs were incubated at $26 \pm 2\,^{\circ}$C, and newly hatched larvae were used for bioassays. All insects were maintained at $26 \pm 2\,^{\circ}$C and 70% relative humidity with a 14 : 10 h light : dark photoperiod.

2.2 Preparation of *Bt* Cry3Aa toxin

Using PCR, 20 *B. thuringiensis* strains from laboratory stocks were screened with primers targeting the *cry3Aa* gene (accession number AY728 479): *BamHI*-F (5′-GGA TCC atg ata aga aag gga gga aga-3′) and *SacI*-R (5′-GAG CTC tta att cac tgg aat aaa ttc a-3′). Using the *BamHI* and *SacI* sites located at the 5′ and 3′ ends of the amplicons, the amplified *cry3Aa* gene was cloned into the pET-28a (Novagen, San Diego, CA) expression vector. To generate adequate *BamHI* and *SacI* sites for cloning the *cry3Aa* gene, the pET28a vector was excised with *XbaI* and *BamHI* and ligated with a linker produced by annealing two oligonucleotides (oligo1: cta gaa ata att ttg ttt aac ttt aag aag gag, and oligo2: gat cct cct tct taa agt taa aca aaa tta ttt). The ligated plasmid was transformed into *E. coli.* BL21 (DE3), and recombinant vectors were verified by sequencing. Selected clones harboring the *cry3Aa* gene were inoculated in LB (Luria-Bertani) medium containing 50 μg μL^{-1} kanamycin and incubated with vigorous shaking (∼250 rpm) at 37 °C. When the OD_{600} was between 0.6 and 1, IPTG was added to a final concentration of 1 mM, and the culture was incubated for three additional hours. Cells were harvested by centrifugation (12 000 g for 1 min) and sonicated in ice-cold 1 M NaCl, 5 mM EDTA and 1% Triton X-100. Cell lysates were centrifuged (12 000 rpm for 10 min), and the precipitate was resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The Cry3Aa protoxin was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by densitometry using a GS-800 calibrated densitometer and Quantity One image analysis software from Bio-Rad Laboratories (Hercules, CA) and bovine serum albumin standards ranging from 0.1 to 2.0 μg.

2.3 Preparation of *T. molitor* rTmCad1p peptide

A 582 bp region from the *TmCad1* cDNA (nucleotides 3963 to 4548), subcloned into pET151-D-TOPO® expression vector (Invitrogen, Carlsbad, CA), was used to generate the pET151-rTmCad1p construct.[22] Transformed ArcticExpress™ (DE3) RP *E. coli* (Stratagene, La Jolla, CA) cells were used for large-batch (20 L) production of the peptide (GenScript Corporation, Piscataway, NJ). The protein was expressed as inclusion bodies and was solubilized and purified by affinity chromatography under hybrid conditions, as previously described.[22] The approximate 30 kDa purified peptide was dialyzed against peptide buffer (10 mM Tris/HCl, pH 8.0, 0.01% Triton-X-100) and concentration evaluated by 10% SDS-PAGE and densitometry comparisons with known BSA standards. Peptide was lyophilized for storage at −80 °C.

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2.4 Insect bioassays

Each bioassay was conducted with 20 neonate larvae per replicate and three replicates per concentration. Larval mortality was determined after 96 h of exposure; larvae were considered dead if they did not respond to contact. All bioassays were performed at 26 ± 2 °C, 70% relative humidity and a 14 : 10 h light : dark photoperiod. Controls included larvae exposed to buffer (PBS, pH 7.4) or rTmCad1p alone.

Assays for neonate SAB larvae were conducted on fresh 5–8 cm long sections of asparagus spears, using a spear dipping method.[35] Briefly, the plant section was dipped for 10 s in the solution, held vertically to allow excess solution to drip off, placed in a drying rack in a fume exhaust hood to air dry for 1 h and then placed into a 9 cm diameter plastic petri dish containing a moistened cotton ball.

Assays for neonate CLB and DLB larvae were conducted on fresh leaf disks of Chinese cabbage using a modified leaf method.[34] Briefly, cabbage leaves were rinsed with water, air dried and cut into the appropriate size (6 cm diameter circle). The pieces were then completely submersed into different concentrations of Cry3Aa toxin for 10 s, air dried and placed onto a 9 cm diameter disk and cushioned with a wet filter to maintain humidity.

To test the effect of rTmCad1p on Cry3Aa toxicity towards SAB, CLB and DLB larvae, neonate larvae of each species were exposed to a single Cry3Aa concentration alone (1.5, 0.2 and 0.5 μg mL^{-1} for SAB, CLB and DLB respectively) or in combination with increasing mass ratios of Cry3Aa : rTmCad1p, including 1 : 1, 1 : 5 and 1 : 50. From these assays, the optimum Cry3Aa : rTmCad1p ratio for Cry3Aa toxicity enhancement was determined to be 1 : 5 for all species. This ratio was used in bioassays to quantify the effect of rTmCad1p on the 50% lethal concentration (LC_{50}) for each species.

2.5 Data analysis

Mortality values were corrected for background mortality using Abbott's formula,[36] and LC_{50} values were calculated by EPA probit analysis using POLO-PC (LeOra Software, Berkeley, CA).[37] The enhancement factor, defined as the ratio of the lethal concentration of Cry3Aa alone to the lethal concentration of Cry3Aa mixed with cadherin peptide, was determined for each of the combinations. Non-overlapping 95% confidence intervals were used to establish significance. Pairwise chi-square analysis was performed to analyze the effect of the enhancer in the dose–response bioassay using log transformed dose data. For bioassays comparing a fixed concentration of toxin with different ratios of rTmCad1p, pairwise chi-square analysis was performed, and mortalities between treatments were considered significantly different if $P < 0.05$.

3 RESULTS

3.1 rTmCad1p enhances Cry3Aa toxicity to SAB, CLB and DLB larvae

To evaluate the Cry3Aa potentiating activity of rTmCad1p, increasing amounts of rTmCad1p were combined with a Cry3Aa toxin concentration causing 10–20% larval mortality (depending on species). As shown in Fig. 1A, rTmCad1p significantly enhanced Cry3Aa toxicity against SAB larvae at low toxin : peptide ratios. Specifically, 1 : 1 and 1 : 5 mass ratios resulted in 80% mortality, as opposed to 16% mortality with Cry3Aa alone. Mortality from exposure of SAB larvae to rTmCad1p peptide alone was not significantly different from controls. Similarly, rTmCad1p also increased Cry3Aa toxicity against CLB larvae (Fig. 1B). In this case, the 1 : 5 Cry3Aa : rTmCad1p ratio resulted in the highest mortality (62%), which was significantly greater than with Cry3Aa alone (8%). Even though Cry3Aa toxin caused relatively higher mortality for DLB larvae (more than 20%) compared with other species, a 1 : 5 toxin : peptide ratio significantly increased toxicity to almost 80%. These data indicated that rTmCad1p significantly enhanced Cry3Aa toxicity against larvae of SAB, CLB and DLB. Based on the levels of enhancement observed, 1 : 5 was selected as the optimum mass ratio of Cry3Aa : rTmCad1p for further testing.

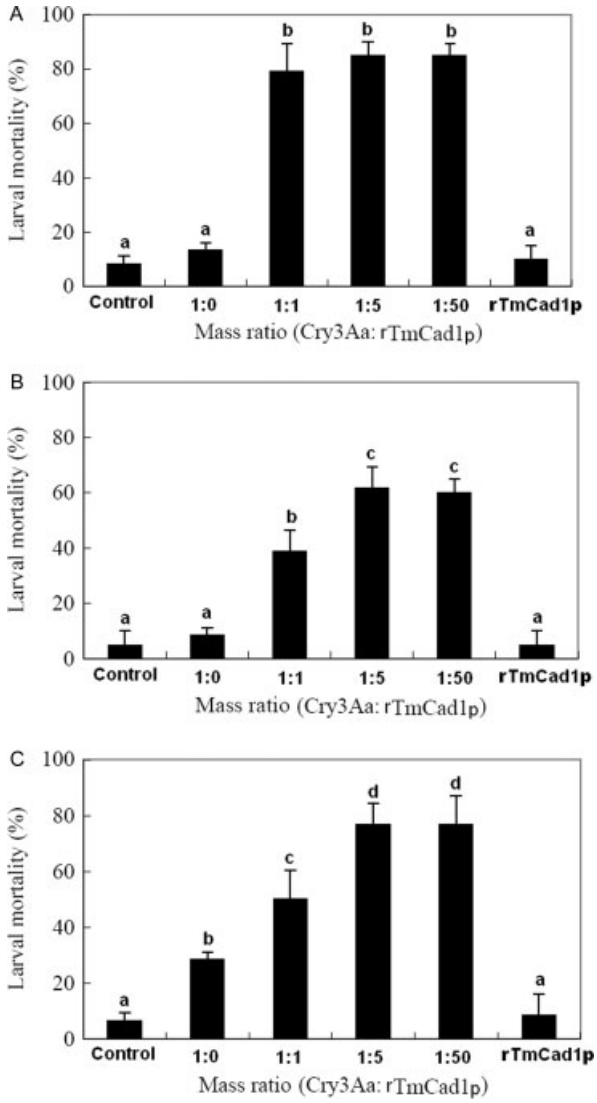

Figure 1. rTmCad1p enhances Cry3Aa toxicity against neonate larvae of SAB (A), CLB (B) and DLB (C). In panels A, B and C, the Cry3Aa concentrations were 1.5, 0.2 and 0.5 μg mL^{-1} respectively. In all panels, PBS buffer control and 10 μg mL^{-1} rTmCad1p were not toxic. Mortality was scored after 96 h of exposure. Different letters above the error bars indicate significant differences between means (chi-square analysis, $P < 0.05$).

3.2 Estimation of Cry3Aa toxicity enhancement by rTmCad1p

In order to accurately quantify the specific Cry3Aa toxicity enhancement effect of rTmCad1p in SAB, CLB and DLB larvae, bioassays were performed to calculate the Cry3Aa LC_{50} values

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Table 1. Effect of rTmCad1p on Cry3Aa toxicity to neonate SAB, CLB and DLB larvae

Treatment	Insect	LC_{50} (95% CL)[a] (μg mL^{-1})	Slope (± SE)	Enhancement factor[b]
Cry3Aa	SAB	3.82 (3.07–4.62)	2.44 (±0.28)	
Cry3Aa + rTmCad1p[c]	SAB	0.25 (0.19–0.31)	2.39 (±0.29)	15.3
Cry3Aa	CLB	1.11 (0.61–1.59)	2.96 (±0.39)	
Cry3Aa + rTmCad1p	CLB	0.14 (0.08–0.20)	2.98 (±0.39)	7.9
Cry3Aa	DLB	1.33 (0.73–1.90)	2.96 (±0.38)	
Cry3Aa + rTmCad1p	DLB	0.31 (0.23–0.38)	2.84 (±0.42)	4.3

[a] LC_{50}, concentration causing 50% mortality; 95% CL, 95% confidence limits.
[b] Enhancement factor = LC_{50} with Cry3Aa alone/LC_{50} with Cry3Aa and rTmCad1p.
[c] A 1 : 5 mass ratio of Cry3Aa toxin : rTmCad1p peptide was used for all Cry3Aa concentrations tested against all three insect species ($n = 60$ larvae per concentration).

for each species (Table 1). CLB and DLB larvae were most susceptible to Cry3Aa, with LC_{50} values of 1.11 and 1.33 μg mL^{-1} respectively (Fig. 2). In comparison, SAB larvae were slightly less susceptible, with an estimated LC_{50} of 3.82 μg mL^{-1}. When Cry3Aa was combined with fivefold mass excess of rTmCad1p, the dose–response curves shifted to those of the lowest Cry3Aa doses (Fig. 2), providing further evidence for the enhancement of Cry3Aa toxicity by rTmCad1p. When comparing the LC_{50} values for Cry3Aa toxin alone or in combination with rTmCad1p, the highest toxicity enhancement was detected for SAB larvae (more than 15-fold), while lower but significant effects were detected for CLB and DLB larvae (7.9- and 4.3-fold respectively).

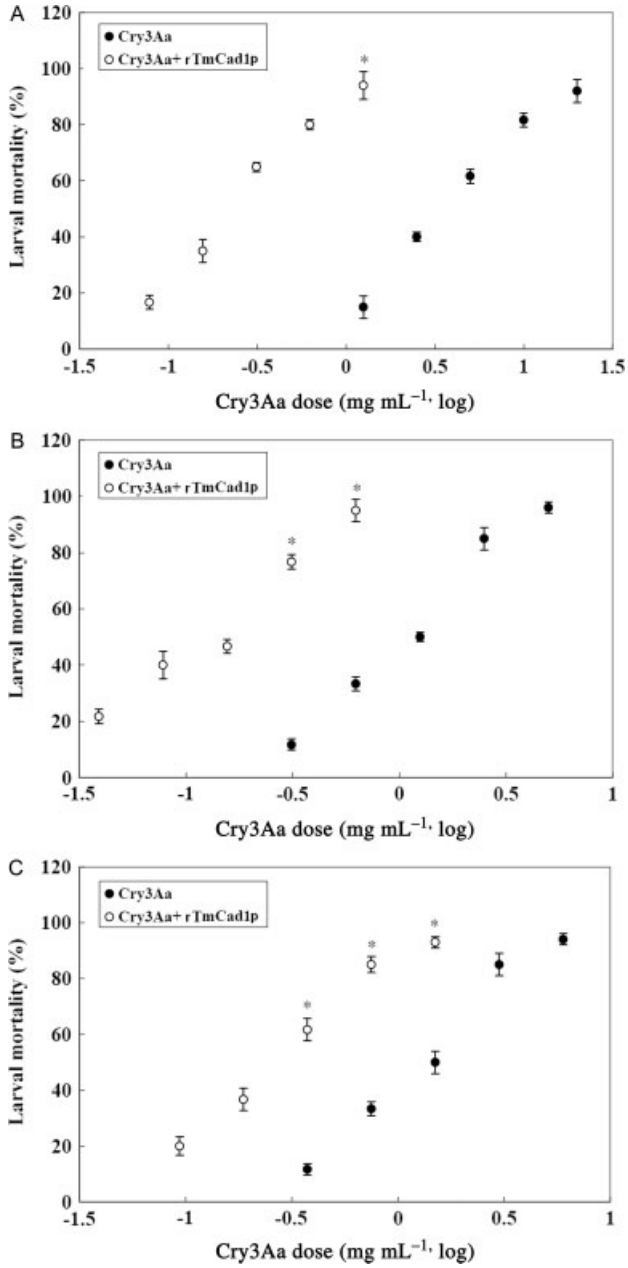

Figure 2. Determination of the Cry3Aa toxicity enhancement by rTmCad1p in neonate larvae of SAB (A), CLB (B) and DLB (C). Suspensions of Cry3Aa toxin with or without fivefold molar excess rTmCad1p were fed to neonate larvae in a spear/leaf dip bioassay. Mortality was scored after 96 h of exposure. Each data point represents the mean ± standard error of the results from a bioassay with 60 larvae per concentration. In panels, an asterisk denotes a significant difference (chi-square analysis, $P < 0.05$) between larval mortality with Cry3Aa treatment only and with Cry3Aa plus rTmCad1p for overlapping toxin doses.

4 DISCUSSION

The present data are the first report on the enhancement of Cry3Aa toxicity against chrysomelid larvae by a peptide from *T. molitor* TmCad1 cadherin containing at least one Cry3Aa binding site. This finding supports recent reports demonstrating Cry toxicity enhancement by polypeptide fragments from host insect cadherins. Furthermore, an optimum molar ratio of toxin to peptide was identified that results in the highest levels of Cry3Aa toxicity enhancement against three important species of beetle vegetable pests.

Several peptides based on putative cadherin receptors have been reported to enhance Cry toxicity against lepidopteran,[29,38] dipteran[28,30] and coleopteran[30] larvae. Even though a polypeptide based on a cadherin from *D. virgifera* was reported to enhance toxicity of Cry3 toxins towards coleopteran pests,[30] this cadherin has not yet been demonstrated to represent a functional Cry3 receptor. In contrast, the protein fragment used in this work is based on a functional Cry3Aa receptor.[22] Furthermore, increased Cry3Aa oligomerization in the presence of rTmCad1p, as reported in Fabrick *et al.*,[22] may be involved in the observed Cry3Aa toxicity enhancement, as this mechanism also has been proposed for the enhancement of Cry1 toxins in Lepidoptera by *M. sexta* cadherin peptides.[39] Previous studies have demonstrated enhancement of Cry toxins by rTmCad1p against coleopteran and lepidopteran larvae and increase in toxicity to a *Bt*-resistant lepidopteran pest have been observed.[31] Therefore, the peptide shows potential for development into a new and important tool for pest management in agriculture.

Cry toxin oligomerization was reported as a critical factor for Cry toxicity.[40] However, the correlation between toxin enhancement and toxin oligomerization has been inconsistent, as a toxin-binding cadherin fragment that induced Cry1Ac oligomerization was demonstrated to reduce toxicity in *H. armigera*.[41] An alternative explanation for the observed Cry toxicity enhancement is that

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cadherin peptides may bind to the microvillae on midgut cells and attract Cry toxin molecules, increasing the probability of toxin interaction with receptors.[27] This hypothesis is consistent with a recently proposed model, whereby low-affinity binding of Cry toxin monomers to binding sites on the brush border epithelium is crucial for toxicity.[42] In the case of rTmCad1p, increased oligomerization was observed in the presence of *T. molitor* BBMV in solution, but *in vivo* events remain to be determined.[22] Although susceptibility to Cry3Aa was not correlated to the fold levels of enhancement, the LC_{50} values for Cry3Aa with rTmCad1p were similar for all three tested species. This observation suggests that differences in cadherin binding may explain diverse susceptibility to Cry3Aa in SAB compared with CLB and DLB. Further research is needed to determine the specific mechanism responsible for Cry3Aa toxicity enhancement by rTmCad1p.

Considering that coleopterans are some of the most damaging pests of many agricultural and forestry crops, there is an urgent need to develop effective biopesticides against these insects. Enhancers of *Bt* toxicity represent an opportunity to improve currently available commercial products into more effective control agents against diverse pests. The present work demonstrates the potential for use of a cadherin-based peptide as an enhancer for Cry3Aa-based products in controlling chrysomelid pests.

5 CONCLUSIONS

The present data support the use of rTmCad1p in combination with Cry3Aa toxin for control of coleopteran vegetable pests. The observation that rTmCad1p can reduce the amount of Cry3Aa required for efficacy suggests that selection pressure by Cry3Aa will be reduced and correlates with the reduction in Cry1Ac resistance in *Pectinophora gossypiella* by rTmCad1p.[31] This discovery provides a novel strategy to enhance insecticidal activity and delay insect resistance in coleopteran pests to *Bt*-toxin-based biopesticides or transgenic crops.

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