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Bacteria-derived pesticidal proteins active against hemipteran pests

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ABSTRACT

Hemipteran pests are among the most important threats to agricultural production. Losses associated with these insects result from both feeding-associated damage and the transmission of plant pathogens by some species. Key among hemipteran pests of agricultural importance are stink bugs, whitefly, aphids and psyllids. While bacteria provide an excellent resource for identification of environmentally benign pesticidal proteins for use against pest insects, relatively few with activity against hemipteran species have been identified. In this comprehensive review including the patent literature, we describe physiological features unique to Hemiptera that may restrict the toxicity of bacterial pesticidal proteins, provide an overview of Hemiptera-active pesticidal proteins and associated structural classes, and summarize biotechnological strategies used for optimization of toxicity against target hemipteran species.

1. Introduction

Bacterial pesticidal proteins (BPPs) are proteins derived from bacteria that are toxic to pestiferous invertebrates including insects and nematodes (Crickmore et al., 2020). While BPPs isolated from different strains of *Bacillus thuringiensis* are the most well-known due to their widespread use in pest control either through bacterial sprays, purified proteins or transgenic plants, BPPs isolated from many other bacterial species also have demonstrated potential (Berry and Crickmore, 2017). Transgenic plants expressing crystal (Cry) proteins that target coleopteran and lepidopteran pests have resulted in an estimated 22 % increase in yield and a 37 % reduction in the use of chemical pesticides (Klümper and Qaim, 2014). The successful use of BPPs in terms of efficacy, sustainability and commercial viability results from multiple factors including the specificity of selected BPPs compared to other management options without deleterious impacts on beneficial or non-target organisms (Koch et al., 2015), the lack of persistence in the environment (Padmaja et al., 2008), and the need for more sustainable approaches (Lechenet et al., 2017).

BPP nomenclature reflects protein structure with 15 distinct structures recognized at the time of writing (Crickmore et al., 2020; Crickmore et al., 2022). Of these, the modes of action of the Cry and cytolytic (Cyt) proteins are the most well characterized. Cry and Cyt proteins are produced by *B. thuringiensis* at the onset of sporulation, and during the stationary growth phase as parasporal crystalline inclusions (Adang et al., 2014; Fernández-Chapa et al., 2019). Once ingested by insects, the parasporal crystalline body is solubilized to release a protoxin, which is then proteolytically activated by midgut proteases. The activated protein binds to specific receptors in the midgut (either protein or lipid for Cry and Cyt respectively) and subsequent pore formation leads to cell disruption and insect death (Jurat-Fuentes and Crickmore, 2017; Jurat-Fuentes et al., 2021; Palma et al., 2014a). The protein domains that bind to gut surface proteins play a key role in determining BPP toxicity.

While BPPs have been successfully deployed for management of coleopteran and lepidopteran pests in particular, they have shown limited field efficacy against agricultural pests in the order Hemiptera. Hemipteran insects, in contrast to lepidopterans and coleopterans, feed on phloem (aphids, whiteflies, mealybugs), xylem (sharpshooters, spittle bugs), or seeds and fruit (stinkbugs). The blood feeding hemipterans, or triatomines, are not considered in the current review. Plant feeding hemipterans cause damage not only by weakening the plant, but also

Abbreviations: BPP, bacterial pesticidal protein.

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causing cosmetic damage (to fruit for example) and by transmitting plant pathogens (Arora et al., 2018; Douglas, 2006; Krinsky, 2019; Novotny and Wilson, 1997; Panfilio et al., 2019; Perilla-Henao and Casteel, 2016). Management of hemipteran pests typically relies on the unsustainable application of chemical insecticides despite the potential for nontarget impacts, due to the lack of effective BPP alternatives (Chougule and Bonning, 2012). While BPPs with toxicity to aphids (Porcar et al., 2009), Asian citrus psyllid (ACP) (Fernandez-Luna et al., 2019), and the western tarnished plant bug (Baum et al., 2012) have been identified for example, and methods established to optimize toxicity (Banerjee et al., 2022; Chougule et al., 2013; Gowda et al., 2016), there is only one example of field deployment of transgenic plants expressing a BPP for hemipteran control (Gowda et al., 2016). Molecular methods for modification of BPPs for enhanced activity against Hemiptera tend to improve toxicity rather than making a non-toxic molecule toxic. Therefore, an important initial requirement is to identify BPPs that are somewhat toxic to a target hemipteran species.

Effective alternative strategies to the application of chemical insecticides are needed particularly for hemipteran pests such as psyllids and stink bugs that have increased in importance as agricultural pests. In this review we discuss the potential bases for the apparent low susceptibility of Hemiptera to BPPs, describe the structural diversity of BPPs with known efficacy against Hemiptera, and provide an overview of strategies to enhance BPP toxicity against hemipteran pests. BPP-based approaches for hemipteran pest management offer a promising approach to combat hemipteran-induced damage and disease transmission toward sustainable food production.

2. Potential bases for limited BPP toxicity against Hemiptera

Relatively few BPPs with toxicity against Hemiptera have been identified (Table 1). Factors that contribute to the low efficacy of BPPs against hemipterans, all of which relate to their specialized feeding habits, include 1) lack of sufficient exposure and selection for toxicity against this group of insects, given the low prevalence of *Bacillus* spp. in plant sap (Chougule and Bonning, 2012), 2) suboptimal environment in the hemipteran gut for BPP activation, processing and / or binding, 3) gut structural considerations including the presence of a filter chamber, and absence of peritrophic membrane / matrix.

2.1. Presence of filter chamber

The guts of the sap-sucking Hemiptera including both phloem-feeders (aphids, whiteflies, mealybugs, planthoppers) and xylem feeders (leafhoppers), contain a filter chamber at the junction between the anterior and posterior midgut (Fig. 1) (Marshall and Cheung, 1974; Rosell et al., 2003). In phloem-feeding hemipterans water is shunted into the anterior midgut and in xylem feeding hemipterans water is pushed into the posterior midgut via aquaporin expressed in the filter chamber to maintain the osmotic balance between hemolymph and the gut (Beuron et al., 1995; Le Caherec et al., 1997; Mathew et al., 2011; Shakesby et al., 2009). Aquaporin has been hypothesized to act as a conduit for water after ions are moved into the cytoplasm through the pores formed by Cry proteins (Endo et al., 2017). The absence of aquaporins outside of the filter chamber, i.e. in the midgut region of sap-feeding insects (Le Caherec et al., 1997; Mathew et al., 2011) and presence of aquaporins in the midgut cells of susceptible insects (Javed et al., 2019; Kuwar et al., 2022; Maruyama and Azuma, 2015) could explain the lower activity of Cry proteins in these species relative to insects in other orders.

2.2. Absence of peritrophic membrane

All Hemiptera lack a peritrophic membrane, a feature of coleopteran and lepidopteran insects that provides protection against microbes and solid food particles (Fig. 1). The peritrophic membrane has been shown to bind Cry proteins and limit their movement into the ectoperitrophic space (Hayakawa et al., 2004; Rees et al., 2009). Once the Cry proteins cross the peritrophic membrane, they interact with gut receptors and the posterior to anterior fluid movement in the ectoperitrophic region (Bolognesi et al., 2001; Bolognesi et al., 2008; Caldeira et al., 2007; Ferreira et al., 2002; Terra et al., 2019; Terra and Ferreira, 2020) may prolong access for Cry proteins to bind before excretion (Fig. 1). The lack of posterior to anterior fluid movement in Hemiptera may result in faster excretion of pesticidal proteins, as the majority of fed Cry1Ac protein was detected in the feces of *Lygus hesperus* (Brandt et al., 2004). Consistent with this scenario, most Cry proteins were observed in the frass of resistant Lepidoptera (Rees et al., 2009), and an increased retention time was observed for modified effective Cry proteins compared to ineffective Cry protein in the midgut of *Anoplophora glabripennis* (Guo et al., 2012). Alternatively, Cry1Ac excretion could result from the lack of binding in the *L. hesperus* gut. In contrast, Cry2Ab was associated with the brush border microvilli of the midgut epithelial cells of *L. hesperus* (Brandt et al., 2004). In the absence of putative receptor proteins in the *L. hesperus* gut, the ability of Cry proteins to bind non-specifically to glycoproteins could explain these findings (Li et al., 2011; Porcar et al., 2009).

2.3. Inappropriate gut enzymes and pH

Cry and Cyt proteins are produced as protoxins and activated by serine proteases in lepidopterans, and by cysteine and aspartic proteases in coleopterans. While cysteine proteases are active in *Nezara viridula* and *Halyomorpha halys* (Lomate and Bonning, 2016; Lomate and Bonning, 2018), the gut membrane and lumen cysteine proteases of *Acyrthosiphon pisum* did not hydrolyze Cry1Ac and Cry3Aa completely (Li et al., 2011). Similarly, *A. pisum* gut contents failed to activate Cry4Aa, which provides a possible explanation for why Cry proteins are not active against these insects (Rausch et al., 2016; Porcar et al., 2009). Additionally, cathepsin L attachment to the gut cell in *A. pisum* may reduce access to the protoxins (Cristofoletti et al., 2003).

Seed- or fruit-eating hemipterans rely heavily on extraoral digestion. Trypsin, chymotrypsin, and aminopeptidase activity are released into plant tissues in the saliva of *H. halys* and *N. viridula* (Cantón and Bonning, 2020; Lomate and Bonning, 2016; Lomate and Bonning, 2018), such that proteins are digested and broken down before ingestion. Ingested materials are then subject to digestion by cysteine proteases in the gut, indicating that few BPPs are likely to survive intact before encountering midgut cells in these insects. Aminopeptidase in saliva (Lomate and Bonning, 2016; Lomate and Bonning, 2018) can inhibit the binding of Cry protein to its gut receptor (Gill et al., 1995; Knight et al., 1994; Sangadala et al., 1994); however, binding of Cry proteins to salivary aminopeptidase has yet to be confirmed. Sap-feeding insects also secrete proteases while feeding, but roles for these enzymes beyond initial cell wall degradation and formation of the salivary sheath are unknown (Carolan et al., 2011; Coudron et al., 2007; Huang et al., 2019; Liu et al., 2016; Nicholson et al., 2012; van Bel and Will, 2016).

The gut pH can impact Cry protein degradation (Yang et al., 2020), and BPP activity by influencing protein solubilization (Domínguez-Arrizabalaga et al., 2020). An optimal pH may also be needed for BPP interaction with gut receptor proteins. The gut pH of *A. pisum* ranges from 5.5 to 8.5 along its length with cathepsin-L optimally active at pH 5.5 (Cristofoletti et al., 2003), while for *N. viridula*, the gut pH ranges from 5.4 to 6.9 (Cantón and Bonning, 2019). In Hemiptera that rely on extraoral digestion, the pH of the plant substrate could impact proteases that activate or degrade BPPs, with plant sap ranging from pH 7.5 to 8.6 for example. This has been demonstrated in *H. halys* and *N. viridula*, where salivary proteases were optimally active at a pH that differs from that of plant substrate (Lomate and Bonning, 2016; Lomate and Bonning, 2018).

The enhancement of BPP toxicity against hemipteran pests through modification to 1) resist gut proteases, 2) act at a different gut pH, and 3)

Table 1
Toxicity of bacterial pesticidal proteins against hemipteran species.

Official Name (old or unofficial name)	Target Species	LC50 μg/mL	% Mortality or Impact	Life Stage	Assay Material	Assay Method	Reference
Cry1Ab	*Diaphornia citri*	~120	–	Adult	PP	MF	Fernandez-Luna et al. (2019)
	Acyrthisiphon pisum	–	25 % at 500 μg/mL	Nymph	PP	MF	Porcar et al. (2009)
	Nilaparvata lugens	190.23 (136.67–243.80)	–	–	PP	MF	Shao et al. (2013b)
Cry1Ac	*A. pisum*	–	Significant at 500 μg/mL	Nymph (2nd instar)	PP	MF	Li et al. (2011)
	N. lugens	198.92 (111.95–255.20)	–	Nymph (2nd instar)	PP	MF	Shao et al. (2018)
Cry1Ba	*D. citri*	~120	–	Adult	PP	MF	Fernandez-Luna et al. (2019)
Cry1Cb2	*Myzus persicae*	9.01 (7.87–10.7)	–	Nymph	Spore-crystal mixture	MF	Torres-Quintero et al. (2022)
Cry2Aa	*Macrosiphum euphorbiae*	–	93 % at 200 μg/mL after 72 h	Adult	PP	MF	Walters and English (1995)
Cry3A	*M. euphorbiae*	–	52 % at 360 μg/mL after 72 h	Adult	PP	MF	
Cry3Aa	*A. pisum*	–	Significant at 500 μg/mL	Nymph (2nd instar)	PP	MF	Li et al. (2011)
Cry3Aa	*A. pisum*	–	60 % at 500 μg/mL	Nymph	PP	MF	Porcar et al. (2009)
Cry4Aa	*A. pisum*	70–100	100 % at 500 μg/mL	Nymph	PP	MF	
Cry11A	*M. euphorbiae*	–	64 % at 350 μg/mL after 72 h	Adult	PP	MF	Walters and English (1995)
Cry11Aa	*A. pisum*	–	100 % at 500 μg/mL	Nymph	PP	MF	Porcar et al. (2009)
Cry73Ba1 (Cry32Wa1)	*M. persicae*	32.7	–	Nymph (2nd Instar)	PP	MF	Palma et al. (2014b)
Mpp51Aa2 (Cry51Aa2)	*Lygus hesperus*	72.9	–	Nymph	PP	MF	Baum et al. (2012)
Mpp64Ba/ Mpp64Ca (Cry64Ba/Cry64Ca)	*Sogatella furcifera*	2.14	–	Nymph (3rd Instar)	PP	MF	Liu et al. (2018)
Mpp64Ba/Mpp64Ca (Cry64Ba/Cry64Ca)	*Laodelphax striatellus*	3.15	–	Nymph (3rd Instar)	PP	MF	
Tpp78Aa1(Cry78Aa1)	*N. lugens*	15.78	–	Adult	PP	MF	Wang et al. (2018)
	L. striatellus	6.89	–	Adult	PP	MF	
Tpp78Ba1(Cry78Ba1)	*L. striatellus*	9.723	–	Nymph (3rd Instar)	PP	MF	Cao et al. (2020)
Pra1Bb1(TIC4771)	*Lygus lineolaris*	–	Demonstrated activity	–	MH	–	Bowen et al. (2020b)
Prb1Bb1(TIC4772)	*L. lineolaris*	–	Demonstrated activity	–	MH	–	Bowen et al. (2020b)
Cyt1Aa	*A. pisum*	–	40 % reduction in average weight compared to control group for doses of 125 μg/ml or higher	Nymph	PP	MF	Porcar et al. (2009)
(CytA)	*A. pisum*	–	Demonstrated activity at 250–1000 μg/ml	–	PP	MF	Loth et al. (2015)
(CytB)	*A. pisum*	–	Demonstrated activity at 250–1000 μg/ml	–	PP	MF	
(CytC)	*A. pisum*	–	Demonstrated activity at 250–1000 μg/ml	–	PP	MF	
Vpb1Ae + Vpa2Ae	*Aphis gossypii*	0.576 (0.51–0.71)	–	Nymph (4th instar)	PP	MF	Sattar and Maiti (2011)
(Vip3)	*Bemisia tabaci*	389 (335–452)	–	Adults		MF	El-Gaied et al. (2020)
Vpb1Ac1 + Vpa2Ae3	*A. gossypii*	0.0875 (0.0342–0.1453)	–	–	PP	MF	Yu et al. (2011)
-(ET37)	*L. hesperus*	–	Inhibitory protein	–	–	–	Baum et al. (2009)
(IRDIG37126)	*Euschistus servus*	–	>50 % at 250 μg/ml	Nymph (2nd Instar)	PP	DB	Beeson IV and Church (2020)
(IRDIG31502)	*E. servus*	–	60 % at1000 μg/ml	Nymph (2nd Instar)	PP	DB	
(TIC809)	*L. hesperus*	–	Inhibitory protein	–	–	–	Baum et al. (2009)
(TIC809)	*L. lineolaris*	–	Inhibitory protein	–	–	–	
(TIC810)	*L. hesperus*	–	Inhibitory protein	–	–	–	
(TIC810)	*L. lineolaris*	–	Inhibitory protein	–	–	–	
(TIC812)	*L. hesperus*	–	Inhibitory protein	–	–	–	
(TIC1362)	*L. lineolaris*	–	mortality at 370 μg/ml	Nymph (Neonate)	PP	SF	Bowen et al. (2020a)

(*continued on next page*)

Table 1 (*continued*)

Official Name (old or unofficial name)	Target Species	LC50 μg/mL	% Mortality or Impact	Life Stage	Assay Material	Assay Method	Reference
(TIC1414)	*L. lineolaris*	–	mortality at 95 μg/ml	Nymph (Neonate)	PP	SF	
(TIC1415)	*L. lineolaris*	–	mortality at 150 μg/ml, survivors stunted	Nymph (Neonate)	PP	SF	
	L. hesperus	–	mortality at 150 μg/ml, survivors stunted	Nymph (Neonate)	PP	SF	
(TIC1498)	*L. lineolaris*	–	mortality at 50 μg / mL, survivors stunted	Nymph (Neonate)	PP	SF	
	L. hesperus	–	100 % at 2300 μg/ml	Nymph (Neonate)	PP	SF	
(TIC1886)	*L. lineolaris*	–	mortality at 124 μg/ml	Nymph (Neonate)	PP	SF	
	L. hesperus	–	mortality at 124 μg/ml	Nymph (Neonate)	PP	SF	
(TIC1922)	*L. lineolaris*	–	mortality at 3000 μg/ml, survivors stunted	Nymph (Neonate)	PP	SF	
(TIC4747)	*L. lineolaris*	–	Demonstrated activity	–	PP	DB	Bowen et al. (2020c)
	L. hesperus	–	Demonstrated activity	–	PP	DB	
	Euschistus heros	–	Demonstrated activity	–	PP	DB	
(TIC7181)	*L. lineolaris*	–	Demonstrated activity	–	PP	DB	

"-": Not available, PP: Purified Protein, MH: microbial host cell derived, MF: Membrane Feeding, DB: Diet based bioassay, SF: Sachet Feeding.

increase binding affinity to gut receptors, is described in Section 4.

3. Hemipteran-active pesticidal proteins

The new nomenclature classifies BPPs into 15 different structural classes, with one additional category (Xpp) for unclassified proteins (Crickmore et al., 2020). Activity against hemipteran species has been shown for different strains of *Bacillus thuringiensis* (Bt), *Dickeya dadantii*, *Xenorhabdus* spp., *Photorhabdus* spp., and *Pseudomonas* spp. (Table 2) (Beeson IV and Church, 2020; Bowen et al., 2020a; Bowen et al., 2020b; Bowen et al., 2020c; Loth et al., 2015). A total of 24 Bt-derived pesticidal proteins are reported to have activity against Hemiptera compared to 44 Bt-derived proteins that are effective against Coleoptera, with very little overlap. Of pesticidal proteins derived from other bacteria some have been shown to be effective against insects in both orders. Despite the predominance of cathepsins in the guts of both Hemiptera and Coleoptera, only four Bt-derived BPPs target both orders (specifically Cry1Ab, Cry1Ba, Cry10, and Mpp51Aa) suggesting that other physiological factors limit toxicity across these two groups (Domínguez-Arrizabalaga et al., 2020; Panneerselvam et al., 2022).

Hemipteran-active Bt pesticidal proteins belong to diverse structural classes based on pfam (Mistry et al., 2021), and CDD (Lu et al., 2020) analyses (Fig. 2)(Crickmore et al., 2022). Those derived from Bt belong to four structural classes: Cry, Cyt, Mpp and Tpp (Table 1) (Bowen et al., 2020a; Crickmore et al., 2020; Fernandez-Luna et al., 2019; Wang et al., 2018). Others include the Pra, Prb, and monalysin family beta-barrel pore-forming BPPs (Table 1, Fig. 2) (Bowen et al., 2020b; Beeson IV and Church, 2020). An overview of the different structural classes that include Hemipteran-active BPPs is provided below.

3.1. Three-domain pesticidal proteins (Cry)

The three domain pesticidal proteins belong to the α pore-forming class and are the best characterized of the BPPs classes, with the first structure published in 1991 (Li et al., 1991; Moar et al., 2017). The activated forms of these proteins have three distinct domains. Domain I is composed of a bundle of alpha helices and is involved in pore formation. Domain II has a beta prism structure with pseudo-threefold symmetry that appears to be related to carbohydrate binding proteins. Domain III has a beta sandwich fold. Domains II and III are involved in receptor binding and specificity of the pesticidal proteins as shown by domain swapping experiments that have changed target specificity (Berry and Crickmore, 2017; Lee et al., 1995; Pigott and Ellar, 2007).

3.2. ETX_MTX2 related pesticidal proteins (Mpp)

Five of the known hemipteran-active pesticidal proteins (Table 1; Fig. 2) possess an ETX_MTX2 domain. The ETX_MTX2 structural group of proteins belong to the β-pore forming class, with a head region and a tail region (Moar et al., 2017). The head region of Mpp proteins have sequence and structural diversity and are likely involved in receptor binding and target species specificity (Moar et al., 2017). The receptor binding domain of hemipteran-active Mpp proteins is discontinuous (Lacomel et al., 2021). The tail region comprised of long beta strands (Berry and Crickmore, 2017), is proposed to be involved in oligomerization and pore formation and is structurally conserved (Moar et al., 2017).

3.3. Toxin_10 related pesticidal proteins (Tpp)

Tpp pesticidal proteins, like Mpps, have head and tail regions (Berry and Crickmore, 2017). In contrast to Mpp proteins, the head region of Tpp proteins contain a beta trefoil (ricin-type beta-trefoil lectin) domain. The receptor binding region of Tpp proteins is located in the beta trefoil domain present toward the *N*-terminus of the head domain (Lacomel et al., 2021). Further, the tail regions of Tpp proteins consist of a Toxin_10 domain.

3.3.1. Beta trefoil domain

The beta trefoil domain is a carbohydrate binding domain presumed to result from triplication of a primitive gene encoding a 40 residue galactoside-binding peptide (Rutenber et al., 1987). The presence of glutamine-any residue-tryptophan $(QxW/F)_3$ is the characteristic feature of a member of the beta trefoil domain family (Hazes, 1996; Hirabayashi et al., 1998). These $(QxW/F)_3$ scaffolds have combined with proteins of diverse functionality, resulting in the evolution of new proteins with novel attributes (Hazes, 1996; Hirabayashi et al., 1998). The beta trefoil domain, which is present in several unrelated BPPs with hemipteran toxicity (Table 1, Fig. 2) may provide carbohydrate binding ability, thus conferring specificity (Hazes, 1996). The beta trefoil domain in hemipteran-active pesticidal proteins may interact with -glycoproteins or glycolipids to facilitate receptor binding or may function during other stages of BPP mode of action (Berry and Crickmore, 2017). The beta trefoil domain is located toward the *N*-terminus of

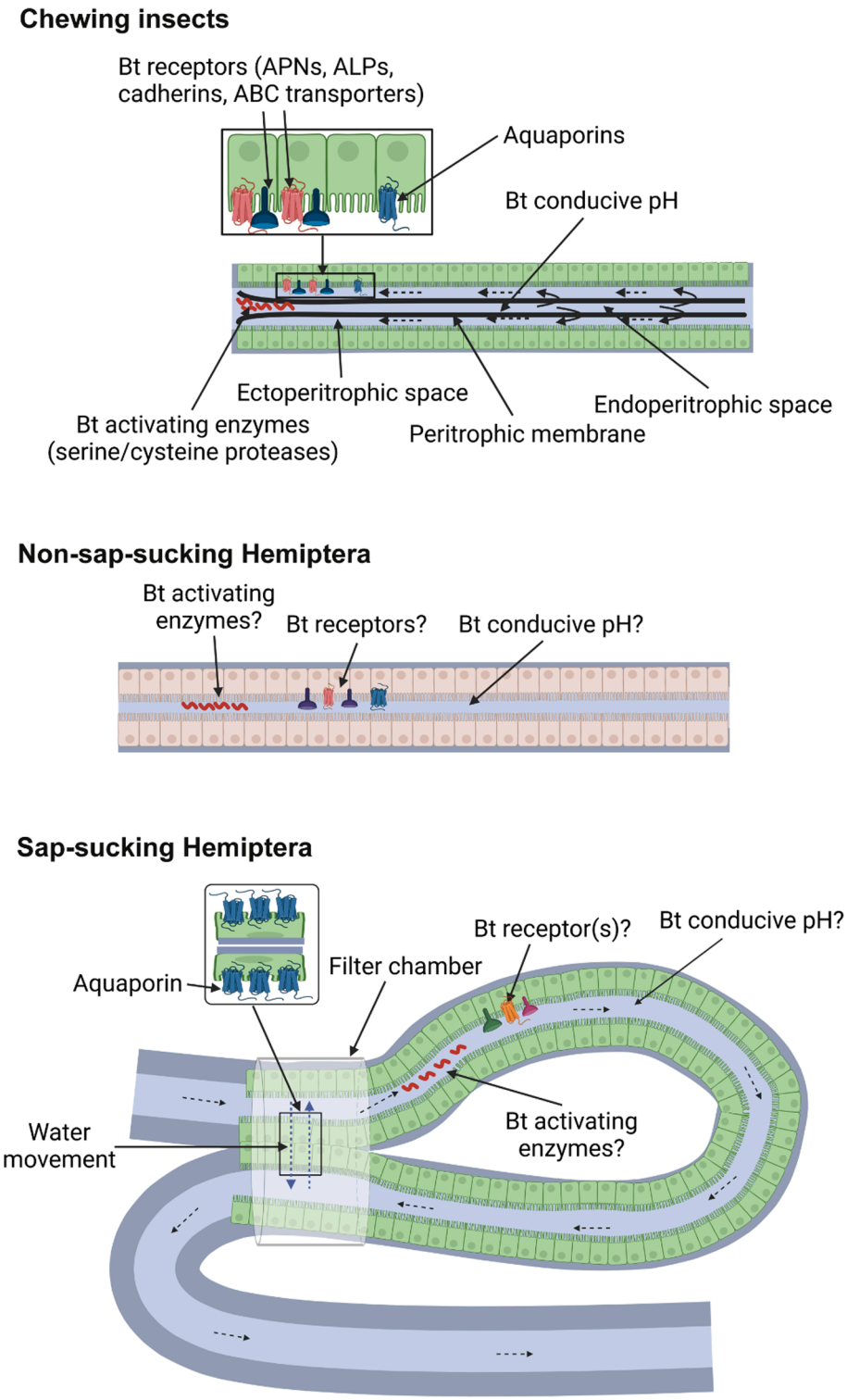

Fig. 1. Features of the hemipteran gut that may impact BPP toxicity**.** Schematics illustrate key aspects important for BPP toxicity for chewing insects such as coleopteran and lepidopteran species, non-sap-feeding Hemiptera such as stink bugs, and plant sap-feeding Hemiptera such as aphids and whitefly. Bt, *B thuringiensis*-derived BPP example. Dashed arrow indicates direction of water movement.

Tpp78Aa1 and Tpp78Ba1, and toward the C-terminus of TIC4747, TIC7181, and Cry73Ba1 proteins (Fig. 2). Interestingly, plant lectins, which are classified into 12 families, including the beta trefoil family, have demonstrated toxicity towards Hemiptera (Macedo et al., 2015). The most likely mechanism of plant lectin toxicity against hemipteran insects is interference with physiological processes as a result of lectin binding to glycoproteins or glycan structures (Macedo et al., 2015). The precise function of the beta trefoil domain and/or $(QxW/F)_3$ motif in the mechanism of action of bacterial pesticidal proteins has yet to be determined.

Table 2
Bacteria that encode insecticidal proteins with hemipteran toxicity.

Species	Pesticidal protein class
Bacillus thuringiensis	Cry, Tpp, Mpp, Cyt, Vip, Vpa, Vpb, Xpp, TICs
Dickeya dadantii	Cyt-like
Pseudomonas sp.	IRDIG
Photorhabdus sp.	Pra/Prb, TICs
Xenorhabdus sp.	Pra/Prb

3.4. *Photorhabdus Insect-Related toxin a component (Pra) and B component (Prb) pesticidal proteins*

Species of *Xenorhabdus* and *Photorhabdus* bacteria have a symbiotic relationship with entomopathogenic nematodes in the genus *Steinernema* and *Heterorhabditis* respectively. The nematodes release these bacteria on entry into the hemocoel of the insect host. The bacteria then release proteins that kill the host insect (ffrench-Constant et al., 2007). These insecticidal proteins include the Pra and Prb proteins, which form a binary pesticidal protein PrAB encoded by two separate bacterial plasmid genes. Waterfield et al. (2005) provided experimental evidence that both Pra (PirA) and Prb (PirB) components are required for insecticidal activity (Waterfield et al., 2005). However, the individual components Pra1Bb1 or Prb1Bb1 are toxic to the hemipteran *Lygus lineolaris* (Table 1) (Bowen et al., 2020b).

Genes encoding Pra and Prb were subsequently found in a variety of other bacterial species (Yang et al., 2017). X-ray crystallography structures are available for Pra and Prb proteins from the gram-negative bacterium *Vibrio parahaemolyticus* (Crickmore et al., 2022; Lee et al., 2015). The combined structural topology of Pra2Aa1(PirA^{vp}) and Prb2Aa1 (PirB^{vp}) from *V. parahaemolyticus* shows similarities to three domain proteins, even though the shared sequence identity is less than 10 % (Crickmore et al., 2022; Lee et al., 2015). Pra2Aa1 folds into an eight-stranded antiparallel β-barrel with jelly-roll topology with structural similarity to Cry domain III. Pra2Aa1 is proposed to play a role in receptor recognition and membrane insertion (Lee et al., 2015). The Prb2Aa1 *N*-terminal domain has seven α-helices and a C-terminal domain with ten β-strands. The *N*-terminus and C-terminus of Prb2Aa1 correspond to Cry domain I (pore formation) and domain II (receptor binding), respectively (Lee et al., 2015). Structural similarities to Cry three-domain proteins suggest that the Pra2Aa1 and Prb2Aa1 complex induces cell death by pore formation (Lee et al., 2015). Hemipteran-active Pra and Prb pesticidal proteins (Pra1Bb1) and (Prb1Bb1) share 32 % and 29.50 % sequence identities with Pra2Aa1 and Prb2Aa1, respectively.

A biotechnological advance for use of this binary pesticidal proteins was fusion of the two proteins by linking their in-frame coding sequences. Fusion proteins were generated either by fusing sequences encoding Pra and Prb proteins from the same or from different bacterial operons and hemipteran activity of some of these fusions was demonstrated (Bowen et al., 2020b) (Table 3).

3.5. *Cytolytic pesticidal proteins (Cyt)*

Cyt proteins have been shown to be active against aphids (Table 1). The Cyt proteins consist of a single domain of α/β architecture with a β-sheet in the center enclosed by two α-helical layers. The central β-sheet is made of six antiparallel β-strands surrounded by an α-helix layer composed of α1 and α2 on one side and α3-α5 on the other (Cohen et al., 2011; Cohen et al., 2008; Li et al., 1996). Sequence alignment has revealed four conserved blocks: block 1, helix α1; block 2, α5 to β5; block 3, β6-β7; and block 4, α6-β8 (Butko, 2003; Xu et al., 2014). Cyt proteins directly interact with saturated membrane lipids such as phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin in the midgut (Rodriguez-Almazan et al., 2011). Two models have been proposed for the mechanism of action of Cyt proteins: a pore formation model, and a detergent-like membrane solubilization model (Butko, 2003). These two models are not mutually exclusive. At low concentrations, Cyt proteins may oligomerize and form a pore. As the Cyt/lipid ratio increases to a critical level, the membrane may not be able to adapt to multiple assembled molecules and break up into protein/lipid complexes as a result (i.e., detergent-like membrane solubilization). Cyt proteins have not been commercialized for use in transgenic crops because their lipid binding and detergent-like membrane solubilization mode of action lacks the desired specificity.

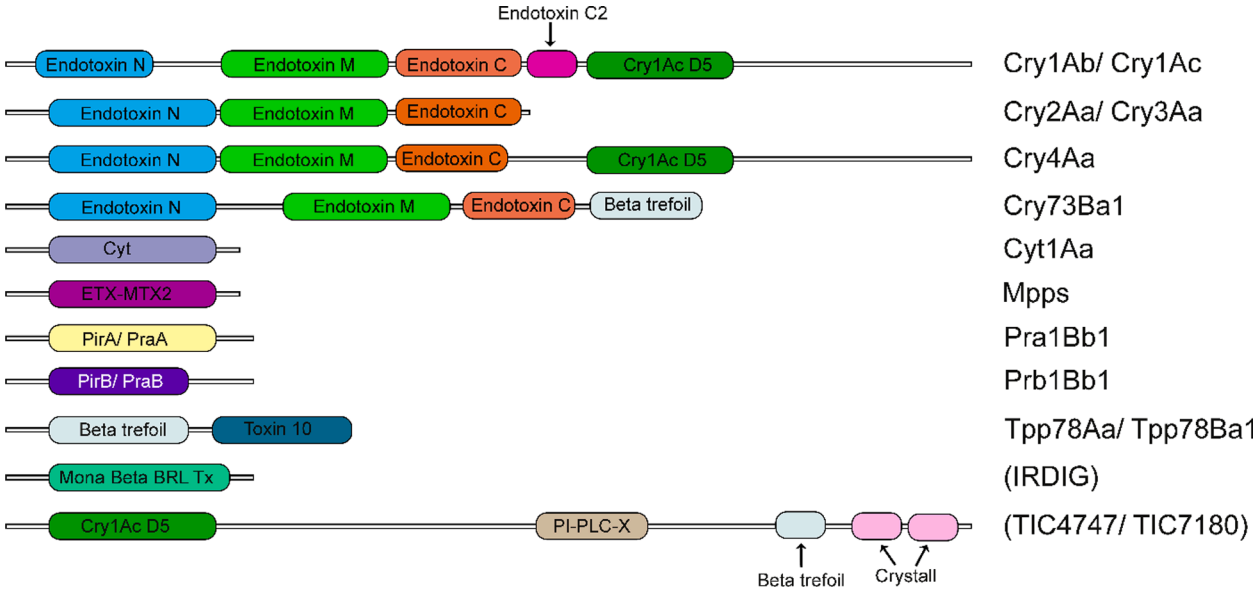

Fig. 2. Structural diversity of Hemiptera-active pesticidal proteins. The hemipteran-active pesticidal proteins belonging to each structural class with pfam and CDD domains as indicated are: **Mpps**: Mpp51Aa1, Mpp64Ba1, Mpp64Ca1, TIC1362, TIC1414, TIC1415, TIC1498, TIC1886 and TIC1922; **3-Domain (Cry)**: Cry1Ab, Cry1Ba, Cry73Ba1, Cry2Aa, Cry3Aa, Cry4Aa; **Tpps**: Tpp78Aa1 and Tpp78Ba1; **Cyt:** Cyt1Aa and Cyt2Aa; **MonaBetaBRL_TX:** IRDIG37126 and IRDIG31502; **TIC4747 and TIC7181** possess Cry1Ac_D5, PI-PLC-X, Beta trefoil_lectin_2 and Crystall domains. Brackets in the figure indicate unofficial protein names.

Table 3
Modifications to bacterial pesticidal proteins for improved toxicity against hemipteran pests.

Name	Modifications	Target species	LC50 (μg/μl)	% mortality	Life stage	Assay material	Assay method	Reference
Cry1Ab	Replacement of loop 1 by GBP P2S (L1-P2S)	*Nilaparvata lugens*	37.82 (30.20–46.38)	–	3rd instar	Purified protein	Artificial diet	Shao et al. (2016)
	Replacement of loop 2 by GBP P2S (L2-P2S)		21.54 (17.83–25.31)					
	Replacement of loop 3 by GBP P2S (L3-P2S)		37.47 (19.82–61.20)					
	Replacement of loop 1 by GBP P1Z (L1-P1Z)		137.77 (116.63–169.43)					
	Replacement of loop 2 by GBP P1Z (L2- P1Z)		77.45 (56.20–112.59)					
	Replacement of loop 3 by GBP P1Z (L3- P1Z)		231.72 (182.03–330.95)					
Cry1Ac	Cry1Ac ricin B-chain (RB) fusion	*Cicadulina mbila*		95	Nymph	Transgenic plants	Cut leaf discs	Mehlo et al. (2005)
Cyt2a	Insertion of GBP into loop 1 (CGAL1)	*Acyrthosiphon pisum*	19.71 ± 5.74 (2.51–21.00)	–	2nd instars	Purified protein	Artificial diet	Chougule et al. (2013)
		Myzus persicae	58.04 ± 2.08 (35.01–65.73)					
	Insertion of GBP into the loop 3 (CGAL3)	*A. pisum*	9.55 ± 2.5 (0.65–12.23)	–				
		M. persicae	42.68 ± 0.4 (17.18–83.04)					
	Insertion of GBP into the loop 4 (CGAL4)	*A. pisum*	11.92 ± 1.99 (0.83–22.43)	–				
		M. persicae	92.75 ± 2.54 (34.67–152.96)					
	Substitution of amino acids by GBP in loop 1 (CGSL1)	*A. pisum*	28.74 ± 2.92 (6.40–93.40)	–				
	Substitution of amino acids by GBP in loop 4 (CGSL4)	*A. pisum*	15.13 ± 0.23 (4.3–25.60)	–				
Mpp51Aa2	[HYS_ Δ(196–201)]	*L. hesperus*	22.4	–	–	Purified protein	Diet based bioassay	Gowda et al. (2016)
	[F46S, Y54H, S167R, S217N, HYS_ Δ(196–201)]		5.9					
	[F46S, Y54H, T93A, S167R, S217N, HYS_ Δ(196–201)]		2.9					
	[F46S, Y54H, S95A, S167R, S217N, HYS_ Δ(196–201)]		2.4					
	[F46S, Y54H, F147A, S167R, S217N, HYS_ Δ(196–201)]		1.1					
	[F46S, Y54H, T93A, F147A, S167R, S217N, HYS_ Δ(196–201)]		1.45					
	[F46S, Y54H, Q149E, S167R, S217N, HYS_ Δ(196–201)]		1.4					
	[F46S, Y54H, S95A, F147A, S167R, S217N, HYS_ Δ(196–201)]		0.8					
	[F46S, Y54H, S95A, F147A, S167R, P219R, HYS_ Δ(196–201)]		9.9					
	[F46S, Y54H, S95A, F147A, S167R, P219R, V251A, HYS_ Δ(196–201)]		0.6					
	[F46S, Y54H, S95A, F147A, S167R, P219R, R273W, HYS_ Δ(196–201)]		1.35					
	[F46S, Y54H, S95A, F147S, Q149E, S167R, P219R, R273W, HYS_ Δ(196–201)]		0.3					
	[F46S, Y54H, S95A, F147A, S167R, P219R, N239A, V251A, HYS_ Δ(196–201)]		0.4					
Mpp51Aa2	[F46S, Y54H, S95A, F147A, S167R, S217N, HYS_ Δ(196–201)]	*L. lineolaris*	223	–	–	Purified protein	Diet based bioassay	Gowda et al. (2016)
	[F46S, Y54H, S95A, F147A, S167R, P219R, HYS_ Δ(196–201)]		8.3					
	[F46S, Y54H, S95A, F147A, S167R, P219R, V251A, HYS_ Δ(196–201)]		4.8					
			5.9					

(*continued on next page*)

Table 3 (*continued*)

Name	Modifications	Target species	LC50 (µg/µl)	% mortality	Life stage	Assay material	Assay method	Reference
	F46S, Y54H, S95A, F147A, S167R, P219R, R273W, HYS_ Δ(196–201)							
	F46S, Y54H, S95A, F147S, Q149E, S167R, P219R, R273W, HYS_ Δ(196–201)		0.85					
	F46S, Y54H, S95A, F147A, S167R, P219R, N239A, V251A, HYS_ Δ(196–201)		1.2					
Mpp83Aa1	Insertion of *Nv* APN binding peptide (43A)	*Nezara viridula*	–	58.33	2nd instar	Purified protein	Artificial diet	Banerjee et al. (2022)
	Insertion of *Nv* gut binding peptide (43 N)			43.33(NS)				
	Replacement by *Nv* APN binding peptide (70–76A)			36.66(NS)				
	Replacement by *Nv* APN binding peptide (172–178A)			53.33				
	Replacement by *Nv* APN binding peptide (208–214A)			40 (NS)				
	Replacement by *Nv* APN binding peptide (224–230A)			61.66				
	Replacement by *Nv* APN binding peptide (269–275A)			65				
	Replacement by Nv BBMV-BP (70–76 N)			53.33				
	Replacement by Nv BBMV-BP (172–178 N)			78.33				
	Replacement by Nv BBMV-BP (208–214 N)			65				
	Replacement by Nv BBMV-BP (224–230 N)			83				
	Replacement by Nv BBMV-BP (269–275 N)			63.33				
IRDIG37126	D18S	*E. servus*	–	100 % mortality at 1000 µg/ml	Nymph	Purified protein	Diet based bioassay	Beeson IV and Church (2020)
	D18P			100 % mortality at 1000 µg/ml				
	D18R & D75E			97 % mortality at 1000 µg/ml				
	D18L & D75E			94 % mortality at 1000 µg/ml				
	D18Q & D75E			100 % mortality at 1000 µg/ml				
TIC6880	Pra + Prb fusion (TIC4771 + TIC4772)	*Nezara viridula*	–	–	–	–	–	Bowen et al. (2020b)
		Euschistus heros	–	–	–	–	–	
		L. lineolaris	–	–	–	–	–	
		L. hesperus	–	–	–	–	–	
TIC9316	Pra + Prb fusion (TIC7575 + TIC7576)	*N. viridula*	–	–	–	–	–	
		E. heros	–	–	–	–	–	
		L. lineolaris	–	–	–	–	–	
		L. hesperus	–	–	–	–	–	
TIC9317	Pra + Prb fusion (TIC7660 + TIC7661)	*N. viridula*	–	–	–	–	–	
		L. lineolaris	–	–	–	–	–	
		L. hesperus	–	–	–	–	–	
TIC9318	Pra + Prb fusion (TIC7662 + TIC7663)	*N. viridula*	–	–	–	–	–	
		E. heros	–	–	–	–	–	
		L. lineolaris	–	–	–	–	–	
		L. hesperus	–	–	–	–	–	
TIC9319	Pra + Prb fusion (TIC7664 + TIC7665)	*N. viridula*	–	–	–	–	–	
		L. lineolaris	–	–	–	–	–	
		L. hesperus	–	–	–	–	–	
TIC9320	Pra + Prb fusion (TIC7668 + TIC7669)	*N. viridula*	–	–	–	–	–	
		E. heros	–	–	–	–	–	
		L. lineolaris	–	–	–	–	–	
TIC9322	Pra + Prb fusion (TIC7666 + TIC7667)	*L. lineolaris*	–	–	–	–	–	
TIC10378	Pra + Prb fusion (TIC710361 + TIC10370)	*E. heros*	–	–	–	–	–	
TIC10380	Pra + Prb fusion (TIC710363 + TIC10372)	*E. heros*	–	–	–	–	–	

-: Not available; GBP: gut binding peptide; BBMV-BP: brush border membrane vesicle binding peptide; HYS_Δ196–201: contiguous HYS deletion in residue range 196–201.

3.6. Monalysin family beta-barrel pore-forming toxins (MonaBetaBRL_TX)

The monalysin family beta-barrel pore-forming toxin domain is present in IRDIG37126 and IRDIG31502 proteins that are active against the brown stink bug (*Euschistus servus*) (Table 1). Monalysins show structural similarities to the epsilon toxin from *Clostridium perfringens* (Cole et al., 2004) and to aerolysin (Parker et al., 1994). The monalysin protein from *Pseudomonas entomophila* is secreted as a pro-protein that requires cleavage to become fully active (Leone et al., 2015). The pro-protein of monalysin forms a stable, doughnut-like 18-mer complex. The 18-mer complex consists of two disk-shaped nonamers adhered together by *N*-terminal swapping of the pro-peptides (Leone et al., 2015). In the pro-protein, the pore forming region is fully buried in the center of the doughnut. Upon activation, the two disk-shaped nonamers dissociate to expose the transmembrane (pore forming) segment, which is deployed for pore formation. Because the monolysin protein lacks a domain for cellular receptor binding, the proposed mechanism of action of monolysin differs from the Cry three domain, Mpp, and Tpp groups. The doughnut-like 18-mer complex may provide an advantage by bypassing a minimum receptor-dependent threshold concentration for oligomerization into the pore-forming complex (Leone et al., 2015).

3.7. Other domains present in hemipteran-active pesticidal proteins

Two novel pesticidal proteins active against Hemiptera, TIC4747 and TIC7181, have been identified (Bowen et al., 2020c) (Table 1). These proteins are unique in possessing a Cry1Ac_D5 domain at the *N*-terminus, PI-PLC-X in the middle, and beta trefoil_lectin_2 and two beta/gamma crystallin domains at the C-terminus.

3.7.1. Cry1Ac_D5

This domain is located at the C-terminus of the protoxin of three domain pesticidal proteins, such as Cry1Ac. In these proteins, domain V (D5) has a beta-roll topology similar to that of domains II and III, and shows structural similarity to the carbohydrate binding modules found in sugar hydrolases. This domain is proposed to provide stability to the Cry1Ac protoxin (Evdokimov et al., 2014).

3.7.2. PI-PLC-x

Phosphoinositide-specific phospholipase C domain X (PI-PLC-X) enzymes contain 2 domains (X and Y) which produce a TIM barrel-like topology containing active site residues (SMART ACC:SM00148). Bacterial PLCs can act on eukaryotic membranes (Meldrum et al., 1991) while PI-PLC enzymes play an important role in signal transduction processes (Meldrum et al., 1991). Phospholipase C is a remarkable signaling moiety, as it can directly modulate three distinct signals: inositol 1,4,5-trisphosphate (IP_3), diacylglycerol, and phosphatidylinositol 4,5-bisphosphate (PIP2). Further, PI-PLC regulates Ca^{2+} signaling (Putney and Tomita, 2012).

3.7.3. Crystallin (Beta/Gamma crystallin)

The βγ-crystallin superfamily has similar domain topology to mammalian eye lens β- and γ-crystallins and are formed from duplicated Greek key motifs (Aravind et al., 2009). The members of this poorly understand family also possess a Ca^{2+}-binding motif.

4. Optimization of bacterial pesticidal proteins for use against Hemiptera

Hemipteran pests appear generally less susceptible to Cry proteins / BPPs than insects of other orders. This has been attributed to lack of activation of Cry proteins in the hemipteran gut due to pH, absence of the appropriate activating enzymes, and / or lack of gut binding (Section 2). Protoxin, activated and solubilized Cry proteins such as Cry1Cb2 have toxicity against the green peach aphid, *Myzus persicae* (Torres-Quintero et al., 2022), and transgenic plant expression of any form can confer resistance. Various molecular and biotechnological methods have been employed to broaden the target range of BPPs to include Hemiptera or to increase the level of toxicity, including the addition of gut binding peptides and site directed mutagenesis of BPPs. The majority of protein modifications have been made for BPPs derived from *B. thuringiensis*, and BPPs modifications that improved toxicity against hemipteran pests are listed in Table 3.

4.1. Enhancing proteolytic activation

Appropriate proteolytic processing of a BPP protoxin into its active form is essential for toxicity and proteolytic activation can also increase solubility (Oppert, 1999). The lack of enzymatic activation of BPPs in the hemipteran gut is a primary factor contributing to the lack of toxicity of some Cry protoxins. Indeed, the major proteases in the gut of *A. pisum* are cathepsin L and cathepsin B type (Carrillo et al., 2011) in contrast to the serine proteases in dipteran species that are susceptible to these BPPs. In an effort to overcome this limitation, enzyme cleavage sites for cathepsin L (FRR) and cathepsin B (FR) were introduced into Cry4Aa to facilitate activation and toxicity against the pea aphid, with modest improvements relative to the native Cry4Aa (Rausch et al., 2016).

4.2. Site-directed mutagenesis

Site-directed mutagenesis is a molecular strategy that creates changes in the DNA sequence to alter specific amino acids in a protein. Regardless of the domain targeted, three outcomes are possible: impaired or diminished toxicity, no change in activity, or enhanced activity compared to the wild type, unmodified BPPs. Enhanced activity is the least likely to occur, although many successful examples have been reported for use against non-hemipteran pest species (Deist et al., 2014). Gain of function site-directed mutagenesis has helped clarify the mechanism of action of some BPPs. Site-directed mutagenesis is also useful for understanding protein interactions with the insect gut (e.g., specificity and receptor binding) and to improve insecticidal toxicity against target pests within the orders Lepidoptera, Diptera and Coleoptera for example (Deist et al., 2014; Vilchez, 2020). While only two studies have used this technique to elucidate function or to enhance toxicity of pesticidal proteins against hemipteran pests, site-directed mutagenesis provides a powerful approach for generating diverse BPPs with hemipteran toxicity.

The most comprehensive study that employed mutagenesis to improve the toxicity of a BPP against a hemipteran was performed with Mpp51Aa2 (formerly Cry51Aa2), which is toxic to *Lygus* species (Baum et al., 2012). Gowda et al. (2016) mutated each residue of Mpp51Aa2 to alanine (one or two at a time) to identify amino acids critical to protein function or that resulted in enhanced insecticidal activity. The variant protein Mpp51Aa2.834_16 (with mutations F46S, Y54H, S95A, F147S, Q149E, S167R, R219R, R273W, and deletion of residues HYS at positions 196–201) showed increased insecticidal activity against *Lygus* sp. This work was a critical part of the development of the transgenic event MON88702, which produces the modified Mpp51Aa2 and has been shown to protect cotton against some hemipteran and thysanopteran pests (Bachman et al., 2017).

In another notable study, variants of a monalysin, IRDIG37126 were generated. Some of the resulting single site and double site mutations such as D18S, D18P, D18R_D75E, D18L_D75E and D18Q_D75E improved the efficacy of this protein against the brown stink bug, *Euschistus servus* (Beeson IV and Church, 2020).

4.3. Gut binding peptides as artificial anchors

One potential reason for the lack of BPP activity against hemipterans is insufficient binding to the gut of the target insect. Therefore, the addition to BPPs of peptides selected for binding to the gut of the target species could enhance the binding and subsequent toxicity of pesticidal proteins. Such gut binding peptides are typically isolated by feeding the target insect on a phage display library, and isolating, amplifying and rescreening phages that bind to the surface of the gut (Mishra et al., 2021).

The addition of gut binding peptides effectively augmented the toxicity of Cyt and Cry proteins against the pea aphid, *A. pisum*, and the rice brown planthopper, *Nilaparvata lugens*. The increased toxicity is hypothesized to result from increased gut binding with the peptide providing an artificial anchor for gut attachment of the BPPs. Modification of the dipteran-active Cyt2Aa by addition of the gut binding peptide GBP3.1 in specific loop regions, resulted in improved binding and toxicity towards *A. pisum* (Chougule et al., 2013). This work also provided information on which regions of Cyt2Aa are important for toxicity; toxicity was lost when loop 3 was modified and toxicity was increased when loop 4 was targeted. Aphid toxicity corresponded with midgut damage observed by transmission electron microscopy (Chougule et al., 2013). Notably, the extent of increased binding of modified Cyt2Aa proteins to the gut did not correlate with toxicity; some modified proteins with strong in vitro binding capacity were unstable on exposure to digestive enzymes in the aphid gut.

Similar work involved the replacement of loop regions of Cry1Ab domain II with the P2S peptide isolated by screening a phage display library for *N. lugens* gut binding peptides. The modified Cry1Ab showed a nine-fold enhancement of activity (Shao et al., 2016) relative to that of native Cry1Ab against *N. lugens* (Shao et al., 2013a). The toxicity of the modified Cry1Ab towards *N. lugens* was associated with extensive damage to the gut epithelium.

To target the southern green stink bug, *Nezara viridula*, Mpp83Aa1 was modified with gut binding peptides selected for binding to recombinant *N. viridula* aminopeptidase N or to brush border membrane vesicles derived from the insect gut and enriched in gut surface proteins (Wolfersberger, 1993). Some of the modified Mpp83Aa1 constructs showed increased binding and enhanced toxicity relative to the native protein (Banerjee et al., 2022). This study introduced the use of recombinant gut surface proteins (aminopeptidase N in this example) as targets for screening phage display libraries allowing for selection of peptides that bind specific gut proteins. Analysis of the gut surface proteome of pests of interest (Tavares et al., 2022) and identification of the most abundant proteins on the gut surface will provide valuable insight into the most abundant gut surface proteins to target when using the peptide modification approach for BPP optimization. However, it is currently unknown whether increased binding to any gut surface protein is sufficient for toxicity, or whether binding to specific BPP receptor proteins is required.

4.4. Fusion of pesticidal proteins with lectin binding domains

An alternative approach for increasing binding of BPPs to the surface of hemipteran gut epithelia is to take advantage of the glycan binding properties of plant-derived lectins. For example, the ricin B-chain, which binds galactose- and *N*-acetylgalactosamine (Houston and Dooley, 1982), was fused with Cry1Ac, expressed in transgenic maize and rice and tested against various insect pests (Mehlo et al., 2005). While this fusion protein showed increased toxicity to the maize leafhopper, *Cicadulina mbila*, relative to Cry1Ac, no toxicity was noted to a second hemipteran, the bird cherry-oat aphid, *Rhopalosiphum padi*. Interestingly, *N*-acetyl galactosamine is a binding partner for Bt- pesticidal proteins (Garczynski et al., 1991; Knowles et al., 1991). Given the abundance of mannose residues in the hemipteran gut (Scheys et al., 2019), the efficacy of mannose binding lectins in enhancing binding and toxicity against hemipteran pests, would be of interest.

5. Concluding remarks and future perspectives:

Hemipteran pests are undoubtedly among the most significant threats to agricultural production, causing losses through both feeding-associated damage and the transmission of plant pathogens. While bacteria-derived pesticidal proteins offer an environmentally friendly alternative to the deployment of potentially damaging chemical insecticides for control of these insects, relatively few proteins with hemipteran toxicity have been identified. However, recently identified hemipteran-active BPPs and biotechnological approaches used to improve BPP efficacy such as addition of gut binding peptides, and mutagenesis show considerable promise.

Although not employed thus far to generate hemipteran-active BPPs, domain swapping using BPPs with known hemipteran toxicity could result in improved Hemiptera-active pesticidal proteins as shown for BPPs active against other insect orders (Deist et al., 2014; Yamamoto, 2022). Site-directed mutagenesis has demonstrated utility toward this end (Gowda et al., 2016), and the use of gut binding peptides holds considerable potential including for use with BPPs beyond Cry proteins. However, screening for Hemiptera-active BPPs is expected to yield additional proteins of interest, and a basal level of toxicity is generally critical for the successful use of these biotechnological strategies for protein enhancement.

Improved sequencing technologies and bioinformatics tools have resulted in the identification of many new pesticidal proteins from a wide range of bacterial sources. Recent advances in the generation of structural information (Jumper et al., 2021) when applied to BPPs will 1) facilitate elucidation of their mechanisms of action, 2) improve the prediction of targets for wild type BPPs and 3) inform strategies for optimized efficacy against specific pests. Taken together, the outlook is promising for future deployment of BPPs to combat Hemiptera-mediated agricultural losses toward the sustainable production of food.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The article describes published research and does not include any original data.

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