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Modification of the Bt toxin Cry4Aa for improved toxin processing in the gut of the pea aphid (Acyrthosiphon pisum)

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CHAPTER 1

INTRODUCTION

Numerous herbivorous arthropods, particularly insects, are capable of causing extensive damage to host plants. Since the adoption of agriculture, decreasing yield loss associated with insects has been an ongoing, ever-present, challenge to humanity's food production. Over time numerous tools have been utilized to manage insect pest populations with differing degrees of success. Historically older methods include the use of nicotine from tobacco plants and other more toxic compounds such as arsenic. These first generation insecticides have been mostly abandoned due to ineffectiveness or excessive toxicity [1,2]. The more recent (1930s) second generation insecticides include many synthetically synthesized organic compounds, such as dichlorodiphenyltrichloroethane (DDT). After their widespread and extensive use it was discovered that some of these compounds have drastic negative environmental impacts and that insect populations are capable of evolving resistance [3,4]. Relatively recently crops have been developed that express microbial toxins derived from the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) [5]. This approach has allowed for the successful management of multiple insect species while decreasing the use of environmentally harmful chemical insecticides, resulting in increased crop yield, preservation of beneficial species, decreased CO_2 emissions (from decreased use of farm machinery to apply insecticides) and decreased human toxicity [5-11].

However, despite the widespread adoption and numerous benefits of Bt crops there is concern that insect populations will develop resistance [12]. In addition, some insect groups, such as the Hemiptera, are significantly less susceptible to these toxins than other insects [13]. Insect resistance management (IRM) is currently receiving great attention to ensure the long term

use of Bt toxins against susceptible pests. A growing field of study is the molecular modification of characterized Bt toxins, to create "designer" toxins, to extend their usage to less susceptible insect groups as well as combat resistance [75].

Insect-Resistant Transgenic Crops Expressing Cry Toxins

It has long been known that the gram-positive entomopathogenic bacterium *Bacillus thuringiensis* produces multiple toxins encoded on large plasmids [14]. *B. thuringiensis* has historically been classified as a soil-dwelling bacterium. The discovery of multiple virulence factors suggests however that *B. thuringiensis* is a specialized insect pathogen that produces toxins to exploit the insect host [15]. Numerous toxins have been discovered in multiple strains of *B. thuringiensis* with efficacy against specific groups of insects [16].

The high degree of specificity and high lethality of Bt toxins make them ideal for incorporation into transgenic plants. This process involves isolation of the toxin gene and incorporation of the gene into the plant genome. This plant transformation can be achieved by either *Agrobacterium tumefaciens*, a bacterium that naturally incorporates foreign genes into the host plant genome, or by a gene gun where plant cells are shot with gold particles coated with DNA. Genetically modified plants expressing these genes confer resistance against specific herbivorous insects [17,18].

The benefit of Bt crops extends to the preservation of beneficial insect species, such as predators and pollinators. This is due to the fact that 1) Bt crops require ingestion of plant tissue in order to be toxic and 2) particular Cry toxins affect specific groups of insects [16]. Therefore non-pest herbivores and predatory insect species are conserved in the presence of transgenic

crops because they do not feed upon the transgenic plant tissue, and the toxin does not affect them.

As with traditional insecticides, Bt toxins expressed in plant tissue represent a strong selection force on insect populations, favoring the development of resistance [19]. Several strains of Bt-resistant insects have been developed in the laboratory, and the extensive use of transgenic plants in the field over several years has led to resistance developing in some insect populations [12, 20-25]. Several management strategies are currently in effect to help preserve the long term use of plants expressing Cry toxins and delay insect-resistance evolution, such as the high-dose refuge strategy (HDR) and pyramiding.

HDR requires a continuous high dose of toxin expressed in plant tissue (LC 99%) and a refuge of non-transgenic plants to allow for survival of some susceptible individuals [19]. The high dose of toxin is expected to make resistance to Bt functionally recessive, with only resistant homozygous individuals surviving on transgenic plants. Any resistant heterozygous progeny produced from mating of the numerous susceptible and rare resistant individuals will be susceptible to the transgenic crops due to high dose, keeping resistant allele frequency low [19,26].

The pyramiding strategy incorporates multiple insect-resistance factors, such as two Cry toxins (such as Cry1Ac and Cry2Ab expressed in cotton) [27]. This is expected to delay resistance because any mutations that confer resistance to one of the Cry toxins would not result in resistance to the other toxin provided the two toxins have different modes of action. The probability of an individual containing a mutation that confers resistance to two unique modes of action should be rare [28-30].

Despite these management practices designed to prolong the practical use of Cry toxins, the evolution to insect resistance is a perpetual concern [12]. At best, HDR strategies ultimately delay as opposed to prevent resistance (as resistant homozygous individuals survive in the refuge), and pyramided transgenic crops can still result in resistance [12]. Discovery and classification of novel Cry toxins as well as understanding the mode of action in insect toxicity is crucial for the continued use of Cry toxins in agriculture.

Cry Toxin Mode of Action

The current list of known *cry* genes is vast, with over 700 cataloged sequences thus far. Cry toxins are classified based on amino acid sequence similarity, which is reflected in the nomenclature. Toxins separated by a different number share up to 40% amino acid sequence similarity, separation by a different upper case letter indicates up to70% similarity, and separation by a lower case letter indicates up to 95% similarity. Hence Cry1Aa and Cry1Ab share between 70% and 95% similarity. This nomenclature is also useful in that it reflects which insect groups will be affected, with more similar toxins affecting a similar range of insects than dissimilar ones. Among the Cry toxins produced by *B. thuringiensis*, the largest group contains three distinct structural domains and are called three-domain (3D) Cry toxins [31,32] (Fig 1).

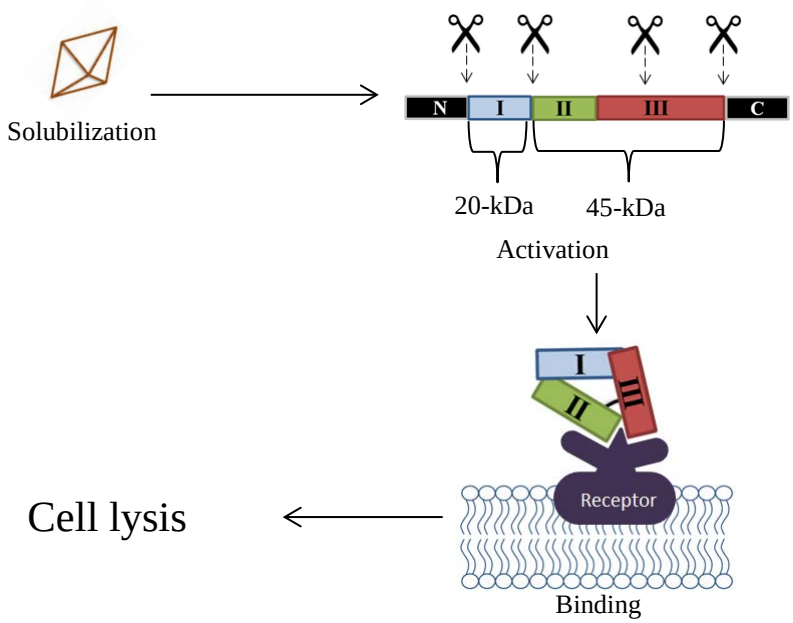

Fig 1. Schematic representation of the major steps involved in Cry toxicity. Modified from Deist et al. [75].

The remainder of this section has been published in a review paper by Deist B, Rausch M, Fernandez-Luna M, Adang M, and Bonning B [75].

The conserved structure of 3D Cry toxins, as well as results from a considerable amount of research, supports a conserved mode of Cry toxin action [32,33]. The first impact of Cry toxins on the insect is cessation of feeding due to paralysis of the gut and mouthparts [34]. In addition to gut paralysis, midgut cells swell, leading to an ion imbalance and death [34]. The molecular events leading to Cry toxin-mediated insect death are controversial [8], but the accepted initial steps are as follows: The Bt Cry . . . proteins require solubilization in the insect midgut to produce protoxins that are typically about 130 -kDa, 70 -kDa. . . . These in turn are proteolytically cleaved at the *C*-terminus and/or at the *N*-terminus by midgut proteases, generating the activated core toxin. The toxin then crosses the peritrophic matrix and binds to receptors in the apical membrane of the midgut cells, with receptor binding being an important determinant of toxin specificity.

Toxin insertion into the epithelial membrane forms ion channels or pores, leading to lysis of the cells, damage to the midgut epithelial tissue, and death of the larva [33,35,36].

Based on their crystal structures, different Cry toxins (Cry1Aa, Cry1Ac, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Ba and Cry8Ea1 [37-39]) have similar folding patterns with three distinct domains [32,37]. Domain I is a seven to eight α-helix bundle comprised of amphipathic helices surrounding the central hydrophobic helix α-5. Domain I is involved in binding and in pore formation [32,37,40]. Domain II consists of three antiparallel β-sheets with exposed loop regions involved in interaction with receptors, while domain III is a β-sandwich of two antiparallel β-sheets involved in receptor binding and possibly membrane insertion.

The role of domain I in membrane insertion and pore formation in the midgut epithelium of the target insect was suggested by the presence of its long, hydrophobic and amphipathic helices [40]. The Umbrella Model proposed by those authors depicts the hydrophobic helical hairpin α4 and α5 as inserting into the membrane and initiating pore formation, while the rest of domain I flattens out on the membrane surface in an umbrella-like molten globule state. Mutant Cry proteins having altered amino acid residues on the putative surface residues of domain I and within the α-helices supported the role of domain I in membrane binding, insertion and pore formation [41].

Domain II is implicated in protein-receptor interactions through the surface-exposed loops at the apices of the three β-sheets. Due to their similarities to immunoglobulin antigen-binding sites, the loops of domain II were suggested to participate in receptor binding. Site-directed mutagenesis and segment swapping analyses provided support for this hypothesis [32,40]. The β-sandwich structure of domain III is also suggested to function in receptor binding; evidence for this role in toxin action comes from domain III swap experiments . . . , and in the case of Cry1Ac, the presence of a

"pocket" in domain III that binds *N*-acetyl galactosamine moieties on protein receptors [42,43]. Domain III is also implicated in maintaining the structural integrity of the toxin molecule by protecting it from proteolysis within the gut of the target organism [37,40].

Low Susceptibility of Hemiptera to Cry Toxins

Until recently herbivorous insects of the order Hemiptera have been considered only minor or secondary pests. With the adoption and widespread use of Bt crops suppressing the former primary pests, and the decreased use of traditional insecticides, hemipteran pests have risen to become a major concern for the agricultural industry [13]. These insects feed on plant phloem tissue through piercing or sucking mouth parts derived from mandibular and maxillary stylets. Phloem is mostly composed of plant products derived from photosynthesis, such as simple sugars. The Hemiptera can cause extensive yield loss on agricultural crops by ingesting phloem and depriving the plant of nutrients [44]. Aphids are currently recognized as major pests in diverse cropping systems [45,46].

Aphids are a major economically important pest of agricultural crops, with the soybean aphid (*Aphis glycines*) costing over $1.6 billion during a 10-year period [45]. In addition to direct yield losses through feeding on plant phloem tissue, aphids transmit numerous plant viruses [47] and excrete nutrient-rich honeydew which provides a medium for fungal growth [48]. The primary approach for controlling aphids relies on chemical insecticides that may have negative environmental consequences and to which aphids can readily develop resistance [44,49].

With the rise of these damaging insect pests the use of Bt crops with resistance against sap-sucking insects would be desirable. However, utilization of this technology for hemipteran

control is difficult due to the limited mortality of these insects when exposed to Cry toxins [13]. For example, some species of aphid experience over a 100-fold difference in toxicity when compared to susceptible insect species [50-52]. The only current exception to this trend is Cry51Aa2, a recently discovered toxin that impacts survival and development in the western tarnished plant bug (*Lygus hesperus*) [53]. Despite this exception the overall low effectiveness of Bt toxins indicates that discovery of natural toxins is an inefficient method to manage hemipteran pests.

Several factors are thought to contribute to the low toxicity observed against aphids, one of which is how *B. thuringiensis* interacts with insects under natural conditions. Spores and crystal toxins that reside in the soil disperse onto leaf surfaces via rain drops splashing toxin and spores onto plant surfaces [54,55]. Herbivorous insects ingest the spores/toxins and become infected while feeding on leaf tissue [15]. The feeding style of aphids effectively bypasses the spores and toxins by piercing into, rather than feeding directly on leaf surfaces. This lack of direct contact resulted in little selection pressure for Hemiptera-active *B. thuringiensis* toxins [32].

In addition the aphid gut environment may not be conducive to the generation of the active toxin from its protoxin form. Although the exact details of the Cry mode of action are unclear, it is generally recognized that the Cry toxin is ingested as a protoxin and becomes proteolytically cleaved into the active toxin [32]. The activated toxin subsequently binds to specific receptors on the insect gut epithelial cells. Conformational changes result in toxin penetration of the gut epithelial membrane to form a pore, followed by cell lysis through osmotic disruption [33]. The aphid gut is mildly acidic in the stomach and neutral in the midgut and hindgut [56]. The major proteases utilized are cysteine proteases of the cathepsin L and

cathepsin B type [57]. In contrast, the susceptible groups, such as Lepidoptera and Diptera, have an alkaline gut environment and utilize serine proteases [32,58], which readily solubilize and activate Cry toxins [59]. The combination of different gut pH and protease content suggests that the aphid gut is a poor environment for activation of Cry protoxins into their active form [60].

Cry Toxin Modification to Target Pests with Low Susceptibility

Current understanding of the Cry toxin mode of action, as well as aphid gut physiology, provides a model to explain the limited toxicity observed in aphids. Fortunately this knowledge can be utilized for the engineering of designer toxins to achieve proper Cry toxin activation and achieve toxicity [75]. This approach has already been demonstrated by modification of previous Cry toxins designed to enhance efficacy in less susceptible insects.

The following paragraph has been published in a review paper by Deist B, Rausch M, Fernandez-Luna M, Adang M, and Bonning B [75].

Walters *et al.* [61] demonstrated the feasibility of this approach by achieving toxicity with modified Cry3A (mCry3A) against the relatively non-susceptible western corn rootworm (*Diabrotica virgifera virgifera*). A chymotrypsin G cleavage site introduced between α-helices 3 and 4 of domain I was cleaved by western corn rootworm gut proteases (Table 1). The introduced cleavage site resulted in enhanced activity, and the activated mCry3A bound specifically to *D.v. virgifera* brush border membrane vesicles (BBMV) [61]. Insertion of protease-recognition sequences at appropriate sites should result in enhanced processing of the protoxin to its active form.

Furthermore, it has been suggested that the proteases of less susceptible insect species could be involved in degradation of Cry toxins. In addition to encoding cleavage sites Cry toxins

can be modified to remove potential sites that could be involved in degradation. Cry1Aa by Bah et al.[62] has been modified to resist degradation in spruce budworm (*Choristoneura fumiferana*) by removal of potential trypsin and chymotrypsin sites, resulting in a 2-4 fold increase in toxicity [62] (Table 1).

By exploiting the major proteases used in the aphid gut, Cry toxins can be modified with sequences encoding cleavage sites at the specific regions required to achieve toxicity. Selection of an ideal and thoroughly characterized Cry toxin for modification is required in order to achieve this goal.

Cry4Aa

Cry4Aa is part of the three-domain Cry toxins produced by *Bacillus thuringiensis* subsp. *israelensis* that is toxic to several mosquito species, and its crystal structure has been resolved [32,63]. Similar to other three-domain Cry toxins the N-terminal domain I contains several amphipathic alpha helices responsible for oligomerization of the active toxin monomers, membrane insertion, and pore formation in the insect gut [63-67]. Domain II is composed of three antiparallel beta sheets; variability of exposed loops has been shown to determine specificity for target insects [63,68-70]. C-terminal domain III is composed of two antiparallel beta sheets, undergoes folding to form a beta sandwich, and is believed to protect the activated toxin from undesirable proteolytic cleavage and may also determine insect specificity [42,63,70,71]. Cry4Aa is synthesized as a 130-kDa protoxin that is converted into protease-resistant 45 and 20-kDa fragments through a 60-65-kDa intermediate. The 45 and 20-kDa fragments are generated through the intramolecular cleavage between α-5 and α-6 helices and re-associate by electrostatic interactions, forming an active toxin monomer [63]. Yamagiwa et al.

[72] produced glutathione S-transferase (GST) fusions of both the 45 and 20-kDa fragments separately. Neither GST-45 nor GST-20 is toxic on its own when assayed against *Culex pipiens* larvae. However, the presence of both fragments results in insecticidal activity [72], indicating that both are needed for toxicity. *In silico* studies indicate that Cry4Aa activated monomers form a trimer with domain I helices α-2 and α-3 interacting with the helices α-4 and α-6 of an adjoining domain I to reduce non-polar interactions, and pore formation occurs via a 90° rotation of α-4/α-5 helices, which are more rigid than other loops in the trimer and contain numerous hydrophobic residues [73].

Feeding assays with Cry4Aa protoxin has resulted in low to moderate toxicity against pea aphids (*Acyrthosiphon pisum*) [74]. However, many Cry protoxins can be activated *in vitro* with commercial proteases. Protoxin activation prior to feeding has shown to achieve toxicity in less susceptible insect species, emphasizing the importance of proteolytic activation in Cry toxicity. Porcar et al.[74] demonstrated increased toxicity of Cry4Aa against pea aphid after incubation with trypsin prior to membrane feeding assays, suggesting proteolytic activation as a limiting step in Cry toxicity against aphids. These findings suggest that cases where solubility and activation limit toxicity have the potential to be rescued by proper treatment prior to feeding.

Research Problem

On the basis of the extensive characterization of Cry4Aa, its toxicity to the pea aphid when trypsin-activated, and our understanding of pea aphid gut physiology, we proposed to construct a modified Cry4Aa that will be processed by aphid proteases for rapid activation of the protoxin. Cathepsin L/B sites were introduced into Cry4Aa to facilitate activation at the site that facilitates removal of amino acids from the N terminus and at the site that separates the 45 and

20-kDa active toxin fragments. Such an aphid-active toxin has potential for use in the production of aphid resistant plants. This work capitalizes on the success of transgenic technology by broadening the host range of a Cry toxin.

Dissertation Organization

Chapter 2 describes the genetic modification of Cry4Aa for enhanced processing in the pea aphid gut. The key findings are summarized in Chapter 3 with general conclusions and discussion of research implications. Supplementary data are in Appendices 1, 2, 3, and 4 include complete nucleotide and peptide sequences of all modified Cry4Aa constructs, raw data from pea aphid feeding assays, summary of feeding assay data analysis, and the second replicate of Cry4Aa exposure to cathepsins in the aphid gut.

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CHAPTER 2
MODIFICATION OF THE BT TOXIN Cry4Aa FOR IMPROVED TOXIN PROCESSING IN THE GUT OF THE PEA APHID (*Acyrthosiphon pisum*)

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Abstract

Aphids are sap-sucking insects (order: Hemiptera) that cause extensive damage to a wide range of agricultural crops. Our goal is to produce a "designer" toxin based on naturally occurring insecticidal crystalline (Cry) toxins produced by the soil-dwelling bacterium *Bacillus thuringiensis* for use against the pea aphid, *Acyrthosiphon pisum*. On the basis that activation of the Cry4Aa protoxin could be a rate-limiting factor contributing to the relatively low aphicidal activity of this toxin, we introduced cathepsin L and cathepsin B cleavage sites into Cry4Aa for rapid activation and generation of the active toxin in the aphid gut environment. Incubation of modified Cry4Aa and aphid proteases *in vitro* demonstrated enhanced processing of protoxin into the active form relative to non-modified protoxin. Aphids fed artificial diet with protoxin at a final concentration of 125 µg/ml showed enhanced toxicity after two days for some of the modified constructs. This is the first study that has facilitated Cry protoxin activation to achieve toxicity against pea aphids. This work highlights the potential for the use of designer toxins for managing aphid populations via transgenic plant resistance.

Keywords: *Bacillus thuringiensis*, *Acyrthosiphon pisum*, Cry4Aa, toxicity, gut physiology

Introduction

Aphids can cause extensive economic losses to agricultural crops, with over $1.6 billion in costs attributed to the soybean aphid (*Aphis glycines*) [1]. Yield losses occur through direct feeding, transmission of numerous viruses [2] and from aphid excrement which provides a medium for fungal growth [3]. A current method for controlling aphids relies on chemical insecticides that have negative environmental consequences and which aphids can rapidly develop resistance [4,5].

Other major insect pests have been successfully controlled by the use of transgenic crops incorporating insecticidal crystal toxins (Cry) isolated from the bacterium *Bacillus thuringiensis* [6-11], resulting in increased yields, and decreased use of traditional insecticides [9-11]. However, incorporating this technology to include aphid control is difficult due limited mortality. Some aphids experience over 100-fold less toxicity when compared to susceptible insect species [12-14]. These differences can be partially attributed to aphid feeding style and *B. thuringiensis* infection in a natural setting. Bt toxins and spores are present in the soil and on leaf surfaces which can be ingested by leaf feeding insects. Aphids use piercing-sucking mouthparts to feed on plant phloem resulting in minimal interaction with the Bt toxin, suggesting that there has been no selection for toxicity against Hemiptera [15].

Cry toxins are initially ingested and solubilized as a protoxin and become activated by insect gut proteases[15]. The activated toxin subsequently binds to receptors on the insect gut epithelium. Toxin conformational changes result in penetration of the gut epithelial membrane by pore formation, followed by cell lysis through osmotic disruption. The Cry pore is formed by an oligomer of the activated toxin [16-18]. Insects experience gut paralysis, limited nutrient

uptake, and extensive damage to epithelium cells, ultimately resulting in death of the insect [19-21].

The gut content of aphids is mildly acidic in the stomach and neutral in the midgut and hindgut. The major gut proteases utilized by the aphid are cysteine proteases of the cathepsin L and cathepsin B type [22]; whereas the susceptible groups, such as Lepidoptera and Diptera, have an alkaline gut content and utilize serine proteases [15,23]. This environment is known to readily solubilize and activate Cry toxins, and suggests that the aphid gut is a poor environment for activation [24,25].

Activation of Cry4Aa prior to insect feeding results in increased activity against pea aphids (*Acyrthosiphon pisum*) [26], suggesting that protoxin activation may be a limiting step in Cry toxicity against aphids [26]. In addition it has been suggested that Cry toxins can be modified to achieve protoxin activation in the gut of the less susceptible insects. Previous research by Walters et al. [27] inserted a chymotrypsin G site between α-helices 3 and 4 of domain I of Cry3A, resulting in cleavage at this site by gut protease in the western corn rootworm (*Diabrotica virgifera virgifera*). Introduction of this cleavage site resulted in increased toxicity due to enhanced activation of the protoxin [27].

Cry4Aa derived from *Bacillus thuringiensis* subsp *israelensis* is a member of the three-domain Cry toxin family, for which the crystal structure has been resolved, and it is toxic to multiple mosquito species [15,28]. Domain I is involved in pore formation in the insect gut [28-32]. Domain II contains residues involved in receptor binding of target insects [28,33-35]. Domain III is also suspected in receptor binding as well as maintaining toxin stability [28,35-37]. Cry4Aa is synthesized as a 130-kDa protoxin that is converted into protease-resistant 45 and 20 -

kDa fragments through a 60-65-kDa intermediate. The 45 and 20-kDa fragments are generated through the intramolecular cleavage and re-associate by electrostatic interactions to form an active toxin monomer [28], hence both these fragments are required for toxicity [38]. A *in silico* study of the active toxin monomers indicates that three monomers associate by domain I to form a trimer, with several helices in domain I forming a pore [39].

In this study, we inserted cathepsin L and B cleavage sites into Cry4Aa to test the hypothesis that these sites will facilitate activation of the Cry4Aa protoxin in the aphid gut resulting in improved toxicity against the pea aphid. Activation of native and modified Cry4Aa protoxin was visualized after exposure to pea aphid proteases both *in vitro* and *in vitro.* In addition, feeding assays of native and modified protoxins were conducted with pea aphids to identify improvement in toxicity against pea aphids. The results of this study can be useful for the production of aphid resistant transgenic plants, providing a crucial management tool for damaging aphid populations.

Materials and Methods

Construction of Modified Cry4Aa-S1

The toxin gene *cry4Aa-S1,* which has been modified for optimal expression of the protein in *E. coli* [40], was used for modification to enhance cathepsin-mediated activation of Cry4Aa. Cry4Aa-S1 has a different genetic sequence but identical amino acid sequence to the 60-65 -kDa intermediate of Cry4Aa [40]. To introduce the cathepsin L and cathepsin B cleavage sites (FRR and FR, respectively) into the *cry4Aa-S1* gene, we used PCR to introduce the modified sequences and overlap extension polymerase chain reaction (OE-PCR) to splice DNA fragments together. For the construct Cry4Aa 2A, sequences encoding the three amino acids ‘FRR’ and two amino acids ‘FR’ were added at distinct sites in domain I of Cry4Aa-S1, at positions 67 and

235 respectively: the sequence introduced at position 235 was immediately upstream of an arginine codon, such that FRR was also encoded at this site (Fig 1). A second construct, Cry4Aa 2S with FRR and FR replacing (rather than adding to) amino acids in Cry4Aa (I65F, D66R, S67R, and N232F, N233R) was also made. The second modification site (location 235) is the region where the 45 and 20 -kDa fragments are separated in Cry4Aa, which is a crucial cleavage site for toxicity. Because of the importance of this site, two additional constructs (Cry4Aa 1A and Cry4Aa 1S) with added or substituted sequences encoding FR at this second site only were made. A total of four modified constructs of Cry4Aa were made (Table 1).

Primers designed to add or substitute cathepsin L/B sequences (Table 2) were incubated with the *cry4Aa-S1* gene in separate PCR reactions using *phusion Hot start II* DNA polymerase (Fermentas UAB, subsidiary of Thermo Fisher Scientific Inc.). Amplified products were visualized by agarose gel and ethidium bromide staining using standard protocols, and bands of expected product size were excised and purified using a QIAquick gel extraction kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's protocol. Purified DNA fragments were quantified by nanodrop (Nanodrop 2000c spectrophotometer, Thermo Scientific, Waltham, MA). Appropriate fragments to produce the addition and substitution *cry4Aa-S1* modified genes were incubated together with end primers (Table 2) encoding restriction sites for *Eco R1* and *Bam H1,* and *phusion Hot start II* DNA polymerase. Amplified products were excised and purified as described above, cloned into pGEX-2T using the restriction sites *Eco R1* and *Bam H1,* and transformed into BL21 Z competent *E. coli* cells. Native (non-modified) Cry4Aa-S1 was also cloned and transformed as described for the modified constructs. Positive clones for each modified construct were identified by colony PCR. The inserts in selected clones

were sequenced by the Iowa State University DNA Facility to confirm the sequence and frame of the modified *cry4Aa-S1* genes.

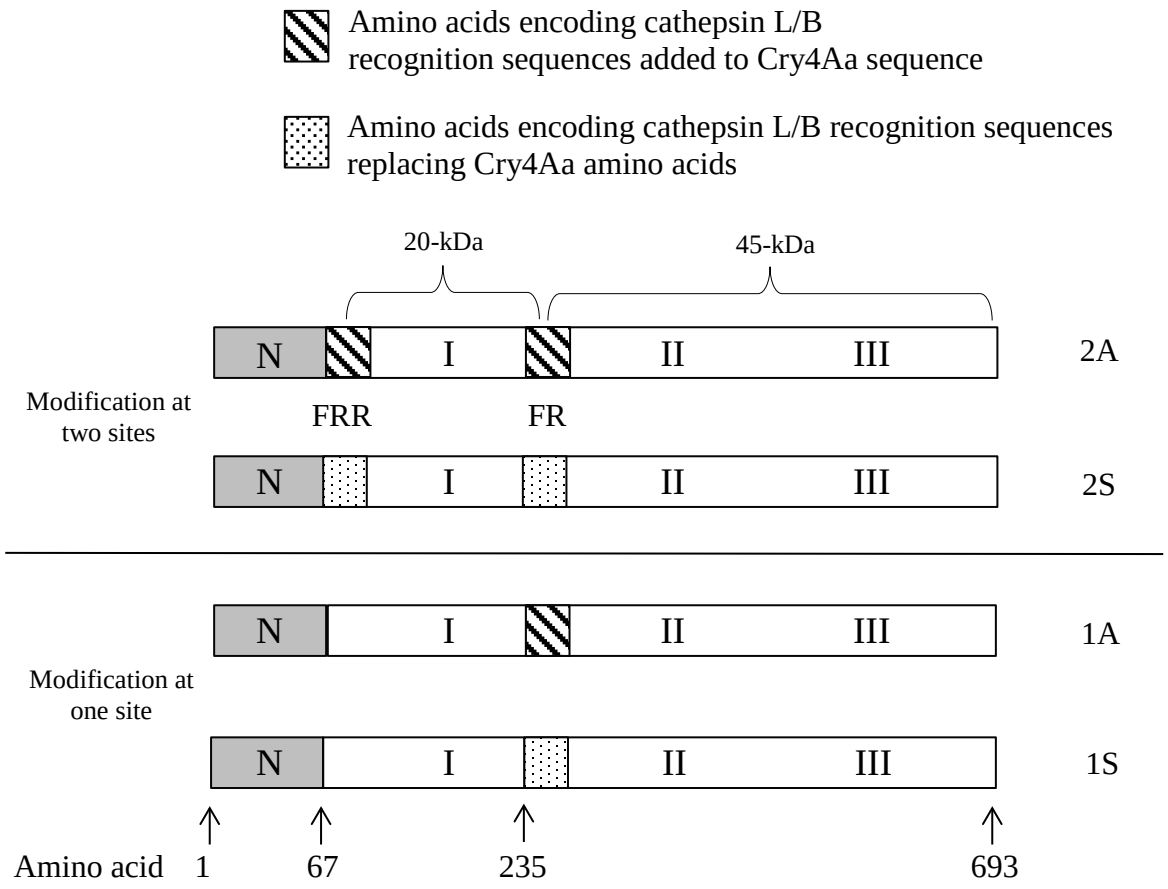


Fig 1. Engineering of Cry4Aa with cathepsin L and B cleavage sites. Amino acid sequences that are recognized by cathepsin L and B proteases (FR and RR respectively) were added to Cry4Aa or replaced existing amino acids, at two locations. Two additional Cry4Aa constructs were modified at the second region only. See Table 1 for details of the modified toxins produced.

Table 1. Modification of Cry4Aa with cathepsin L/B-specific cleavage sites.

Construct	Type	Modification
Cry4Aa 2A	Addition FRR and FR	Between S67 and G68; Between N237 and R238
Cry4Aa 2S	Substitution FRR and FR	Replaced I65F, D66R, S67R Replaced N233F, N234R
Cry4Aa 1A	Addition FR	Between N237 and R238
Cry4Aa 1S	Substitution FR	Replaced N233F, N234R

Table 2. Construction of modified Cry4Aa constructs. (A) primers used in OE-PCR reactions. Primers 0F and 5R contain *Bam H1* and *Eco R1* restriction sites respectively (grey highlights) for use in cloning. (B) OE-PCR reactions, with separate reactions indicated by [ ].

A

Primer number	Primer sequence
0F	5’ TAGGATCCATGAACCCGTACCAAAAC 3’
1F	5’ATGGATCCATGAACCCGTATCAAAATAAAAACGA 3’
2F	5’ATTCGAAACGTTCATCGATTCATTCCGTCGTGGTGAACTGTCGGCATACACCATC 3’
3F	5’TCGAAGCGTATCTGAAAAACAATTTCCGTCGTCAGTTCGACTATCTGGAAC 3’
4F	5’ACGGTGGTGACTTCGAAACGTTCTTCCGTCGTGGTGAACTGTCGGCATACACCATC 3’
5F	5’ TGAAATTCGAAGCGTATCTGAAATTCCGTCGTCAGTTCGACTATCTGGAAC 3’
1R	5’ TGAATCGATGAACGTTTCGAAGT 3’
2R	5’ ATTGTTTTTCAGATACGCTTCGA 3’
3R	5’ GAACGTTTCGAAGTCACCACCGT 3’
4R	5’ TTTCAGATACGCTTCGAATTTCA 3’
5R	5’ TAGAATTCTCACACGGTTTCCAGTTTTTG 3’

B

Construct	OE-PCR Reactions
Cry4Aa 2A	[0F + [1F+1R] [2F+2R] [3F+5R] + 5R]
Cry4Aa 2S	[0F + [1F+3R] [4F+4R] [5F+5R] + 5R]
Cry4Aa 1A	[0F + [1F+2R] [3F+5R] + 5R]
Cry4Aa 1S	[0F + [1F+4R] [5F+5R] + 5R]

Expression and purification of modified Cry4Aa

The expression vector pGEX-2T contains an ampicillin-resistance gene, a *lacI* promoter, and a glutathione S-transferase (GST) tag with a thrombin cleavage site. Expression of the *lacI* promoter by IPTG results in expression of GST-toxin fusion proteins. Cultures of each modified and native Cry4Aa/pGEX-2T *E. coli* were prepared in 10 ml Terrific Broth (12 g tryptone, 24 g yeast extract, 0.4% glycerol, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, $1L^{-1}$) with 50 µg/ml Carbenicillin, and grown overnight at 37 ºC with shaking at 250 rpm. These cultures were used to inoculate 1 L of Terrific Broth with Carbenicillin (50 µg/ml). Cultures were grown to an OD of 0.5. Expression of the GST-Cry4Aa-S1 fusions was induced with 0.06 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 20°C with shaking (220 rpm) for 5 hours. Culture flasks were covered in aluminum foil to prevent photodegradation of IPTG. Cultures were spun down at 1,560 *g*, at 4°C for 20 minutes and stored at -20°C. Cells were resuspended in 30 ml of PBS pH 7.3 with 300 µl of 100 mM phenylmethanesulfonylfluoride (PMSF) protease inhibitor. Expressed protein was released from cells by sonication (60 Sonic Dismembrator, Thermo Fischer Scientific International, Waltham, MA) at a setting of 6 for 1 min, with chilling on ice for 3 min. This procedure was repeated 5 times. Cell debris was removed by centrifugation at 23,210 *g* for 10 min at 4°C. A volume of 1 ml of Glutathione Sepharose ® (GSH) 4B beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), which binds to the GST-toxin fusion proteins, was added to the cell lysate and rocked gently for one hour at 4°C. GSH beads were separated from the cell lysate by centrifugation (1,560 *g*, 4°C, 5 minutes) and transferred to a column. Cry4Aa was liberated from the GSH beads with 50 units of thrombin and incubation at 4°C overnight. Five 500-µl fractions of purified Cry4Aa-S1 protoxin were collected and viewed for purification by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) alongside samples of the cell lysate, flow through, and a wash of the column following fraction

collection. Fractions showing intense bands of the correct size were pooled and concentrated by using Amicon Ultra-0.5 Centrifugal Filter Devices with a 3-kDa cut-off (Merck Milipore Ltd., Co Cork, IRL) according to the manufacturer's protocol. For aphid-feeding assays, purified protoxin buffer was exchanged with 10 mM Tris pH 7.5 using Amicon Ultra-0.5 Centrifugal Filter Devices (3-kDa) according to the manufacturer's protocol. Protein concentration was determined using a Bradford Assay with bovine serum albumin as a standard.

Pea aphid rearing

Pea aphids (*Acythosiphon pisum*) were maintained in an environmental chamber (L:D 24:0 h, temp 22°C, 70% RH) on fava bean (*Vicia faba*) plants (Peaceful Valley Farm and Garden Supply, Grass Valley, CA) grown in flower pots. Aphids were transferred to fresh plants by adding recently germinated plants and having aphid infested leaves touch the verdant leaves. Old plants were discarded 2-3 days later.

Processing of modified toxins by aphid gut cathepsins under *in vitro* conditions

Aphids were placed in a dissecting well containing 50 µl of 30 mM sodium acetate pH 6.0. Ten aphid guts were dissected in a 30-minute time period, pooled and snap frozen in liquid nitrogen. A total of 250 aphid guts were prepared in this way. Samples were thawed on ice and pooled. Gut tissue was homogenized using a pestle and centrifuged at 16,000 *g* for 25 minutes at 4°C. The supernatant was drawn off and concentrated using Amicon Ultra-0.5 Centrifugal Filter Devices (3-kDa) according to the manufacturer's protocol and labeled as the lumen fraction. The gut pellet was resuspended in 200 µl 30 mM sodium acetate pH 6.0 and labeled as the membrane fraction. The protein concentration of each fraction was determined by Bradford

Assay using bovine serum albumin as a standard. Both fractions were snap frozen in liquid nitrogen and stored at -80°C.

To examine the processing of the native and modified Cry4Aa-S1 protoxins under *in vitro* conditions, a 5:1 ratio (gut sample protein: protoxin, w/w) for the lumen and membrane gut fractions in a volume of 20µl was used. A total of 1 µg of gut lumen or membrane sample was used for each reaction. Cysteine protease activators ethylenediaminetetraacetic acid (EDTA) and cysteine were added to final concentrations of 3 mM to both the lumen and membrane reactions. Metal ions may bind to cysteine residues in the enzyme, negatively impacting enzymatic activity either directly, by binding to the active site cysteine, or indirectly by binding to other cysteine residues and inducing conformational changes. Chelation of metal ions by EDTA or provision of cysteine as an alternative metal ion binding substrate therefore results in cysteine protease activation. A separate set of lumen and membrane gut sample reactions were included in the absence of activators. Samples were incubated for 1 hour at room temperature for cysteine protease activation. A total of 200 ng of native or modified Cry4Aa-S1 protoxin was then added to each sample and incubated at room temperature while shaking at 250 rpm for 3 hours. Negative controls included modified protoxins that were incubated in the absence of gut proteins or activators. Reactions were stopped with 5X Laemmli buffer and heated to 100°C for 5 min. Western blot visualization of processed protoxin was conducted using polyclonal Cry4Aa-S1 antiserum (1:5000 dilution). This antiserum was raised in rabbits (New Zealand White) by injecting bacterially expressed Cry4Aa S1 (Iowa State University Hybridoma Facility). The secondary antibody was HRP-conjugated anti-rabbit IgG (1:5000). Immunoreactive bands were detected by incubating the nitrocellulose membrane in Hyglo Chemiluminescent HRP detection reagent for 1 minute and exposure to X-ray film using standard procedures. Solubilized native

and modified protoxins (200 ng) were used as negative control samples. Trypsin-activated native Cry4Aa-S1 (200 ng) activated with 5% trypsin (Sigma-Aldrich Co. LLC., St. Louis, MO) for 3 hours at 37°C was used as a positive control.

Aphid toxicity assays

Filter-sterilized 2X complete aphid artificial diet [41] was placed on Parafilm® stretched thinly across a 3-cm cell culture plate with a 1-cm hole and covered with a second layer of Parafilm®. This created a pocket of diet which aphids could feed upon by piercing the membrane with their stylets. The 2X complete aphid diet was diluted 1:1 with10 mM Tris pH 7.5. Native and modified Cry4Aa-S1 in 10 mM Tris pH 7.5 were mixed separately with complete aphid diet to a final concentration of 125 µg/ml. A volume of 100 µl of toxin/diet mixture was added to each feeding dish. A total of 15 second-instar pea aphids were transferred to each plate and incubated at 22°C. Aphid mortality was scored every 24 hours for 4 days. Complete aphid diet diluted to 1X with 10 mM Tris pH 7.5 was included as a negative control and complete diet with trypsin-activated Cry4Aa-S1 toxin (prepared as described above) was included as a positive control. Trypsin was removed following toxin activation using benzamidine sepharose beads (GE Healthcare Bio Sciences, AB, Sweden) according to the manufacturer's protocol prior to use in feeding assays. Three replicates were conducted for each control and treatment groups, except in the trypsin control, which included two replicates. A binomial comparison was used to analyze the feeding assay data in Excel 2010 (Microsoft, Redmond, WA). Calculated z-scores, representing the number of standard deviations above or below the mean were used to generate p-values for each comparison. Generated p-values from the binomial comparison analysis were used in a multiple comparison analysis. For multiple

comparisons a Bonferroni adjustment was used to calculate the revised threshold (0.05 threshold divided by 21 comparisons) of $p<0.002$ to indicate significant differences.

Processing of modified toxins by aphid gut cathepsins under *in vivo* conditions

Protocols for these experiments were based on Li et al. [25]. Native Cry4Aa-S1 and modified Cry4Aa-S1 protoxins showing increased aphicidal activity were fed to aphids at a concentration of 300 ng/µl in complete artificial diet along with blue food coloring (McCormick and Co. Inc, Hunt Valley, MD), with 2 µl food coloring per 100 µl of diet. Nine feeding dishes were set up for each treatment as described above with 20 third-larvae transferred to each plate and incubated at 22°C overnight. A total of 60 aphid guts showing blue coloration were excised in 20 µl 10 mM Tris pH 7.5 for the native- and modified- protoxin-fed groups, and snap frozen in liquid nitrogen. Aphid guts were thawed on ice, homogenized with a pestle and centrifuged at 16,000 *g* for 25 minutes at 4°C. Lumen and membrane fractions were isolated as described above. Western blot visualization of 60 aphid guts were conducted as described above.

Results

Synthesis and expression of modified Cry4Aa toxins

PCR amplification of fragments containing addition and substitution modifications yielded bands of expected size (Fig 2A). Incubation and amplification of appropriate fragments using OE-PCR resulted in product bands of expected size, indicating complete synthesis of modified *cry4Aa* genes (Fig 2B). Native and modified *cry4Aa* were successfully cloned into pGEX-2T and transformed into BL21 Z competent *E. coli* cells as described in the methods. Sequencing from the Iowa State University DNA facility verified that positive clones contained the correct sequences in the correct reading frame for all constructs.

Nat ive Cry4Aa toxins were induced and purified as described above, resulting in purified protein of the expected size that reacts positively with Cry4Aa antiserum through western blot detection. Purified modified Cry4Aa constructs resulted in two prominent bands at 60 and 65 - kDa in polyacrylamide gels (Fig 3), both of which were detected by western blot. Trypsin activation of the modified Cry4Aa protoxins resulted in processing into the expected 45 and 20 - kDa bands required for toxicity (data not shown), indicating that the functional regions of the toxins were intact.

***In vitro* impacts of pea aphid gut proteases on modified and native Cry4Aa protoxins**

Incubation of negative controls (modified protoxins incubated without aphid gut proteases or EDTA and cysteine) resulted in stable protoxin with no activation observed (Fig 4), except for Cry4Aa 1A which showed a faint 45-kDa band. Exposure of native Cry4Aa protoxins to pea aphid lumen gut proteases in the presence of protease activators resulted in partial activation as indicated by the presence of the 45-kDa band. The absence of activators resulted in decreased activation as only a faint 45-kDa band was observed. In contrast the processing of several modified Cry4Aa constructs by pea aphid lumen gut proteases in the presence of protease activators was enhanced as indicated by a prominent 45-kDa band of similar intensity to the positive control (Cry4Aa digested with 5% trypsin). Cry4Aa 2S showed a similar level of activation as the native Cry4Aa. Protoxin processing was decreased in the absence of protease activators for all modified protoxins except for Cry4Aa 1A which showed a prominent 45-kDa band. The 45-kDa toxin band was not detected on exposure of native or modified Cry4Aa protoxins to pea aphid membrane gut proteases in the presence of protease activators. Exposure to membrane proteases in the absence of activators revealed several non-specific bands as well as a 45-kDa band.

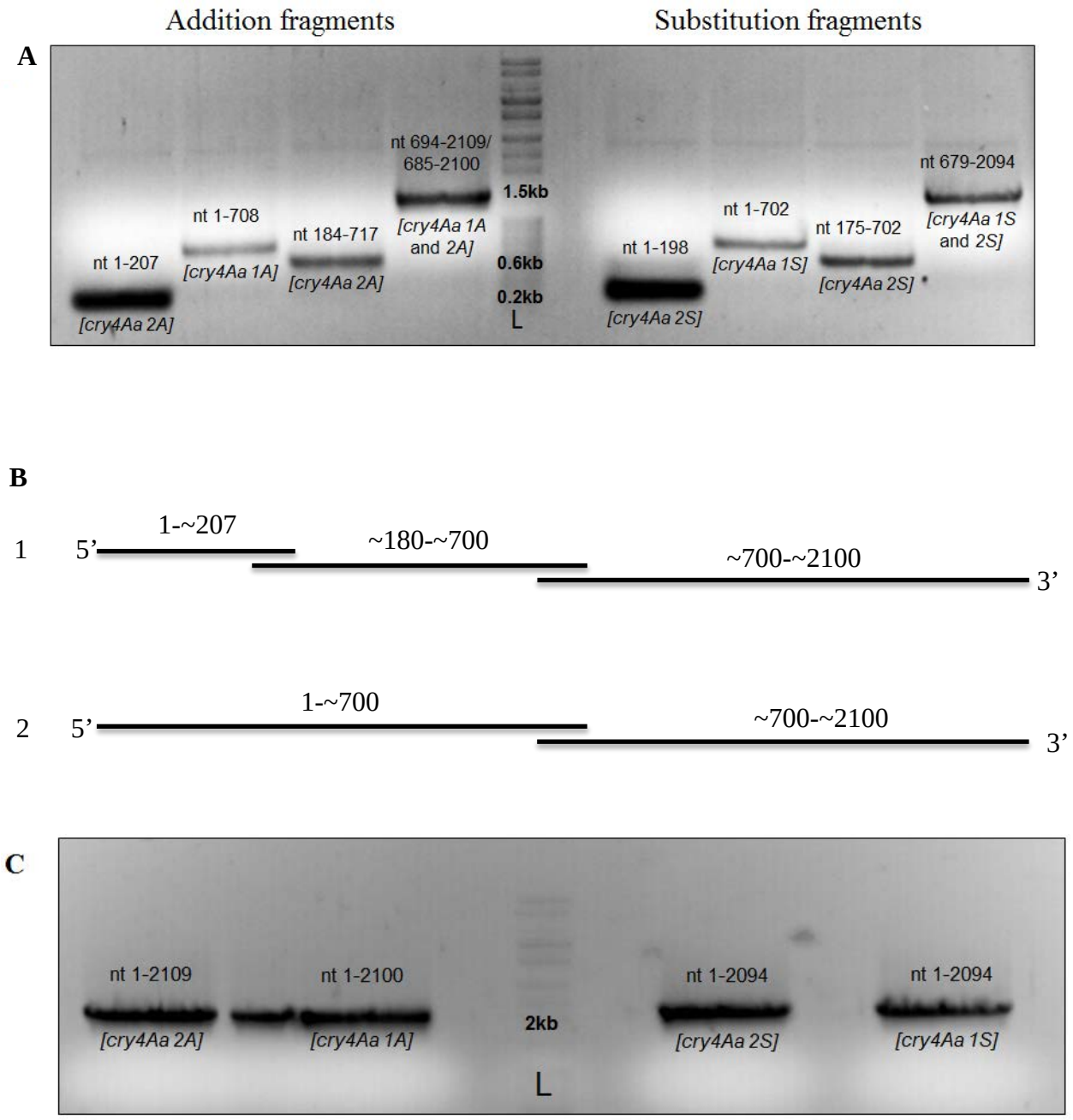


Fig 2. Construction of modified Cry4Aa-S1. (A) PCR products with nucleotides encoding cathepsin L/B cleavage sites added to existing sequence, or replacing existing sequence. (B) 1, assembly of fragments to generate constructs with modifications at two sites. 2, assembly of fragments to generate constructs with modification at one site. (C) Overlap extension polymerase chain reaction (OE-PCR) products with complete modified *cry4Aa* genes. Nucleotide numbers (nt) are indicated. [A] indicates sites incorporated by addition, [S] indicates sites incorporated by substitution of sequence, [1] indicates one site modified, [2] indicates 2 sites modified. L, size ladder.

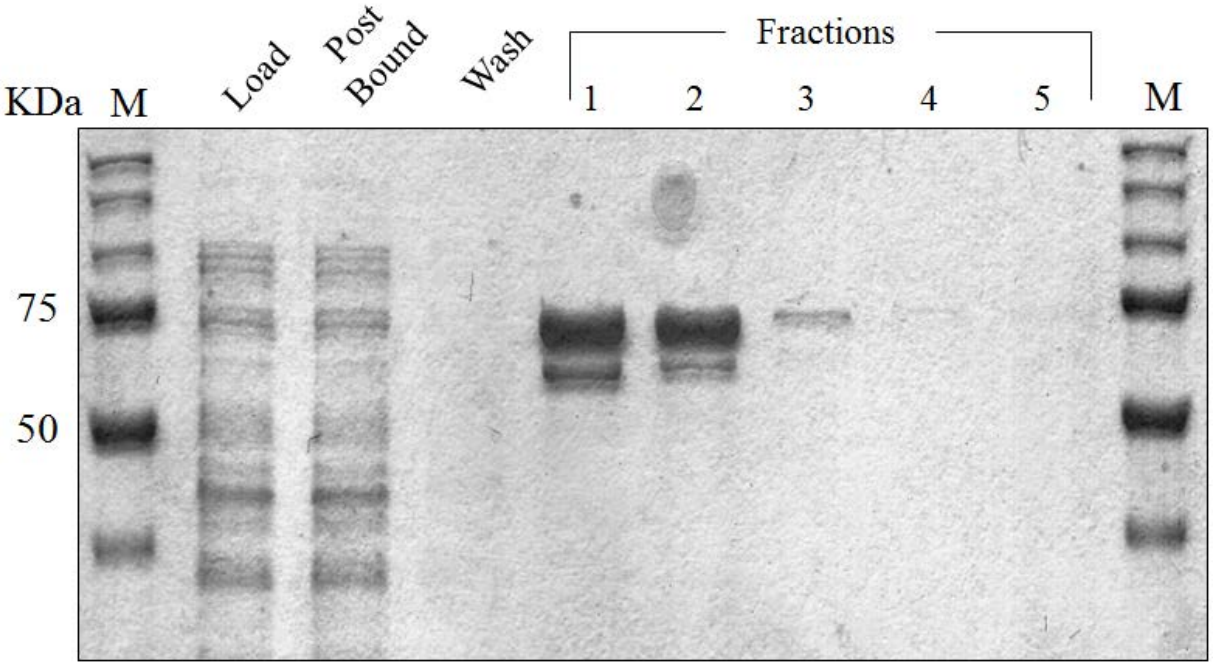
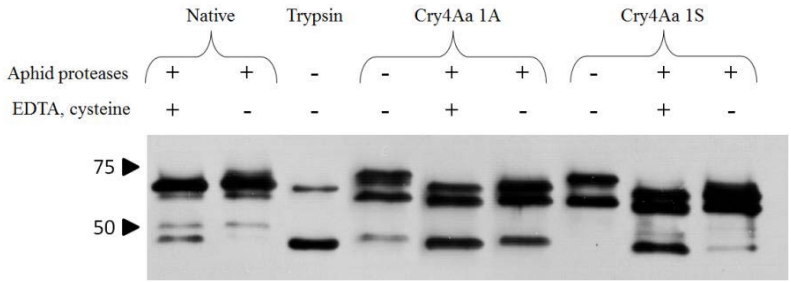


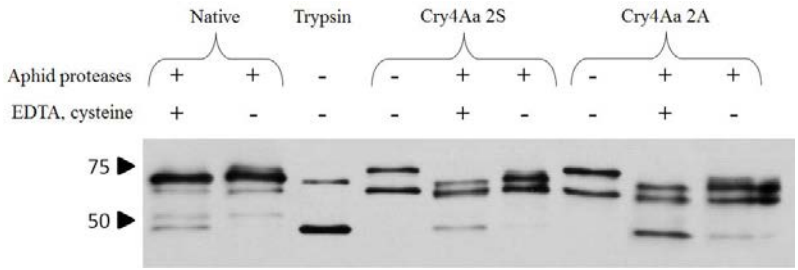
Fig 3. Purification of Cry4Aa 2A. BL21 *E. coli* cells were induced to express modified Cry4Aa/pGEX-2T. The fusion protein was purified using glutathione Sepharose® 4B beads. Five 500 µl fractions were collected following overnight incubation with thrombin. Fractions showing intense bands on denaturing SDS PAGE were pooled and concentrated for experimental use. M, molecular mass markers. Load, cell lysate. Post Bound, cell lysate after incubation with Sepharose ® 4B beads. Wash, rinse of Sepharose® 4B beads with 5ml PBS pH 7.3

Aphid toxicity assays

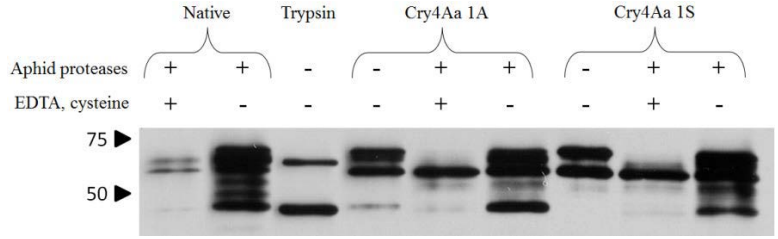
When making multiple comparisons a Bonferroni adjustment is used to determine a revised threshold. In the following analyses $p<0.002$ indicate significant differences. Low mortality was observed in the Tris (15.6 %) and native Cry4Aa (17.8%) controls after two days of feeding, in line with previous research that showed that Cry4Aa exhibits low toxicity against pea aphids (Fig 5) [15]. Pea aphids fed on trypsin-activated Cry4Aa showed significantly increased mortality after one day of feeding (21%) relative to Tris (z-score = -3.19, p = 0.0014) but was not greater than that for aphids fed native Cry4Aa (z-score = -2.77, p = 0.006). In contrast trypsin-activated native Cry4Aa showed significantly increased toxicity against the pea aphid after two days of

Lumen fraction





Membrane fraction



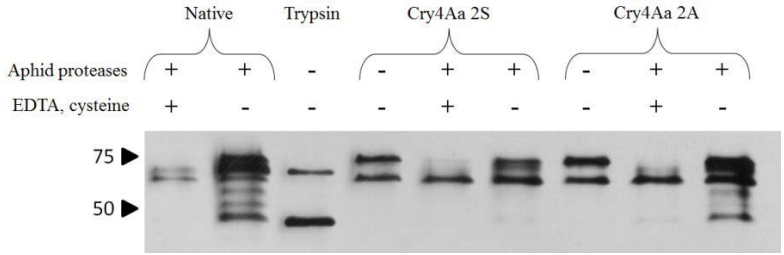


Fig 4. *In vitro* activation of modified Cry4Aa by aphid gut proteases. Protoxins were incubated with the lumen or membrane extracts from the pea aphid gut and hydrolyzed products detected by western blot using Cry4Aa antibodies. Protoxin activation profiles were generated in the presence or absence of cathepsin activators (EDTA and cysteine, 3 mM) as indicated. Native Cry4Aa digested with 5% (w/w) trypsin and modified Cry4Aa not exposed to proteases or activators were included as controls. Native; native Cry4Aa. Trypsin; native Cry4Aa exposed to 5% w/w trypsin, positive control.

feeding (63 %) relative to the Tris (z-score = -4.26, p = 0.00002) and native Cry4Aa treatments (z-score = -4.03, p = 0.0001). Among the modified Cry4Aa protoxins, aphids fed Cry4Aa 2A showed similar mortality when compared to Tris (z-score = -2.46, p = 0.014) and native Cry4Aa (z-score = -2.01, p = 0.044) after one day of feeding. However, after two days of feeding, aphids in the Cry4Aa 2A treatment showed significantly increased toxicity (51 %) relative to Tris (z-score = -3.58, p = 0.0003), and to native Cry4Aa (z-score = -3.33, p = 0.0009) and was similar to aphid mortality in the trypsin-activated Cry4Aa treatment (z-score = 1.04, p = 0.3). (Fig 5). Mortality in the Cry4Aa 1S treatment was not significantly different from mortality in the native Cry4Aa after one or two days of feeding (day one: z-score = -2.0, p = 0.004; day two: z-score = -1.47, p = 0.14) or trypsin- activated Cry4Aa treatments (day one: z-score = 0.92, p = 0.35; day 2: z-score = 2.74, p = 0.006). Mortality in the Cry4Aa 2S treatment was not significantly different from the Tris (day 1: z-score = -1.7, p = 0.09; day two: z-score = -0.28, p = 0.78) and native Cry4Aa treatments (day 1: z-score = -1.2, p = 0.24); day two: z-score = 0, p = 1). Mortality in the Cry4Aa 1A treatment also was not significantly different from the Tris (day one: z-score = -1.4, p = 0.17; day two: z-score = -0.81, p = 0.42) and native Cry4Aa treatments (day one: z-score = -0.85, p = 0.4); day two: z-score = -0.53, p = 0.60).

Modified toxin processing in the pea aphid gut

Native Cry4Aa in complete aphid diet remained stable, indicating that toxin processing did not occur in aphid diet during the feeding assay (Table 6). Native Cry4Aa solubilized in 10 mM Tris pH 7.5 resulted in protoxin, as well as a faint 50 -kDa band. Trypsin activation of native Cry4Aa resulted in a prominent 45 -kDa band (Fig 6).

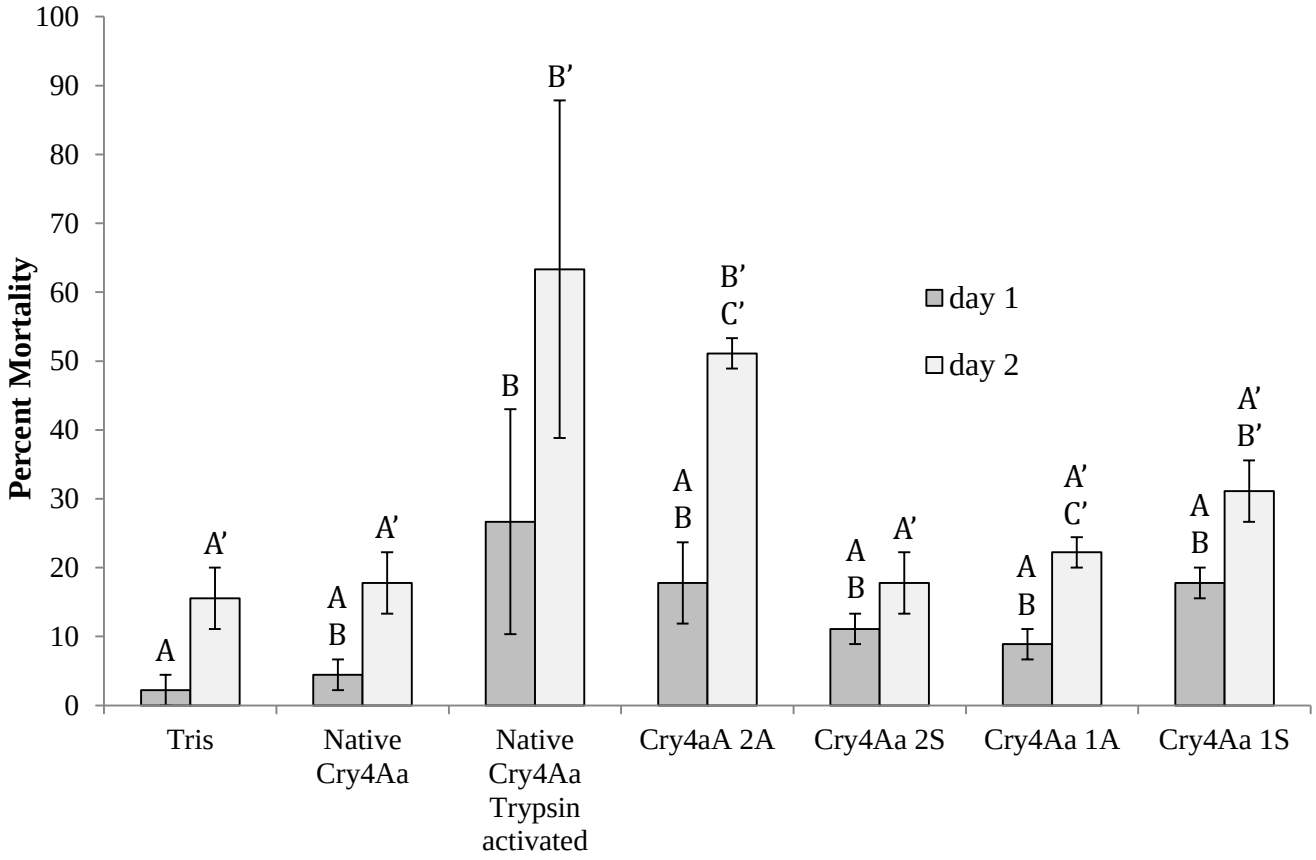


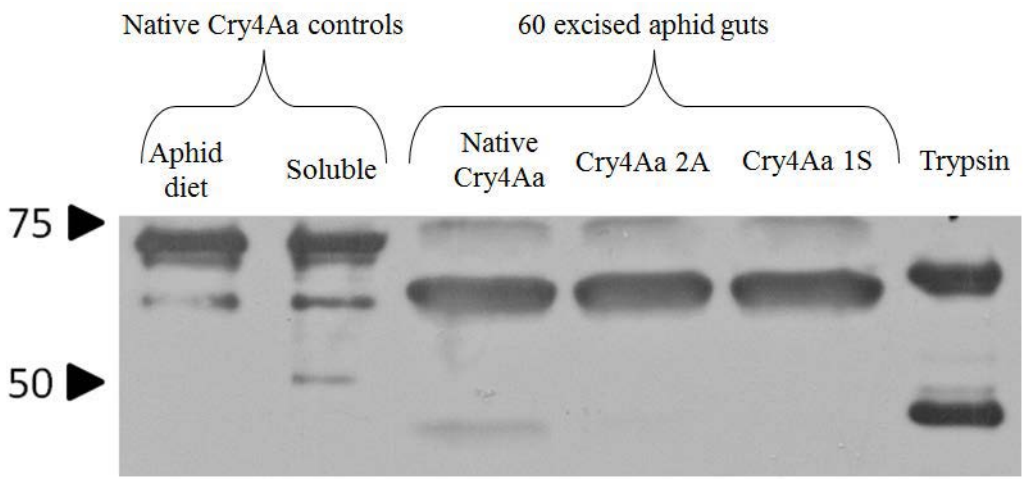
Fig 5. Impact of modified Cry4Aa on aphid survival. Mortality (%) of pea aphids after one and two days feeding on Tris buffer, pH 7.5, native Cry4Aa protoxin, native trypsin-activated Cry4Aa, and modified Cry4Aa protoxins is shown (mean $\pm$ SE). Mortality from treatments with different letters are significantly different (Bonferroni adjustment, $p < 0.002$ is significantly different) by binomial comparisons.

	Day 1		Day 2	
Treatment	**% average mortality**	**SE**	**% average mortality**	**SE**
Tris	2.2 %	2.2 %	15.6 %	4.4 %
Native	4.4 %	2.2 %	17.8 %	4.4 %
Trypsin-activated Native	26.7 %	16.3 %	63.3 %	24.5 %
Cry4Aa 2A	17.8 %	5.9 %	51.1 %	2.2 %
Cry4Aa 2S	11.1 %	2.2 %	17.8 %	4.4 %
Cry4Aa 1A	8.9 %	2.2 %	22.2 %	2.2 %
Cry4 Aa 1S	17.8 %	2.2 %	31.1 %	4.4 %

Table 6. Aphid mortality at 24 and 48 hours after exposure to modified Cry4Aa. Average % daily mortality during 48 hour feeding assay (% average mortality, SE: standard error).

Gut lumen and membrane fractions prepared from 60 aphids fed native Cry4Aa resulted in the majority of the toxin remaining in the protoxin form and very little processing as indicated by the faint 45 -kDa band (Fig 6). Even less *in vivo* processing was seen for the modified toxins compared to the native Cry4Aa in either the membrane or the lumen gut protease fractions. Faint 45 -kDa bands are seen for Cry4Aa 2A, but not for Cry4Aa 1S. A second replicate of this experiment was conducted on 57 excised aphid guts and yielded similar results in the lumen fraction, while native Cry4Aa was not detected in the membrane fraction (Appendix 4).

Lumen fraction



Membrane fraction

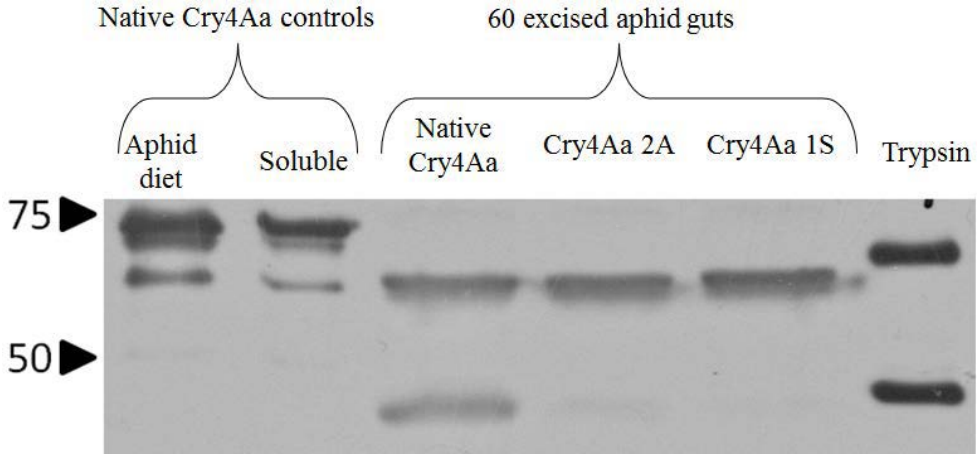


Fig 6. Detection of modified Cry4Aa following exposure to cathepsins in the aphid gut. Aphids were fed overnight, and lumen and membrane fractions from dissected guts were separated by SDS PAGE and analyzed by western blot for toxin profiles. A second replicate of 57 aphid guts yielded similar results in the lumen fraction, and native Cry4Aa was not detected in the membrane fraction. Native Cry4Aa controls: aphid diet, Cry4Aa exposed to aphid diet overnight. Soluble, Cry4Aa solubilized in PBS pH 7.4. Trypsin, native Cry4Aa exposed to 5% w/w trypsin, (positive control). Note: none of the controls were exposed to aphid gut proteases.

Discussion

Purification of native and modified toxins

Purification of all modified Cry4Aa constructs resulted in two prominent proteins of similar size that also appeared in western blots (Fig 3). All modified and native Cry4Aa protoxins treated with trypsin produce the expected 45 and 20 -kDa bands when viewed by SDS-PAGE. Based on our experimental results, the functional regions of the protoxin (domains I, II, and III) appeared to be intact.

In vitro *processing of modified toxins*

Modified protoxins exposed to pea aphid gut lumen proteases, in the presence of protease activators, resulted in increased activation relative to the native Cry4Aa, demonstrating that insertion of cathepsin cleavage sites can be used to facilitate activation *in vitro* (Fig 4). Among our modified Cry4Aa protoxins we observed increased activation in the lumen fraction *in vitro* in all but the Cry4Aa 2S construct. A possible explanation for the lack of activation in Cry4Aa 2S is that the substitution of several amino acids at the two regions may interfere with cleavage by proteases. Protein folding or adjoining residues have the potential to interfere with the protease binding site. The residues at these regions may be crucial to ensure exposure of the cleavage sites to proteases; hence removal of those residues attenuates activation.

Exposure of native and modified protoxin *in vitro* to membrane proteases, in the presence of activators, did not produce a prominent 45-kDa band. Also, the top band of the protoxins appears weaker, suggesting degradation. In the absence of activators, protease exposure resulted in non-specific cleavage, as indicated by multiple bands as well as the 45-kDa band (Fig 4). In the aphid gut the majority of proteases are membrane–associated [25]. Although the major

proteases, present in the aphid gut are cathepsin L and B, there are other types, such as GPI anchored aminopeptidase (APN) and alkaline phosphatase (ALP) [42]. Our data suggest that Cry4Aa protoxins experience greater protease exposure in the membrane fraction, resulting in over-digestion in the presence of activators, hence no detection of a 45-kDa band, while in the absence of activators the basal activity of the membrane proteases is sufficient to cause partial degradation resulting in multiple bands.

Toxicity of modified toxins to aphids

Pea aphids experienced increased mortality, compared to controls, when exposed to trypsin-activated Cry4Aa and Cry4Aa 2A (Fig 5). Mortality caused by trypsin-activated Cry4Aa and Cry4Aa 2A were similar. These results correlate with findings from the *in vitro* experiment, which found enhanced processing of Cry4Aa 2A when incubated with lumen proteases, and the model of Cry toxicity, with protoxin activation being crucial for toxicity and modification being able to facilitate this activation in less susceptible insects. Our findings suggest that modification of Cry4Aa can be used to achieve toxin activation in the pea aphid gut and that this activation is sufficient to achieve toxicity in the pea aphid.

In the feeding assays, only aphids fed on Cry4Aa 2A had mortality that was significantly greater than the mortality of aphids fed on native protoxin, and it was similar to the trypsin-activated toxin treatment at 48 hours. Notably, the other modified Cry4Aa constructs, which also showed increased activation *in vitro*, did not show enhanced toxicity relative to native Cry4Aa. Addition of amino acids, as opposed to substitution of amino acids, maintained all residues utilized by the native toxin and hence all the potential residues crucial for proper folding, association with the insect membrane, and pore formation. Upon protoxin activation,

these constructs would contain all residues required to initiate these downstream effects. A substitution construct, however, lacks amino acids that are present in the native toxin. In addition to possibly inhibiting activation, the loss of these residues may interfere with further downstream events that are needed for toxicity.

Porcar et al. [26] demonstrated that Cry4Aa protoxin has low to moderate toxicity against pea aphids. However, activation with trypsin prior to feeding generates the active 45 and 20 - kDa toxin fragments. Feeding with active toxin greatly increases toxicity against pea aphids, consistent with the fact that protoxin activation by insect proteases is crucial for toxicity [15,43,44]. We also observed increased toxicity against pea aphids with trypsin-activated Cry4Aa.

The feasibility of facilitating protoxin activation in less susceptible insect species by introducing sites at the appropriate regions in the protoxin has been demonstrated previously. Walters et al. [27] introduced a chymotrypsin/cathepsin G recognition site in between α-3 and α-4 in domain I of Cry3A (mCry3A), which substantially increased toxicity against western corn rootworm neonates. This resulted in the rapid proteolytic cleavage of the 67-kDa protoxin into the 55-kDa active toxin and association of the active toxin with the gut membrane of western corn rootworm (*Diabrotica virgifera virgifera*) *in vivo*. In addition, this activation also facilitated increased specific binding to western corn rootworm brush border membrane vesicle (BBMV). Walters et al. [27] concluded that the enhanced toxicity was due to the introduction of cleavage sites, which increased activation and subsequent binding to midgut cells. The results from our study are in line with the findings of Walters et al. [27]. By exploiting the major proteases utilized in the aphid gut (cathepsin L and B) and modifying a known Cry toxin that, when activated, is toxic against pea aphids we found enhanced protoxin activation *in vitro* (in pea

aphid lumen proteases) which correlates with increased toxicity observed for some of our modified Cry4Aa constructs.

In vivo *processing of modified toxins*

Despite the increased toxicity observed in Cry4Aa 2A we did not observe improved processing of the protoxin *in vivo.* Cry4Aa 2A showed only a faint 45 -kDa band *in vivo* that was less intense than the native protoxin, indicating that native Cry4Aa was activated to a greater extent than Cry4Aa 2A. This confounding result emphasizes our limited understanding of the fate of Cry toxins post-binding. Current research is focused on the steps involved in leading to toxicity [45], but nothing is known about the fate of the toxin post lysis. Our data indicate that modified Cry4Aa protoxins do experience increased activation *in vitro* by pea aphid lumen proteases in the presence of protease activators (EDTA and cysteine), and this correlates with increased toxicity of Cry4Aa 2A against pea aphids. It is our interpretation that rapid association with the membrane and pore formation, after activation by lumen proteases, allows Cry4Aa 2A to escape degradation by membrane proteases. Insertion of the pore-forming regions of the Cry toxin would likely prevent proteolytic degradation and maintain the ability to cause cell lysis. The enhanced activation achieved in Cry4Aa 2A translates into more numerous pores forming on epithelium cells, causing cell lysis. After this cell lysis event the post lysed Cry4Aa 2A is now likely exposed to membrane proteases. Either proteases previously associated with the recently lysed cell or membrane proteases from nearby adjoining epithelial cells may subsequently degrade the exposed post-lysed Cry toxin. In contrast, the limited activation of native Cry4Aa may be insufficient to cause cells lysis, or at least cause less cell lysis than Cry4Aa 2A. The limited amounts of activated native Cry4Aa remain protected from membrane proteases by never becoming post-lysed Cry4Aa and remain intact to be detected by western blot. This is a possible

scenario that may explain what appeared to be greater activation of the native protoxin than our modified toxin. This interpretation is also supported by our results *in vitro,* which showed that membrane proteases are involved in degrading Cry4Aa toxins.

Alternatively, the modifications made may have interfered with protease activation either by conformational changes reducing accessibility, or by adjoining residues interfering with the protease binding. Protease specificity can be influenced not only by the target amino acids but also by adjacent residues. In trypsin proteases the substrate binding site is deep and narrow, with a negatively charged aspartate at the bottom. Cleavage can only occur with amino acids that have long side chains and are positively charged; only arginine and lysine are appropriate for this site [46]. Adjoining residues have the potential to interfere with these stringent requirements and limit protease cleavage. It is possible that the residues adjoining the inserted cathepsin L/B cleavage sites may also be involved with limiting protease access and result in limiting activation. However we find this unlikely as Cry4Aa 2A demonstrated enhanced *in vitro* activation and increased toxicity.

Among our *in vitro* and *in vivo* experiments we did observe some activation of the native Cry4Aa which is surprising given that the major proteases utilized by the pea aphid are cathepsin L and B [22]. No other regions throughout the native Cry4Aa peptide sequence contain the sequences FR that could be cleaved by cathepsin L, while there are two regions that contain the sequences RR that may be cleaved by cathepsin B; R355 and R356, as well as R481 and R482.Trypsin-like proteases have been detected in the pea aphid genome as well as trypsin-like mRNAs, however it is unknown if these proteases are expressed in the gut [47]. Trypsin-like protease activity was not detected in the pea aphid gut in a previous study [22].

Conclusions

Previous research has been focused on Cry protoxin activation, with susceptible insects cleaving the protoxin at specific sites and activation being a precursor to toxicity [15,44,48]. Insects that are less susceptible to these toxins often lack the proteases required for activation or do not achieve sufficient activation for toxicity [25]. Toxin activation prior to feeding can result in toxicity in these insects [26], suggesting that activation can be a rate-limiting step in less susceptible insects.

Our hypothesis that Cry4Aa modified with cathepsin L and B cleavage sites will result in protoxin activation was partially supported by our *in vitro* exposure of modified Cry4Aa to aphid gut proteases. Furthermore, the increased processing sites correlated with increased toxicity for Cry4Aa 2A. Additional modification of Cry4Aa, such as removal of potential sites involved in degradation or addition of peptides for improved binding to the gut [49], may be required to reach levels of toxicity appropriate for use of modified Cry4Aa in transgenic plants. Expanding the currently used Bt transgenic technology to include toxins active against aphids would facilitate environmentally benign management of these pests. Employing our current understanding of Cry toxin mode of action and aphid gut physiology will be required to create novel designer toxins specific against aphids. The data from our study demonstrate the feasibility of this approach and emphasize the need for further research on the Cry toxin mode action to provide additional regions for modification to achieve enhanced toxicity.

Author Contributions

MR conducted all laboratory experiments. BD made the anti-Cry4Aa antiserum and assisted with aphid gut dissections. NPC was involved with experimental design. BCB contributed to discussion of results and data analysis.

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CHAPTER 3

CONCLUSIONS

The successful use of Cry toxins through transgenic crops has been exemplary for managing insect pest populations resulting in multiple benefits to growers and the environment [1-7]. Due to the suppression of these original pests and the decreased use of insecticides several hemipteran insects have risen to the status of primary pests [8]. The challenge of utilizing the current generation of transgenic crops against these hemipteran pests is the limited susceptibility of these insects to Cry toxins [8]. Findings from our study, as well as others, have established that the continued use, and benefits, of transgenic crops can be expanded to include hemipteran pests by the molecular fine-tuning of these toxins [8].

In Chapter 2 we demonstrated the potential for modifying Cry toxins by inserting cathepsin L and cathepsin B cleavage sites into Cry4Aa to facilitate activation of the protoxin in order to achieve increased toxicity against pea aphids. We concluded that addition of cleavage sites at two regions involved in activation does result in enhanced activation *in vitro* by aphid lumen gut proteases, and this correlates with enhanced toxicity in membrane feeding assays. Our findings are in line with Walters et al. [[9] who found that introduction of a chymotrypsin/cathepsin G recognition site in Cry3A (mCry3A) substantially increased toxicity against western corn rootworm neonates. In addition, we observed possible degradation of native and modified Cry4Aa protoxins by aphid membrane proteases. In order to explain the observed increased toxicity, despite the apparent degradation, we propose that upon activation by lumen proteases the modified Cry4Aa associates with the aphid gut membrane and forms a pore, which may protect the toxin from degradation by membrane proteases. Upon causing cell lysis

by osmotic disruption [10] the post-lysed Cry4Aa toxin is subsequently degraded on exposure to membrane proteases.

Future research should focus on alternative methods of Cry toxin modification to further enhance toxicity against aphids. Earlier research has suggested that toxin degradation could be involved in limiting toxicity in some cases [11-15]. Removal of putative sites involved in degradation could further increase toxicity against aphids. Previous research indicated that this is an appropriate strategy to prevent degradation. Cry1Aa has been modified for increased toxicity against spruce budworm by mutating potential trypsin and chymotrypsin sites involved in degradation, resulting in a 2-4 fold increase in toxicity [16].

An alternative approach to enhance toxicity would be to facilitate improved binding to the gut membrane. Addition of gut binding residues has proven to be a viable strategy to enhance and broaden toxicity [17-20]. For example, replacing domain II of Cry1Ba with domain II from Cry1Ia resulted in increased toxicity against western corn rootworm [21]. Although we achieved increased toxicity against pea aphid by exploiting protease recognition sites, the possibility of improving toxicity further, by incorporating aphid gut binding peptides should not be dismissed. Indeed, the efficacy of this approach for use against the pea aphid has been demonstrated using Cyt2Aa as a model toxin [22].

The data we have generated contribute to the knowledge base of how Cry toxins can be modified to achieve toxicity in pea aphids, which can be expanded to other hemipteran pests. In addition we have generated a model to explain some of our more confounding results, forming the basis for future research. Transgenic plant expression of modified Cry toxins designed to target aphids and other hemipteran pests is a viable option for their management.

An important consideration for production of aphid resistant transgenic plants is use of an appropriate promoter for expression of the transgene. Promoters from viruses (cauliflower mosaic virus 35S promoter), bacteria (*Agrobacterium tumefaciens* Ti plasmid mannopine synthetase *mas* promoter), or plants (*Arabidopsis thaliana* Act 2 promoter) are commonly used for transgenic plants designed to protect against leaf feeding insects. These promoters drive transgene expression throughout plant tissues [22]. For transgenic plant protection against aphids, expression of the transgene in plant phloem is required as the phloem constitutes the food source for the aphid. Phloem-specific promoters such as sucrose synthase I from rice and maize have been used for heterologous expression in the phloem [23,24].

The results of this project are relevant to both the scientific community and to the general population. We hope that our findings will prove useful in expanding the utility of transgenic crops for crop protection, thereby contributing to the food requirements of a growing world population.

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APPENDIX 1
NUCLEOTIDE AND PEPTIDE SEQUENCE OF MODIFIED Cry4Aa CONSTRUCTS

The regions modified with cathepsin L/B sequences are highlighted in grey

cry4Aa 1A nucleotide sequence

GGATCCATGAACCCGTATCAAAATAAAAACGAATATGAAACGCTGAACGCATCGCAGAAAAAACTGAACATCTCCAACAACTA
CACCCGTTACCCGATCGAAAATTCACCGAAACAGCTGCTGCAATCGACCAACTACAAAGATTGGCTGAACATGTGCCAGCAAA
ACCAGCAATACGGTGGTGACTTCGAAACGTTCATCGATTCAGGTGAACTGTCGGCATACACCATCGTGGTTGGCACCGTTCTG
ACGGGTTTTGGTTTCACCACGCCGCTGGGTCTGGCACTGATTGGCTTTGGCACCCTGATCCCGGTCCTGTTCCCGGCTCAGGAC
CAAAGCAATACCTGGTCTGATTTTATTACCCAGACGAAAAACATCATCAAAAAAGAAATCGCGAGTACCTACATCTCCAACGCC
AACAAAATTCTGAACCGTAGCTTCAACGTTATCTCTACGTATCATAATCACCTGAAAACCTGGGAAAACAACCCGAACCCGCAG
AACACGCAAGATGTGCGTACCCAGATCCAACTGGTTCATTACCACTTCCAGAACGTGATCCCGGAACTGGTTAATAGCTGCCCG
CCGAACCCGTCTGATTGTGACTACTACAACATCCTGGTGCTGAGCAGCTATGCGCAGGCAGCAAATCTGCATCTGACCGTCCTG
AACCAAGCAGTGAAATTCGAAGCGTATCTGAAAAACAATTTCCGTCGTCAGTTCGACTATCTGGAACCGCTGCCGACCGCAAT
CGATTACTACCCGGTCCTGACGAAAGCTATCGAAGATTATACCAACTACTGTGTGACCACGTACAAAAAAGGTCTGAACCTGAT
CAAAACCACGCCGGACTCCAACCTGGATGGTAACATCAACTGGAACACCTACAACACGTACCGTACCAAAATGACCACGGCAG
TTCTGGACCTGGTCGCTCTGTTCCCGAACTACGATGTCGGTAAATACCCGATTGGTGTGCAGAGCGAACTGACCCGCGAAATCT
ACCAAGTGCTGAACTTCGAAGAAAGCCCGTACAAATATTACGACTTCCAGTACCAAGAAGATTCTCTGACGCGTCGTCCGCACC
TGTTTACCTGGCTGGATAGTCTGAACTTCTACGAAAAAGCACAGACCACGCCGAACAACTTTTTCACGTCCCATTACAACATGTT
CCACTACACCCTGGACAACATTTCACAGAAAAGTTCCGTTTTCGGTAACCACAACGTCACGGATAAACTGAAATCTCTGGGTCT
GGCTACCAATATTTATATCTTTCTGCTGAACGTTATCTCGCTGGACAACAAATACCTGAACGATTACAACAACATCAGCAAAAT
GGACTTTTTCATCACGAATGGCACCCGTCTGCTGGAAAAAGAACTGACGGCGGGCAGTGGTCAGATCACCTATGATGTGAACA
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ATATCCTGTCGTTCATCAAAAGCCTGTCTATCCCGGCAACGTACAAAACCCAGGTGTACACGTTCGCATGGACCCATTCATCGG
TTGATCCGAAAAACACGATCTATACCCACCTGACCACGCAGATCCCGGCAGTTAAAGCTAACAGTCTGGGCACCGCGTCCAAA
GTCGTGCAAGGTCCGGGCCACACCGGCGGTGATCTGATCGACTTCAAAGATCATTTCAAAATCACCTGCCAGCACTCAAATTTT
CAGCAATCGTACTTCATTCGTATCCGCTATGCAAGTAATGGCTCCGCAAACACCCGTGCCGTTATCAACCTGTCTATTCCGGGC
GTCGCAGAACTGGGTATGGCACTGAACCCGACCTTCAGTGGTACGGACTACACCAACCTGAAATACAAAGATTTCCAGTATCT
GGAATTTAGCAATGAAGTGAAATTTGCCCCGAATCAAAACATTAGTCTGGTGTTCAACCGCTCCGACGTTTACACCAACACCAC
GGTCCTGATTGATAAAATCGAATTTCTGCCGATTACCCGTAGCATCCGTGAAGACCGTGAAAAACAAAAACTGGAAACCGTGT
GAGAATTC

Cry4a 1A peptide sequence

MNPYQNKNEYETLNASQKKLNISNNYTRYPIENSPKQLLQSTNYKDWLNMCQQNQQYGGDFETFIDSGELSAYTIVVGTVLTGFGF
TTPLGLALIGFGTLIPVLFPAQDQSNTWSDFITQTKNIIKKEIASTYISNANKILNRSFNVISTYHNHLKTWENNPNPQNTQDVRTQIQL
VHYHFQNVIPELVNSCPPNPSDCDYYNILVLSSYAQAANLHLTVLNQAVKFEAYLKNNFRRQFDYLEPLPTAIDYYPVLTKAIEDYTNY
CVTTYKKGLNLIKTTPDSNLDGNINWNTYNTYRTKMTTAVLDLVALFPNYDVGKYPIGVQSELTREIYQVLNFEESPYKYYDFQYQED
SLTRRPHLFTWLDSLNFYEKAQTTPNNFFTSHYNMFHYTLDNISQKSSVFGNHNVTDKLKSLGLATNIYIFLLNVISLDNKYLNDYNNI
SKMDFFITNGTRLLEKELTAGSGQITYDVNKNIFGLPILKRRENQGNPTLFPTYDNYSHILSFIKSLSIPATYKTQVYTFAWTHSSVDPKN
TIYTHLTTQIPAVKANSLGTASKVVQGPGHTGGDLIDFKDHFKITCQHSNFQQSYFIRIRYASNGSANTRAVINLSIPGVAELGMALNP
TFSGTDYTNLKYKDFQYLEFSNEVKFAPNQNISLVFNRSDVYTNTTVLIDKIEFLPITRSIREDREKQKLETV

NUCLEOTIDE AND PEPTIDE SEQUENCE OF MODIFIED Cry4Aa CONSTRUCTS

The regions modified with cathepsin L/B sequences are highlighted in grey

cry4Aa 2A nucleotide sequence

GGATCCATGAACCCGTATCAAAATAAAAACGAATATGAAACGCTGAACGCATCGCAGAAAAAACTGAACATCTCCAACAACTA
CACCCGTTACCCGATCGAAAATTCACCGAAACAGCTGCTGCAATCGACCAACTACAAAGATTGGCTGAACATGTGCCAGCAAA
ACCAGCAATACGGTGGTGACTTCGAAACGTTCATCGATTCATTCCGTCGTGGTGAACTGTCGGCATACACCATCGTGGTTGGCA
CCGTTCTGACGGGTTTTGGTTTCACCACGCCGCTGGGTCTGGCACTGATTGGCTTTGGCACCCTGATCCCGGTCCTGTTCCCGG
CTCAGGACCAAAGCAATACCTGGTCTGATTTTATTACCCAGACGAAAAACATCATCAAAAAAGAAATCGCGAGTACCTACATCT
CCAACGCCAACAAAATTCTGAACCGTAGCTTCAACGTTATCTCTACGTATCATAATCACCTGAAAACCTGGGAAAACAACCCGA
ACCCGCAGAACACGCAAGATGTGCGTACCCAGATCCAACTGGTTCATTACCACTTCCAGAACGTGATCCCGGAACTGGTTAAT
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GACCGCAATCGATTACTACCCGGTCCTGACGAAAGCTATCGAAGATTATACCAACTACTGTGTGACCACGTACAAAAAAGGTCT
GAACCTGATCAAAACCACGCCGGACTCCAACCTGGATGGTAACATCAACTGGAACACCTACAACACGTACCGTACCAAAATGA
CCACGGCAGTTCTGGACCTGGTCGCTCTGTTCCCGAACTACGATGTCGGTAAATACCCGATTGGTGTGCAGAGCGAACTGACC
CGCGAAATCTACCAAGTGCTGAACTTCGAAGAAAGCCCGTACAAATATTACGACTTCCAGTACCAAGAAGATTCTCTGACGCGT
CGTCCGCACCTGTTTACCTGGCTGGATAGTCTGAACTTCTACGAAAAAGCACAGACCACGCCGAACAACTTTTTCACGTCCCAT
TACAACATGTTCCACTACACCCTGGACAACATTTCACAGAAAAGTTCCGTTTTCGGTAACCACAACGTCACGGATAAACTGAAA
TCTCTGGGTCTGGCTACCAATATTTATATCTTTCTGCTGAACGTTATCTCGCTGGACAACAAATACCTGAACGATTACAACAACA
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TAACTACTCACATATCCTGTCGTTCATCAAAAGCCTGTCTATCCCGGCAACGTACAAAACCCAGGTGTACACGTTCGCATGGAC
CCATTCATCGGTTGATCCGAAAAACACGATCTATACCCACCTGACCACGCAGATCCCGGCAGTTAAAGCTAACAGTCTGGGCAC
CGCGTCCAAAGTCGTGCAAGGTCCGGGCCACACCGGCGGTGATCTGATCGACTTCAAAGATCATTTCAAAATCACCTGCCAGC
ACTCAAATTTTCAGCAATCGTACTTCATTCGTATCCGCTATGCAAGTAATGGCTCCGCAAACACCCGTGCCGTTATCAACCTGTC
TATTCCGGGCGTCGCAGAACTGGGTATGGCACTGAACCCGACCTTCAGTGGTACGGACTACACCAACCTGAAATACAAAGATT
TCCAGTATCTGGAATTTAGCAATGAAGTGAAATTTGCCCCGAATCAAAACATTAGTCTGGTGTTCAACCGCTCCGACGTTTACA
CCAACACCACGGTCCTGATTGATAAAATCGAATTTCTGCCGATTACCCGTAGCATCCGTGAAGACCGTGAAAAACAAAAACTG
GAAACCGTGTGAGAATTC

Cry4a 2A peptide sequence

MNPYQNKNEYETLNASQKKLNISNNYTRYPIENSPKQLLQSTNYKDWLNMCQQNQQYGGDFETFIDSFRRGELSAYTIVVGTVLTG
FGFTTPLGLALIGFGTLIPVLFPAQDQSNTWSDFITQTKNIIKKEIASTYISNANKILNRSFNVISTYHNHLKTWENNPNPQNTQDVRT
QIQLVHYHFQNVIPELVNSCPPNPSDCDYYNILVLSSYAQAANLHLTVLNQAVKFEAYLKNNFRRQFDYLEPLPTAIDYYPVLTKAIED
YTNYCVTTYKKGLNLIKTTPDSNLDGNINWNTYNTYRTKMTTAVLDLVALFPNYDVGKYPIGVQSELTREIYQVLNFEESPYKYYDFQ
YQEDSLTRRPHLFTWLDSLNFYEKAQTTPNNFFTSHYNMFHYTLDNISQKSSVFGNHNVTDKLKSLGLATNIYIFLLNVISLDNKYLND
YNNISKMDFFITNGTRLLEKELTAGSGQITYDVNKNIFGLPILKRRENQGNPTLFPTYDNYSHILSFIKSLSIPATYKTQVYTFAWTHSSV
DPKNTIYTHLTTQIPAVKANSLGTASKVVQGPGHTGGDLIDFKDHFKITCQHSNFQQSYFIRIRYASNGSANTRAVINLSIPGVAELG
MALNPTFSGTDYTNLKYKDFQYLEFSNEVKFAPNQNISLVFNRSDVYTNTTVLIDKIEFLPITRSIREDREKQKLETV

NUCLEOTIDE AND PEPTIDE SEQUENCE OF MODIFIED Cry4Aa CONSTRUCTS

Regions modified with cathepsin L/B sequences highlighted in grey

cry4Aa 1S nucleotide sequence

GGATCCATGAACCCGTATCAAAATAAAAACGAATATGAAACGCTGAACGCATCGCAGAAAAAACTGAACATCTCCAACAACTA
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Cry4a 1S peptide sequence

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NUCLEOTIDE AND PEPTIDE SEQUENCE OF MODIFIED Cry4Aa CONSTRUCTS

Regions modified with cathepsin L/B sequences highlighted in grey

cry4Aa 2S nucleotide sequence

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Cry4a 1S peptide sequence

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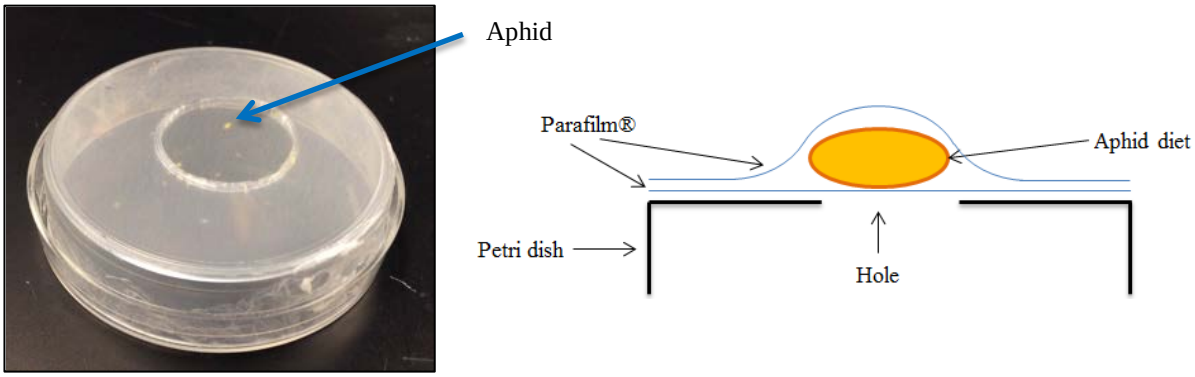
APPENDIX 2
PEA APHID EXPOSURE TO NATIVE AND MODIFIED Cry4Aa PROTOXINS BY MEMBRANE FEEDING ASSAY

Photograph and schematic of feeding dish used for pea aphid feeding assays. Thinly stretched Parafilm® covers a 3-cm hole in the petri dish; a second layer of Parafilm® covers a pocket of aphid diet. Aphids placed in the dish pierce the Parafilm® with their stylets to feed.



APPENDIX 3
DATA ANALYSIS

	Alive	Dead	Total	Estimated Mortality
Tris	38	7	45	0.16
Native	37	8	45	0.18

Example of Binomial comparison data output between Native Cry4Aa and Tris control	Pooled	0.17
	z-score	-0.28
	p-value	0.78

Day 1 aphid mortality multiple comparisons of p-values generated from Binomial comparisons Revised threshold adjustment for multiple comparisons following Bonferroni adjustment (0.05/21),with $p<0.002$ indicating significant difference.

	Tris	**Native**	**Trypsin-activated**	**2A**	**2S**	**1A**
Tris	X	X	X	X	X	X
Native	0.5571	X	X	X	X	X
Trypsin-activated	0.0014	0.0055	X	X	X	X
2A	0.0139	0.0442	0.3572	X	X	X
2S	0.0909	0.2377	0.0812	0.3684	X	X
1A	0.1674	0.3980	0.0397	0.2148	0.7253	X
1S	0.0139	0.0442	0.3572	1	0.3684	0.2148

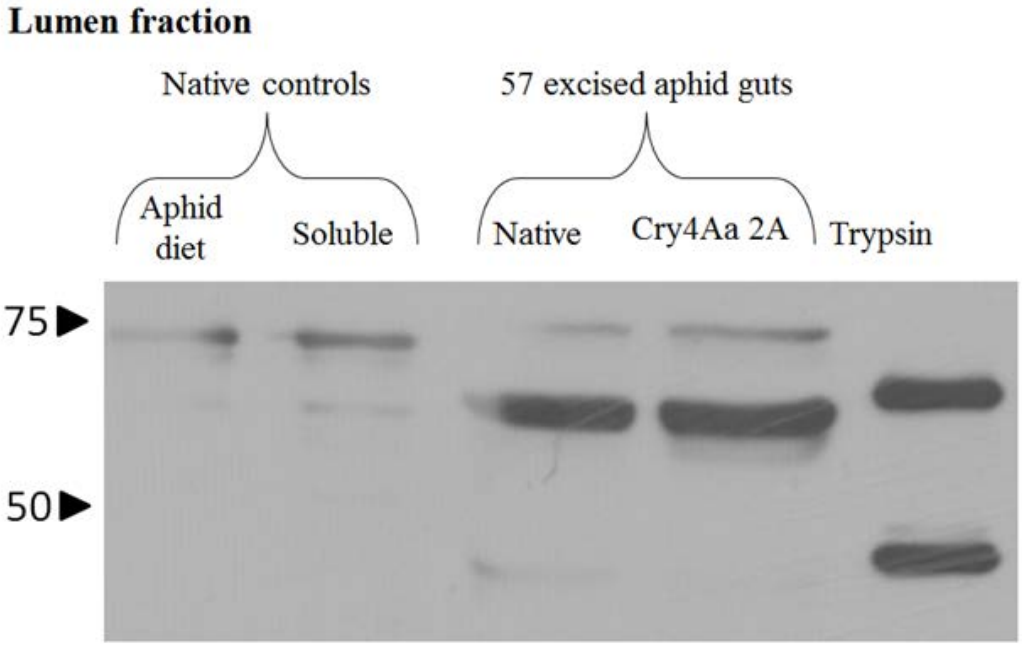
DATA ANALYSIS

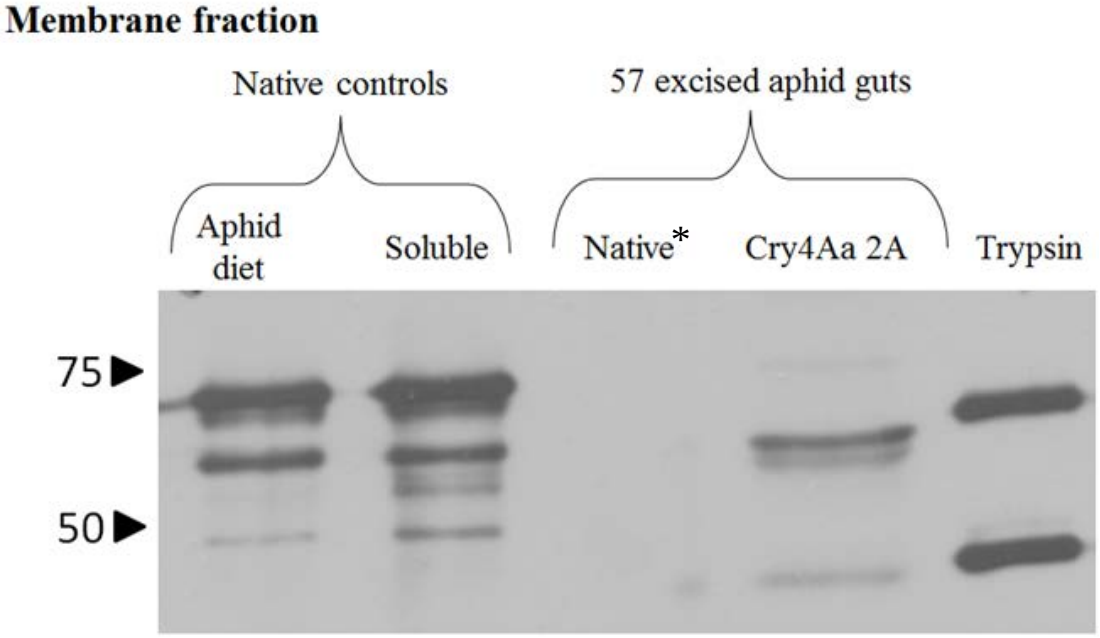
Day 2 aphid mortality multiple comparisons of p-values generated from Binomial comparisons Revised threshold adjustment for multiple comparisons following Bonferroni adjustment (0.05/21),with $p<0.002$ indicating significant difference.

	Tris	**Native**	**Trypsin-activated**	**2A**	**2S**	**1A**
Tris	X	X	X	X	X	X
Native	0.7773	X	X	X	X	X
Trypsin-activated	<0.0001	<0.0001	X	X	X	X
2A	0.0003	0.0009	0.2962	X	X	X
2S	0.7773	1	<0.0001	0.0010	X	X
1A	0.4191	0.5982	0.0003	0.0045	0.5982	X
1S	0.0811	0.1411	0.0059	0.0538	0.1411	0.3404

APPENDIX 4
DETECTION OF MODIFIED Cry4Aa FOLLOWING EXPOSURE OTO CATHEPSINS IN THE APHID GUT-SECOND REPLICATE

57 guts excised from pea aphid fed native Cry4Aa and Cry4Aa 2A at 300µg/ml overnight at 22ºC





* Bubble may have prevented protein transfer to membrane used for western blot