CHAPTER 15

Transgenic Plants and Plant Biotechnology

SUMMARY

Traditionally, crops have been enhanced through careful selective breeding for desirable traits. With the great leaps made in molecular biology, it is now possible to introduce new genes into plants to give them desirable traits and characteristics. Plants containing foreign genes are called transgenic plants.

Plants cells are totipotent, which means a single cell can differentiate into various tissues. Scientists are able to manipulate plant cells in culture and then regenerate entire plants. Plant cells can be grown in liquid culture or on a solid medium and derived from apical meristems, root tips, pollen grains, eggs, and embryos. Plant hormones can be added to the plant tissue culture medium to elicit desired effects, such as the addition of auxins, which promote differentiation and growth. Many different clones can be produced from plant tissue cultures.

Plant engineering can not only produce crop plants that have greater yields or production of novel products, but could also have resistance to herbicides, insect pests, and environmental stresses. Genes containing desirable traits are identified from other organisms and then inserted into the genome of the plant. Often this process involves the use of the Ti plasmid from *Agrobacterium tumefaciens* to deliver the genes. The Ti plasmid is normally responsible for causing tumor growth in plants. Biotechnologists can render the Ti plasmid harmless and insert transgenes into the portion of the plasmid that gets transferred to the plant host during a normal infection. That portion also recombines with the plant's chromosome, thus delivering a gene of interest directly into the plant's genome. To ensure proper expression in the plant, the transgene is constructed downstream of a plant promoter. The promoter may be constitutive or inducible.

Particle bombardment utilizes a particle gun as a method to deliver transgenes into plant tissue. Basically, gold or tungsten particles are coated with the gene of interest and loaded into a particle gun. The gun is fired at a piece of plant tissue using either air, helium, or electrical charge. Some of the coated metals make it into the cytoplasm of cells where DNA is dissolved and integrates into the genome. This method is less specific than using the Ti plasmid to deliver transgenes, however.

Regardless of the delivery method, detection for the presence of the gene of interest occurs using a selectable marker. Two of the most popular selectable markers are the gene for resistance to the antibiotic neomycin and the gene for luciferase production. Luciferase can also be used to quantify gene expression *in vivo*, thus acting as a reporter gene. In crop plants that are used for human consumption, there is much debate about the presence of the selectable marker. Many people are concerned that the marker could potentially cause an allergic reaction in individuals who consume transgenic plant products. One method to eliminate this issue is to remove the selectable marker or reporter gene after the presence of the transgene has been confirmed. The Cre/*loxP* system is widely used to accomplish this task. Cre is a recombinase protein that recognizes *loxP* sequences in the DNA. Selectable markers and reporter genes are constructed with *loxP* sites on either end. Once the presence of the transgene has been confirmed, the Cre recombinase promotes the deletion of the marker or reporter using the *loxP* sequences.

Once a transgenic plant has been created, several levels of testing must be completed. Specifically, the level of expression of the transgene is monitored. Also, the product of the transgene may harm the plant and/or may be harmful to the ecosystem. Finally, the transgene must be transferred to a plant that has greater crop yields. This transfer is accomplished by back-crossing in traditional plant breeding experiments. Additionally, various government regulatory agencies set up rigorous guidelines that must be followed.

Before the transgenic plant is made commercially available, it must pass rigorous testing and approval from the agencies.

Transgenic plants have already been produced with herbicide and insect resistance, as well as some that are tolerant to various environmental stresses. Herbicides are often necessary to rid the fields of weeds. However, most are not discriminatory against crop plants. Glyphosate, which is the active ingredient in many herbicides, targets the biosynthesis pathway for aromatic amino acids. Transgenic crops have been engineered with a mutant version of an enzyme that is resistant to the action of glyphosate. This yields a transgenic plant that is resistant to the action of glyphosate.

Unlike herbicides, insecticides are more hazardous to humans. Insect-resistant plants have also been created, which eliminate the need for spraying fields with insecticides. The gene for a toxin from the soil bacterium *Bacillus thuringiensis* was engineered into plants under the control of plant promoters. This toxin (Bt toxin, also called Cry) acts specifically on the digestive system, where it produces holes. When insects consume plant tissue that is expressing the Bt toxin, they succumb to its effects. Stress tolerance can also be increased in plants by introducing a pathway for trehalose synthesis. This sugar moderates the effects of stress in drought-tolerant plants, fungi, and bacteria by absorbing and releasing water molecules. Introduction of the pathway into crop plants has yielded plants that are more resistant to similar conditions.

Novel genes can also be determined in plants using functional genomics. The goal is to identify new genes or pathways that could be engineered to greatly improve plants of human interest. Several techniques exist to identify these genes. Genes may be silenced by RNAi to determine their effects on the entire plant. Additionally, gene knockouts can be made using mutagenesis, either random or targeted. Fast neutron mutagenesis induces random DNA deletions. TILLING (targeting-induced local lesions in genomes) creates point mutations that are identified using mismatched hybrid PCR.

The use of genetically modified crops is controversial. The safety of the food produced from transgenic crops is of utmost importance. Many people are concerned about the reporter genes, selectable markers, such as antibiotics, and other elements used in the process of creating a transgenic plant. Claims of allergic reactions to foods have been made against companies such as Starlink, which accidentally released transgenic corn into the food supply; this corn ended up in taco shells on grocery shelves. Extensive testing was performed on the affected individuals, but no evidence was found to support the individuals'claims.

Environmental groups have also laid claims against transgenic plants. Specifically, monarch butterfly caterpillars that are exposed to pollen from transgenic crops expressing Bt toxin are stunted in growth or die off at higher rates than their unexposed counterparts. The study to test these effects was actually extremely flawed in various ways. First, the amount of pollen on milkweed, the preferred food source for the caterpillars, was not controlled. Other variables, such as weather and natural movement of the caterpillars, were also not controlled in the original experiment. New, more controlled studies have shown that only certain types of Bt toxin are harmful to the caterpillars. These harmful toxins are no longer in production within the United States.

Case Study Delivery of Intrahemocoelic Peptides for Insect Pest Management

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To feed the growing global population, food production must be sustainable. Approximately 10% to 20% of high value crops are lost because of herbivorous insects. Stored foods are also subject to contamination and damage by insects and mites. In addition to the food industry, insects and mites pose health risks. For example, mosquitos are vectors to the virus that causes dengue fever, the protozoan that causes malaria, and a multitude of other microorganisms of medical concern. Bed bugs are resurging because of insecticide resistance. More than 500 species of insects and mites are resistant to insecticides. Part of the pest management strategy is to utilize insect-specific insecticidal peptides.

In this review article, the authors discuss the recent work toward the development of hemocoel-targeting toxins and recent advances in pest management strategies.

Venom from scorpions, wasps, cone snails, anemones, lacewings, and others could potentially be developed into insect-specific toxins. Provide a general survey of the available toxins and any strategies for targeting specific insects.

The targets of neurotoxins are usually ion channels, specifically for calcium, sodium, potassium, and chloride. To be effective, these neurotoxins need to work on nerves and, therefore, require a delivery system. Arachnid-derived insecticidal peptides are of particular interest because of their diversity. Arthropod-derived neuropeptides, enzymes, and hormones help regulate developmental processes but sometimes need to be given in higher doses to counteract the insects' own regulatory networks.

Many insecticides are not orally toxic and, therefore, need some sort of delivery strategy to reach the target areas. What delivery strategies are currently being used or have potential for use?

Carrier mechanisms are needed to move insecticidal proteins from the gut into the body cavity (hemocoel). Lectins bind to a wide range of carbohydrates (monosaccharides or complex polysaccharides) and resist proteases that may be present in a wide range of organisms. Lectins bind to gut glycoproteins, and the snowdrop lectin (GNA) can even pass into the hemolymph from the oral cavity. Lectin could possibly serve as a vehicle for movement of insecticidal molecules from the oral cavity into the hemocoel. Additionally, plant viruses that enter and persist in the hemocoel of insects could be used as a potential delivery method. Entomopathogenic fungi have also been engineered to be effective delivery tools.

How might GNA be specifically used as a delivery tool?

GNA is *Galanthus nivalis* agglutinin, which can pass into the insect hemolymph once orally delivered. GNA has been fused with *Manduca sexta* allatostatin to deliver this neuropeptide to the hemolymph of the tomato moth. GNA has also been fused to a spider neurotoxin *Segestria florentia* toxin 1 (SFI1), which targets lepidopteran and hemipteran insect pests. The SFI1-GNA fusion, again, directs the toxin to the hemolymph. Hv1a is derived from Australian funnel-web spiders and is orally toxic against one tick species and highly toxic against multiple different insect species. Hv1a-GNA fusions can be delivered orally but direct the toxin to the central nervous system. ButalT-GNA fusions target the ButalT toxin to the hemolymph of a wide range of target insects.

Are there any viral proteins that could be used as delivery systems?

Yes. A luteovirus coat protein fused to a toxin has efficiently delivered the toxin to specific areas within insect targets. For example, viral coat protein fused to GFP (CP-P-GFP) was fed to aphids and the results imaged by fluorescence microscopy. The fusion protein was observed in pericardial cells, indicating effective translocation of the coat protein fusion from the gut to the hemolymph and then to the pericardial cells in an attempt by the insect system to remove it. CP-P-Hv1a fusions resulted in significant mortality of four aphid species.

Insecticidal toxins can be targeted to the hemocoel or other target locations within the pests by using effective delivery strategies. The strategies discussed in this review article include the use or protein, viral, and fungal-derived delivery systems to target the hemocoel or central nervous system, and even the pericardial cells of insects and other pests. The result is specific and rapid killing of the pest.

Review

Cell PRESS

Delivery of intrahemocoelic peptides for insect pest management

Bryony C. Bonning and Nanasaheb P. Chougule

Department of Entomology, Iowa State University, Ames, IA 50011, USA

The extensive use of chemical insecticides for insect pest management has resulted in insecticide resistance now being recorded in >500 species of insects and mites. Although gut-active toxins such as those derived from *Bacillus thuringiensis* (Bt) have been successfully used for insect pest management, a diverse range of insect-specific insecticidal peptides remains an untapped resource for pest management efforts. These toxins act within the insect hemocoel (body cavity) and hence require a delivery system to access their target site. Here, we summarize recent developments for appropriate delivery of such intrahemocoelic insect toxins, via fusion to a second protein such as a plant lectin or a luteovirus coat protein for transcytosis across the gut epithelium, or via entomopathogenic fungi.

Introduction

Current status of insect pest management

With the world population projected to increase to >9 billion by 2050 [1], production of food in a cost-effective and environmentally sustainable manner is a high priority. A doubling of current food production will be required to sustain the future population at projected levels. However, an estimated 10–20% of major crops worth billions of dollars are lost to herbivorous insects, representing a major constraint to achieving this goal. In addition, post-harvest losses resulting from insect and mite-associated damage of stored food, cause estimated losses of 30%, valued globally at >100 billion US dollars [2]. Not only do arthropods negatively affect agriculture, they also negatively affect human health and welfare through infliction of injury and transmission of diseases. Bed bugs are of significant public health importance with their recent resurgence attributed in part to increased international travel and resistance to multiple pesticides [3,4]. Mosquito-vectored dengue virus and malaria have spread rapidly during the past decade into highly populated urban areas resulting in a dramatic rise in the numbers of clinical cases [5,6]. There are some 50 million dengue hemorrhagic fever infections per year resulting in 500 000 hospitalizations [7], and 250 million cases of malaria per year, leading to some 1 million deaths worldwide [8,9]. An estimated 2 billion US dollars has been spent annually on malaria control in recent years and costs associated with morbidity are massive. Vector control is one of the most effective strategies used to prevent the spread of mosquito-borne diseases [8].

Driven primarily by the significant deleterious impact of arthropods on the production of food and fiber and the associated economic losses, multiple research entities focus on arthropod management and crop protection solutions. However, the management of arthropod pests for protection of both agriculture and public health remains reliant primarily on the application of chemical insecticides. There are a number of disadvantages associated with their use including development of resistance by pest populations, deleterious impacts on non-target organisms, environmental pollution, and potential effects on human health [10]. Hence, there is ongoing pressure to develop target-specific, environmentally friendly, and biodegradable pest management tools.

Pest-tolerant transgenic plants provide a more sustainable approach for crop protection. Toxins derived from Bt have been highly effective for the management of lepidopteran (moth) and coleopteran (beetle) pests when delivered by transgenic plants [11]. Indeed, since their initial introduction in the early 1990s, transgenic plants have been widely adopted with 67% of corn and 77% of cotton planted in the US in 2012 expressing Bt toxins [12]. As a result, insecticide use and crop production costs have both been reduced. However, resistance to Bt toxins has been documented [13,14] and Bt toxins are not sufficiently toxic for management of sap-sucking hemipteran pests [15–17] without modification [18], with a few notable exceptions [19]. In some cases, the reduced application of chemical insecticides on Bt crops has resulted in increased populations of hemipteran pests [20,21].

RNAi has the potential to be used for the development of target-specific management methods for insect pests and the practical application of this approach for arthropod control has been demonstrated [22–24]. However, the efficacy of RNAi following oral delivery of silencing RNA appears to be restricted to Coleoptera.

In this review, we outline recent work conducted towards exploitation of toxins that act within the hemocoel for insect pest management, including significant new advances.

Insecticidal peptides that lack oral toxicity

The venom from a wide range of predatory species (e.g., scorpions, wasps, predaceous mites, cone snails, anemones,

Corresponding author: Bonning, B.C. (bbonning@iastate.edu).
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lacewings, and parasitoids), provides an outstanding resource for isolation of insect-specific neurotoxins [25,26]. These insecticidal neurotoxins typically target sodium, potassium, calcium, or chloride channels. With few exceptions, these neurotoxins are not orally active and require appropriate delivery systems to access their target site, the nerves. Arachnid venoms, which are complex peptidic libraries, have received particular attention [27]. Based on the number of species and number of toxins present in the venom of those examined, there are an estimated 0.5–1.5 million arachnid-derived insecticidal peptides [25]. There are predicted to be at least 10 million bioactive spider-venom peptides [28]. Of the 800 peptides in the ArachnoServer 2.0 Database, 136 are insecticidal with 38 being insect selective, 34 nonselective, and 64 of unknown phyletic selectivity [25]. Arthropod-derived neuropeptides, enzymes, and hormones that function to regulate insect development and maintain homeostasis (e.g., diuretic hormones, and juvenile hormone esterase) also constitute peptides with potentially insecticidal effects when delivered outside their normal physiological timeframe. Although these endogenous regulators provide insect specificity, a major drawback is that high concentrations may be required to overcome natural regulatory mechanisms that restore appropriate physiological levels within the insect. Although a few peptides (e.g., proctolin and *Aedes aegypti* trypsin modulating oostatic factor, TMOF) are transported at low levels across the insect gut epithelium [29], the impact of misexpression of the majority of these insecticidal agents has been assessed through the use of recombinant baculoviruses as delivery vehicles (reviewed in [30,31]). The target specificity of these naturally occurring arthropod-derived proteins, peptides, and toxins is particularly appealing for the development of novel pest management technologies if appropriate delivery systems can be devised (Box 1).

Box 1. Barriers to delivery of peptide toxins

Insect cuticle: The insect cuticle (an apolar lipid matrix), which covers the exterior of the insect as well as the fore- and hindgut, presents a major barrier to the direct application of insecticidal peptides for pest management. The development of neuropeptide analogs that can be directly delivered through the insect cuticle holds promise as a method for overcoming this obstacle [58], and the use of entomopathogenic fungi for toxin delivery via the cuticle has been demonstrated [57].

Peritrophic membrane (PM): The PM, composed of chitin and proteins, that lines the midgut of many insects serves to protect the midgut epithelium from mechanical damage and provides a barrier against pathogens, such as baculoviruses. Pores in the lepidopteran PM range from 21 to 29 nm and passage across the PM is driven primarily by hydrostatic forces. Although this membrane is not thought to present a significant barrier to the movement of most proteins and peptides from the gut lumen to the surface of the epithelial cells, coexpression of the *Aed. aegypti* TMOF with a baculovirus-derived chitinase that disrupts the PM had a significantly greater impact on larvae of the tobacco budworm, *Heliothis virescens*, compared to lines expressing the transgenes separately [29].

Stability in the gut: Although insect neuropeptides such as kinins, pheromone-biosynthesis-activating neuropeptide, and allatostatin, have potential for use in pest management, the rapid degradation of such peptides by proteases in the insect gut and hemolymph presents a major obstacle [59]. Peptidase-resistant analogs made through production of biostable analogs or polyethylene glycol polymer conjugates of the insect kinins have been developed to enhance peptide stability, and resulted in pyrokinin-mediated antifeedant activity and mortality in the pea aphid, *Acyrthosiphon pisum* [58].

Protein removal from the hemocoel: Once in the hemocoel, insecticidal peptides may be removed by the pericardial cells or degraded by proteolytic enzymes. The pericardial cells are specialized cells involved in regulation of hemolymph composition. These cells synthesize and secrete some hemolymph proteins while actively removing others via filtration and receptor-mediated endocytosis (e.g., lysozyme, horseradish peroxidase, hemoglobin, ferritin, and juvenile hormone esterase). Novel insecticidal peptide or toxin fusion proteins active within the hemocoel also risk clearance by pericardial cells from the hemolymph. The determinants for endocytosis into the pericardial cells are largely unknown, thus, the potential for clearance of any given fusion protein has to be tested empirically.

Potential carrier proteins: proteins that move from the insect gut into the hemocoel

Numerous papers describe the movement of a diverse range of proteins from the insect gut into the hemocoel in a broad range of arthropods (Table 1, Box 2) [32]. These proteins and peptides range widely in molecular mass and include bovine serum albumin (BSA), immunoglobulins (IgG), and teratocyte-secreted protein (TSP)14. Some of the proteins that transcytose across the gut epithelium of insects (e.g., IgG, albumin, and horse radish peroxidase), also transcytose across mammalian epithelial cells.

Analysis of the mechanisms underlying protein transepithelial transport in insects has been facilitated by use of isolated midgut epithelia of *Bombyx mori* in conventional Ussing chambers, along with the use of fluorescent probes and confocal microscopy to distinguish between transcellular and paracellular transport pathways [29]. These analyses confirm that the efficiency of transport of these proteins tends to be low. For example, about 1% of BSA is transcytosed with the majority targeted to lysosomes in the silkworm, *B. mori* [26].

Lectins as peptide transport vehicles

Lectins are carbohydrate-binding and protease-resistant proteins that are widely distributed in animals, plants, and microorganisms [33]. These proteins carry out various biological functions by binding reversibly to specific monosaccharides or complex glycans through noncatalytic domains. In plants, lectins play an important role in defense against insect herbivores and a broad spectrum of plant lectins has been tested for insecticidal activity against agriculturally important lepidopteran, coleopteran, dipteran, and hemipteran pests [34–36]. Lectins negatively affect multiple physiological processes by binding to glycoproteins in the gut membrane. Along with binding to the insect gut, certain plant lectins such as the snowdrop lectin, *Galanthus nivalis* agglutinin (GNA), can pass intact into the insect hemolymph following oral delivery [37]. GNA binds an insect gut membrane receptor glycoprotein, aminopeptidase N [38], which may mediate entry into the cell by receptor-mediated endocytosis, followed by transcytosis of a portion of the endocytosed lectin. In the insect circulatory system, GNA has been detected in hemolymph, Malpighian tubules, fat bodies, ovarioles, and the central nerve cord [39].

The movement of GNA from the gut into the hemocoel provides a mechanism for the effective oral delivery of toxins to their site of action, allowing for exploitation of

Table 1. Selected proteins that move from the insect gut into the hemocoel and mechanism of transport where known. For additional examples and references see [32]

Protein	Mass (KDa)	Mechanism (Ref)	Insect (Order)
Immunoglobulin	150		*Aedes aegypti* (Diptera)
			Ostrinia nubilalis (Lepidoptera)
			Acheta domestica (Orthoptera)
BSA	66	T [60,61]	*Gromphadorhina portentosa* (Blattaria)
			Helicoverpa zea (Lepidoptera)
			Heliothis virescens (Lepidoptera)
			Ach. domesticus (Orthoptera)
Albumin	66	T [60,61]	*Glossina morsitans* (Diptera)
			Bombyx mori (Lepidoptera)
Casein	32		*Lygus hesperus* (Hemiptera)
Urease	91		*B. mori* (Lepidoptera)
Horseradish peroxidase	40	T, P [62]	*G. morsitans* (Diptera)
			Sarcophaga falculata (Diptera)
GFP	27		*L. hesperus* (Hemiptera)
GNA	50[a]		*Adalia bipunctata* (Coleoptera)
			Nilaparvata lugens (Hemiptera)
			L. oleracea (Lepidoptera)
			Chrysoperla carnea (Neuroptera)
TSP14	14		*Hel. virescens*, (Lepidoptera); *Manduca sexta* (Lepidoptera)
AaIT	8		*S. falculata* (Diptera)
Cobra-derived neurotoxin	7		*S. falculata* (Diptera)
TMOF	76	T, P [29]	*Schistocecra gregaria* (Orthoptera)
			Aed. aegypti (Diptera)
Pheromone biosynthesis activating neuropeptide (PBAN)	3.6		*H. zea* (Lepidoptera)
Luteovirus coat proteins	22	T [50]	*Acyrthosiphon pisum* (Hemiptera)

[a]Mass of tetramer composed of four identical subunits. Abbreviations: P, paracellular transport; T, transcytosis.

insect-specific toxins that are ineffective when administered orally (Figure 1). A number of fusion proteins containing insect-specific peptides and GNA have been produced and investigated for insecticidal properties (Table 2).

GNA effectively delivers the neuropeptide *Manduca sexta* allatostatin (GNA-Manse-AS) to the hemolymph of the tomato moth, *Lacanobia oleracea*, resulting in suppressed feeding, growth retardation, and reduced survival. A fusion combining GNA with the insecticidal spider-venom-derived neurotoxin *Segestria florentia* toxin 1 (SFI1) is insecticidal to both lepidopteran and hemipteran insect pests. Ingestion of SFI1–GNA resulted in 100% mortality of first instar larvae after 6 days of feeding, whereas no effect was observed in SFI-1- or GNA-fed insects. The ability of GNA to act as a carrier protein to deliver SFI1 into the hemolymph of these insects was demonstrated by

Box 2. Mechanisms of transcytosis

Transcytosis is the movement of proteins from one side of a cell to the other within membrane-bound vesicles [63]. This is distinct from paracellular transport, which is the movement of proteins (such as proctolin [29]) between cells that is regulated by the permeability of tight junctions in mammals and the more leaky septate junctions in insects (see Figure 1 in main text). Although the mechanisms underlying transcytosis of proteins from the gut into the hemocoel of insects are poorly understood [32], transcytosis has been more extensively studied in mammalian systems. In *Drosophila*, transcytosis plays an important role in creation of morphogen gradients that drive developmental processes [64,65], but protein movement from the gut to the hemocoel has not been examined.

Proteins internalized via receptor-dependent or -independent endocytosis in clathrin-coated or clathrin-free vesicles are targeted to the endosome. A drop in pH to 5.5–6 within the endosome results in conformational changes in some receptors causing ligand release. From here, ligands and receptors may be transported to the apical membrane (retroendocytosis), the basolateral membrane (transcytosis), or remain attached to receptors to continue along the endolysosomal pathway (see Figure 1 in main text). In mammals, the best characterized endocytosis motifs of cargo proteins are the Tyr-based YXXΦ (where Y is tyrosine, X any amino acid, and Φ a bulky hydrophobic amino acid) and dileucine-based motifs. In some cases the subsequent fate of the ligand appears to depend on post-translational processing. For example, ubiquitination is required for transcytosis of endocytosed Delta in *Drosophila* [64,66]. A key question that remains to be answered is where and how transcytosed cargo is sorted.

In the mammalian system, albumin, low-density lipoproteins (LDL), metalloproteases, and insulin are transcytosed across endothelial cells in a fast, selective, and tightly regulated manner [63]. Albumin and LDL serve as carriers of metabolites with LDL being the primary carrier of cholesterol. LDL and albumin are transported by endocytosis and transcytosis according to whether the cell itself will use the plasma proteins and molecules that they carry, or whether they will be made available to adjacent tissues. The signals that regulate this differential transport are unknown.

Caveolae (~70 nm diameter vesicles), which were hypothesized to function in protein trafficking in mammalian cells, now appear to provide primarily non-transport functions [67]. Although caveolae are endocytosed to fuse with the early endosome, they are largely static with internalization only occurring under specific conditions.

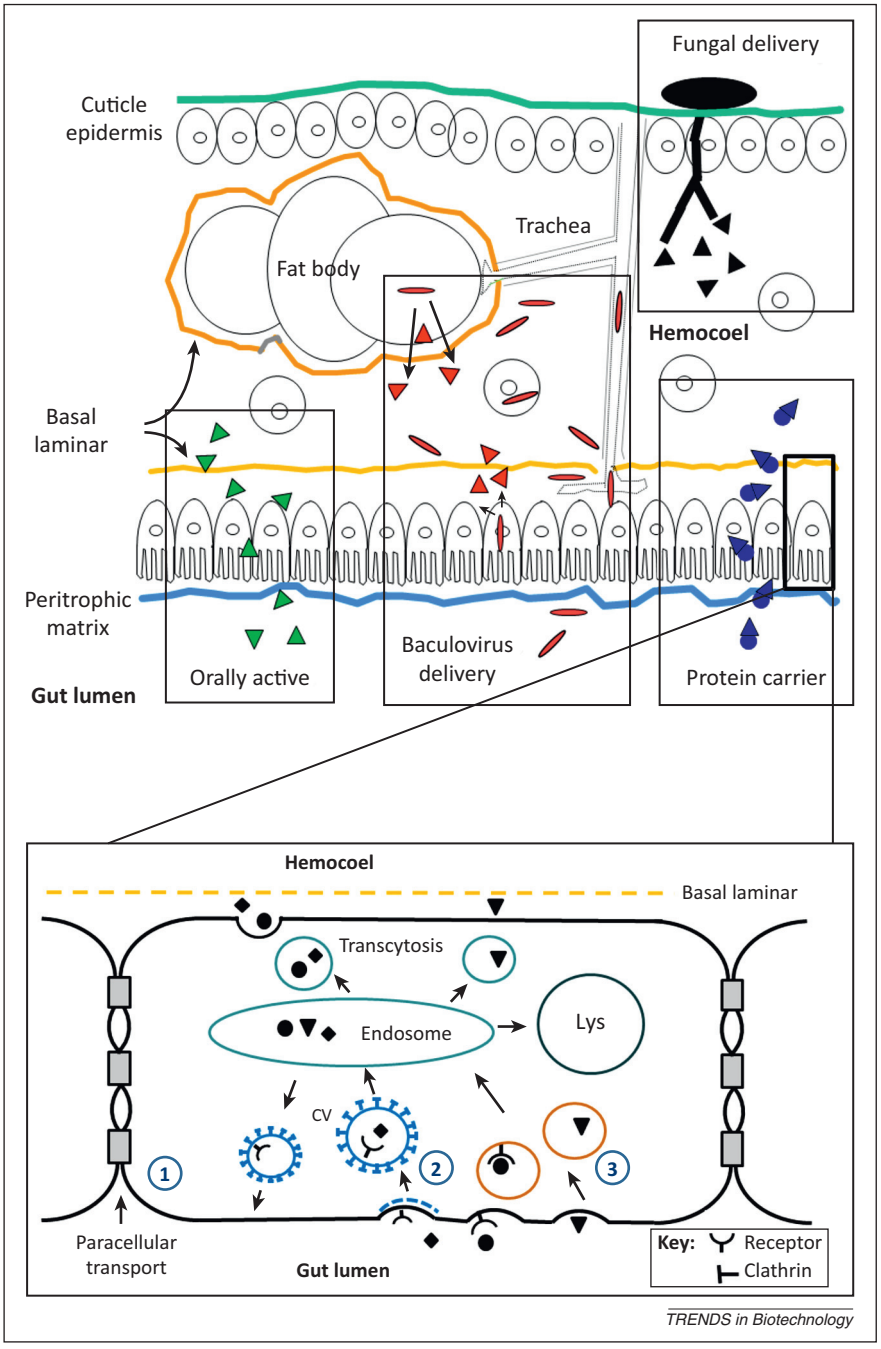

Figure 1. Delivery of intrahemocoelic toxins for management of insect pests. In a few cases such as teratocyte-secreted protein (TSP)14, intrahemocoelic toxins (depicted as triangles) are orally active and can be delivered directly from transgenic plants. For intrahemocoelic toxins that are not orally active, a protein carrier (depicted by circles) such as a lectin (e.g., the snowdrop lectin, *Galanthus nivalis* agglutin, GNA) or the coat protein of an insect-vectored plant virus [e.g., Pea enation mosaic virus (PEMV) vectored by the pea aphid] can be used to deliver toxins into the hemocoel [39,50]. Alternatively, insect pathogens can be used as vectors for expression of the toxin and secretion into the hemocoel of the infected insect. Baculoviruses (depicted as rods) infect the midgut epithelium and other tissues such as the fat body. Virus-expressed toxin secreted into the hemocoel results in death of the host insect [31]. Entomopathogenic fungi (depicted at top, right) such as *Metarhizium anisopliae* can also be engineered for release of intrahemocoelic toxins into the hemocoel [52]. In contrast to other modes of delivery, fungal delivery is via the cuticle, rather than via the gut of the insect. Inset: Mechanisms of transport across the insect gut epithelium. (1) Paracellular transport: movement of proteins such as proctolin via intercellular septate junctions. Endocytosis (receptor-mediated or receptor-independent) via (2) clathrin-coated vesicles (e.g., PEMV coat protein, or albumin), or (3) independent of clathrin (e.g., ferritin). Endocytosed vesicles fuse with the endosome and are sorted for trafficking to the lysosome for degradation, apical membrane (receptor recycling), or basolateral plasma membrane (transcytosis). Not shown: in mammalian systems, vesicles from the endosome may fuse with trans-Golgi network secretory vesicles, for secretion from the cell [68]. Gray boxes, cell junctions. Abbreviations: cv, clathrin-coated vesicle; Lys, lysosome.

immunoblot detection of GNA-immunoreactive proteins of the same molecular mass as the intact fusion. The SFI1–GNA fusion protein was also highly toxic against *Myzus persicae* and the rice brown planthopper, *Nilaparvata lugens*. However, in the case of *N. lugens* most of the toxicity was attributed to GNA.

Proteolytic degradation of GNA-based fusion proteins in the insect gut has reduced the efficiency of toxin delivery to the hemolymph [40,41]. Although GNA and the insecticidal peptides were resistant to proteolysis in the insect gut, the linker sequences used for the GNA fusion proteins were susceptible to proteolysis, demonstrating the importance

Table 2. Insecticidal effects of plant lectin–intrahemocoelic toxin fusion proteins

Lectin–toxin fusion	Target insect	Fusion protein mortality[a] (%)	Control mortality[b] (%)	Refs
	Lepidoptera			
GNA-Manse-AS	*Lacanobia oleracia*	–	–	[69]
SFI1-GNA	*L.oleracia*	100	>10	[40]
ButaIT-GNA	*L.oleracia*	25	0	[41]
ButaIT-GNA	*Spodoptera littoralis*	50	20	[38]
Hv1a-GNA	*Mamestra brassicae*	85	20	[39]
	Hemiptera			
ButaIT-GNA	*Nilaparvata lugens*	92	64	[41]
SFI1-GNA	*Myzus persicae*	51	<10	[70]
SFI1-GNA	*N. lugens*	100	76	[70]
	Coleoptera			
ButaIT-GNA-His	*Tribolium castaneum*	48	0	[38]
	Diptera			
ButaIT-GNA	*Musca domestica*	75	25	[38]

[a]Mortality data are for the highest concentrations of lectin–toxin fusion tested.

[b]Control mortality indicates toxicity of either GNA or toxin alone at the highest concentration tested.

of using protease-resistant linkers for efficient toxin delivery into the hemocoel.

The insecticidal ω-hexatoxin-Hv1a (Hv1a), derived from the venom of an Australian funnel-web spider (*Hadronyche versuta*) specifically inhibits insect but not mammalian voltage-gated calcium channels [42,43]. Hv1a is highly toxic by injection towards many different insect pests including species from the orders Lepidoptera, Coleoptera, Diptera, and Dictyoptera, and is ineffective after oral ingestion [39,44]. However, Hv1a is orally toxic against one tick species (*Amblyomma americanum*), which may be related to differences in gut physiology associated with blood feeding [45]. The spider-derived toxins such as Hv1a that contain a disulfide pseudoknot are classified as inhibitor cystine-knot (ICK) motif toxins. The cystine-knot in these neurotoxins results in strong chemical, thermal, and biological stability, contributing to their persistence and making them particularly attractive for use as model toxins [27,46]. Fusion of Hv1a to GNA results in oral delivery of the toxin to its site of action, the central nervous system, in *Mamestra brassicae* [39]. GNA-mediated delivery into the hemolymph and central nerve cord has been demonstrated by immunoblotting as well as fluorescence microscopy. Hv1a–GNA caused 40% mortality after 4 days in feeding bioassays, with surviving insects dying before pupation. Feeding second instar larvae cabbage leaf discs coated with 0.2% Hv1a–GNA caused 85% mortality after 10 days. Similarly, fusion of a toxin derived from the red scorpion *Mesobuthus tamulus* to GNA (ButaIT–GNA) caused increased mortality against Lepidoptera, Coleoptera, Hemiptera, and Diptera [38]. Delivery of ButaIT–GNA in *L. oleracea* hemolymph has been demonstrated by immunoblotting. Taken together, these studies highlight that plant lectins can be transcytosed across the insect gut epithelium, and have potential for delivery of intrahemocoelic toxins to their target sites.

Plant virus coat protein (CP)-mediated delivery

A large number of plant viruses are vectored by sap-sucking insects (Hemiptera) including aphids, whiteflies, leafhoppers, plant hoppers, and thrips, with more than half of these viruses vectored by aphids [47]. The persistently transmitted viruses (i.e., plant viruses that enter and persist in the hemocoel of the insect vector) are ingested during vector feeding on plant sap, and then move from the gut of the vector into the hemocoel before being transmitted to other plants via the salivary glands [48]. These plant viruses typically do not replicate in the insect vector. The ability of the virus to move from the gut into the hemocoel by transcytosis is of particular interest for the delivery of insect-specific toxins into the insect hemocoel (Box 3). It is expected that plant viruses have evolved to avoid being

Box 3. BSA and transcytosis of plant viruses

Albumin, including BSA, which functions to transport steroids, fatty acids, and hemin in mammals, is transcytosed across both mammalian and insect gut epithelia (see Table 1 in main text). The mechanism of albumin transcytosis in mammals is therefore of particular interest. Albumin has three domains and a remarkable propensity to bind numerous ligands at different sites, providing a depot for some, and carrier for other ligands [71]. In mammals, albumin binds the megalin/cubilin receptor, which is subsequently internalized in clathrin-coated vesicles.

On the basis that megalin, a 600-kDa transmembrane protein in the LDL receptor family, functions as a receptor for multiple proteins (a 'scavenger receptor') including albumin in mammals, Casartelli *et al.* investigated the potential role of an insect megalin homolog as receptor for BSA in cultured columnar cells of the silkworm, *B. mori* [58]. Although the megalin homolog colocalized with fluorescein isothiocyanate (FITC)-labeled BSA, and BSA uptake was reduced in the absence of Ca^{2+}, which is required for megalin-mediated endocytosis, the function of megalin as a BSA receptor was not definitively shown.

Nonenzymatically glycated albumin binds to two different receptors in mice, a lectin-like receptor and a receptor assumed to specifically bind albumin, causing increased permeability of the endothelium for glycated albumin relative to native albumin [72]. The presence of BSA resulted in increased plant virus movement from the gut into the hemocoel of the aphid vector and subsequent transmission of the virus [73]. This raises the possibility that binding of BSA to the plant virus could allow for entry of the BSA–virus complex via two sets of receptors, namely the plant virus receptor and BSA receptor. Notably, several other proteins also facilitated plant virus uptake and/or transmission by the aphid vector, including casein, lysozyme, cytochrome C, carbonic anhydrase, and two plant lectins. Of these proteins, casein and the plant lectins may also transcytose across the aphid gut epithelium (see Table 1 in main text), and if they bind to the plant virus, may similarly provide an additional receptor site to facilitate virus transport.

targeted to the lysosome following receptor-mediated endocytosis into epithelial cells. Based on transmission electron micrographs, the movement of the luteoviruses across the gut epithelium of the aphid vector is mediated by clathrin-coated vesicles [49]. These vesicles form tubular transport structures that release virus into the hemocoel. A similar clathrin-coated-vesicle-mediated process occurs for virus movement from the hemocoel across the accessory salivary gland into the duct of the aphid salivary gland.

The efficacy of the use of luteovirus coat protein–toxin fusions against aphids has been demonstrated [50]. The CP of the Pea enation mosaic virus (PEMV) followed by the proline-rich region at the N terminus of the CP readthrough domain was fused to GFP (GFP). Following feeding on this fusion protein (CP–P–GFP) but not on GFP alone, green fluorescence was seen in the pericardial cells of the pea aphid. This result demonstrates that: (i) CP–P transported GFP from the gut across the aphid gut epithelium; (ii) the fusion protein was removed from the hemolymph by the pericardial cells; and (iii) the virion structure was not required for transcytosis of the CP. Feeding on the CP–toxin fusion, CP–P–Hv1a in membrane feeding assays or via transgenic *Arabidopsis*, resulted in significant mortality of four species of aphid. These aphids included the economically important green peach aphid, *Myzus persicae*, and the soybean aphid, *Aphis glycines*. Hence, the luteovirus CP can cross the gut epithelium of multiple aphids, including aphid species such as *A. glycines* that do not vector PEMV. Similarly, the CP of other luteoviruses such as Barley yellow dwarf virus [51] and Soybean dwarf virus (N. Pal, unpublished data) are also effective for toxin delivery.

A major advantage of plant virus CP-mediated toxin delivery is the specificity, and the expected efficiency of transport from the gut into the hemocoel. We expect to find that these viruses have an efficient mechanism to promote transcytosis across the gut epithelial cells into the hemocoel of the vector. Following identification of the PEMV receptor in the aphid gut, it is conceivable that PEMV CP could be modified for binding to homologous proteins for transcytosis into the hemocoel of non-vector insects.

Insect pathogen delivery systems

In addition to the use of baculoviruses as vectors for delivery of insect toxins [30,31], an entomopathogenic fungus, specifically *Metarhizium anisopliae*, has also been engineered for expression of *Androctonus australis* insect toxin (AaIT) [52,53]. There are some 1000 species of entomopathogenic fungi that have narrow host ranges and target virtually all insect species. In contrast to other insect pathogens, these fungi do not require ingestion by the host but can directly penetrate the cuticle. There are several entomopathogenic fungal products already in use for biological control purposes [52]. The efficacy of *Met. anisopliae* was first increased by engineering the fungus to overexpress one of its own cuticle degrading proteases, Pr1 [54]. Subsequent work showed that expression of AaIT improved efficacy against the tobacco hornworm, *Man. sexta*, mosquitoes and the coffee berry borer beetle, *Hypothenemus hampei* [55]. Co-expression of Pr1 and AaIT in the entomopathogenic fungus *Beauveria bassiana* did not result in further improvement of the mycoinsecticide however as the toxin was degraded by Pr1 in the hemocoel [56].

B. bassiana was also engineered to express *Aed. aegypti* TMOF which inhibits synthesis of gut trypsin or *Man. sexta* diuretic hormone [57]. Expression of diuretic hormone reduced both the lethal dose and lethal time of the fungus against the wax moth, *Galleria mellonella*, and expression of TMOF reduced the fecundity of blood-fed female mosquitoes.

Concluding remarks

The vast majority of insecticidal intrahemocoelic toxins lack the ability to traverse the insect gut epithelium to reach their target sites. Although the use of baculoviruses for delivery of such toxins has been studied extensively and shown to be effective, recombinant baculovirus insecticides have yet to be adopted for pest management. Transgenic entomopathogenic fungi have now been developed for more rapid killing of the targeted pest and show promise for pest suppression.

The first demonstration of the use of a protein-based carrier system for delivery of insecticidal peptides into the insect hemocoel was lectin-based, and since then, the oral activity of many GNA-based fusion proteins has been described against multiple insect orders. Key among recent developments for delivery of intrahemocoelic toxins to

Box 4. Outstanding questions

- Where and how is transcytosed cargo sorted? Similar to the specific delivery of drugs and genes for gene therapy in humans [64], the transcytosis pathway can be exploited for the specific delivery of insecticidal peptides from the gut into the hemocoel for insect pest management. Indeed, significant progress has been made in improving delivery systems for human therapeutics through use of the transcytosis pathway. However, remarkably little is known of the molecular mechanisms of transcytosis in insects. Understanding of these mechanisms will be crucial for exploitation of this pathway for insect pest management purposes to take full advantage of insect-specific peptides that act within the hemocoel.
- Is transcytosis of plant viruses more efficient than transcytosis of ingested proteins such as plant lectins? If so, what is the basis for the increased efficiency of transepithelial transport? The transcytosis of some proteins (e.g., IgG or albumin) is relatively inefficient. As a result of the importance of transcytosis of luteoviruses into the hemocoel of the aphid vector for virus transmission, it is expected that the virus has evolved for efficient uptake into the gut epithelial cells, along with a mechanism to avoid being targeted to the lysosome. Understanding of these processes will facilitate optimization of the delivery of intrahemocoelic toxins.
- Can insect-specific intrahemocoelic toxins from microbes be isolated for practical application of this technology through insect-resistant transgenic plants for pest management? The use of insect-specific toxins derived from microbes rather than higher organisms for pest management has become a priority for agricultural industry on the basis of increased public acceptance. Hence, further research is needed to identify suitable toxins from prokaryotes, with entomopathogenic nematode-associated bacteria providing an excellent resource [74]. Alternatively, artificial toxins designed with detailed knowledge of toxin mechanism and receptor structure could provide viable options for future use in pest management.

insects has been the demonstration that luteovirus CPs can be used for delivery of heterologous proteins into the insect hemocoel from transgenic plants. Based on knowledge of receptor binding, this plant virus CP-mediated technology may be adaptable for delivery of toxins to non-vector insects via transgenic plants.

Significant strides have also been made through more comprehensive analyses of the mechanisms involved in transepithelial protein transport specifically through the use of cultured cells from the *B. mori* midgut along with fluorescent labels and current imaging technologies. Further advances are expected in this area through the use of similar approaches to study protein movement from the gut into the insect hemocoel (Box 4).

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