

Review

Delivery methods for peptide and protein toxins in insect control

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Abstract

Since the introduction of DDT in the 1940s, arthropod pest control has relied heavily upon chemical insecticides. However, the development of insect resistance, an increased awareness of the real and perceived environmental and health impacts of these chemicals, and the need for systems with a smaller environmental footprint has stimulated the search for new insecticidal compounds, novel molecular targets, and alternative control methods. In recent decades a variety of biocontrol methods employing peptidic or proteinaceous insect-specific toxins derived from microbes, plants and animals have been examined in the laboratory and field with varying results. Among the many interdependent factors involved with the production of a cost-effective pesticide—production expense, kill efficiency, environmental persistence, pest-specificity, pest resistance-development, public perception and ease of delivery—sprayable biopesticides have not yet found equal competitive footing with chemical counterparts. However, while protein/peptide-based biopesticides continue to have limitations, advances in the technology, particularly of genetically modified organisms as biopesticidal delivery systems, has continually progressed. This review highlights the varieties of delivery methods currently practiced, examining the strengths and weaknesses of each method.

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1. Arthropod pests and methods of their control

Arthropod pests destroy about 25% of the world's annual crop production (Oerke, 1994), contribute to the loss of nearly 20% of stored food grains (Bergvinson and Garcia-Lara, 2004), damage human structures to the cost of millions of dollars (Elzen and Hardee, 2003), and transmit an array of human and veterinary pathogens (Gubler, 1998a–c; Gratz, 1999). Considering the cost induced by disease transmission alone, there has long been strong impetus to develop effective means to control these pests, and in recent decades because of the increased development of pest resistance to chemical pesticides, the search for alternative methods has received tremendous attention.

Arthropods such as mosquitoes, ticks, sandflies, tsetse flies, fleas, midges and triatomid bugs are disease vectors for a multitude of human pathogens including malaria, sleeping sickness, plague, filariasis, dengue, dengue hemorrhagic fever, West Nile encephalitis, Japanese encephalitis, yellow fever, and Rift Valley fever (Gubler, 1998a–c; Gratz, 1999). Malaria and dengue fever produce more than 300 million cases and 2 million deaths annually (Gubler, 1998a–c), and the mosquito-transmitted West Nile virus is quickly becoming endemic to the United States along with similar flaviviruses in Australia (Petersen and Roehrig, 2001). Ticks, the primary facilitators of vector-borne illness in the US and other temperate regions of the Northern hemisphere, transmit the bacterial pathogens responsible for Lyme disease, Ehrlichiosis, Rocky Mountain spotted fever and tularemia (Gayle and Ringdahl, 2001; Parola and Raoult, 2001; Randolph, 2001; Walker, 2003).

Since the introduction of DDT in the 1940s, insect pests have been controlled almost exclusively with chemical insecticides (Casida and Quistad, 1998). Fast acting, cheap to produce, relatively easy to deliver, and highly potent, chemical insecticides have been viewed with extreme optimism; problems

associated with these compounds did not begin to become apparent to most scientists until almost two decades after their introduction. These limitations included poor species specificity—leading to losses in beneficial insect species, disequilibrium of ecosystems resulting in elevation of minor pests to major pests, toxicity in vertebrate species including birds, fish, and mammals—and resistance development of target organisms. In response to the environmental threat that these compounds pose, DDT and many other chlorinated insecticides were banned from agricultural use in many countries in the 1970s, and alternative classes of chemical compounds were developed. These compounds, some of which still have poor mammal insect specificity ratios, include carbamates, organophosphates, synthetic pyrethroids, neonicotinoids, synthetic growth regulators and metabolic disrupters. Despite the development of this array of chemical compounds, and although there has been a trend in developed countries toward compounds that offer minimal mammalian toxicity and greatly reduced environmental impact, very often these compounds have been prohibitively expensive for use in developing countries, necessitating the continued search for alternative sources of pesticidal compounds.

Thus coincident with the development of chemical alternatives, research and development of biologically based pesticides had also begun. Since the evolution of insecticide resistance, and awareness of environmental and human health impact of some chemical pesticides, the impetus to produce alternative control methods and new biopesticides has brought to light a splendid array of compounds originating from insect predators and pathogens, and from plant defensive compounds (Copping and Menn, 2000).

Of all these biologically based alternatives, various subspecies of the bacterium, *Bacillus thuringiensis* (*Bt*) and associated toxins have emerged as the primary commercial powerhouses. In the US, though still less than 2% of the market, sprayable

Bt formulations have penetrated cotton, fruit and vegetable, aquatic, and other insecticide markets, and in the last decade new *Bt* formulations have consistently grown in a few fruit and specialty vegetable markets; *Bt* has remained the mainstay for Lepidoptera control in organic production throughout the world (Whalon and Wingerd, 2003).

Nevertheless, after decades of research and development, the repertoire of commercially available pesticides is modest and the need for alternatives remains. Utilization of a limited number of pharmacological targets—for example acetylcholinesterase targeting by all carbamates and organophosphates, and Na⁺ channels targeting by all pyrethroids—has facilitated the development of resistance to one or more classes of chemical; by 1992 more than 500 species of insects and mites, including 95 species of mosquito and nine species of tick, had developed resistance (Feyereisen, 1995; Brogdon and McAllister, 1998). Such resistance has been exacerbated by the shrinking availability of many chemical pesticides, caused by increased regulatory restriction of insecticide use, market removal of insecticides no longer registered for public health use, and reduced profits of certain compounds. These combined factors perpetuate pest-induced worldwide losses of food, feed and fiber of several billion dollars each year (Elzen and Hardee, 2003), and may profoundly affect the reemergence and control of vector-borne diseases (Kondrashin and Rooney, 1992; Krogstad, 1996; Rodhain, 1996; Brogdon and McAllister, 1998; Attaran et al., 2000; Neale, 2000).

The application of insect resistance management—seeking to understand and prevent the development of resistance—in combination with judicious application of pesticides, may serve to preserve useful pesticides by slowing, preventing or reversing development of resistance in pests. In fact, in the hopes of extending the effective life of genetically modified (GM) plant and other biopesticide products, some governments have mandated resistance management programs such as the co-planting of transgenic with wild-type crop varieties (Macdonald and Yarrow, 2003). Nevertheless, since some level of resistance development should be anticipated for any insecticide (Elzen and Hardee, 2003), the development of new pesticides with specificity for and effectiveness against pest species, coupled with minimal non-target toxicity and rapid environmental degradation will continue to be in demand.

2. Sources of peptidic/protein biopesticides

The term biopesticides describes a plethora of pest control techniques including the application of microbial organisms, entomophagous nematodes, plant-derived pesticides, secondary metabolites from micro-organisms, insect pheromones applied for mating disruption, monitoring or lure-and-kill strategies, and genes used to increase the resistance of crops to insect, fungal, viral or herbicide damage (Copping and Menn, 2000). A variety of peptides and proteins have been used to produce biopesticides, biopesticidal microbes, and pest-resistant crops. These compounds derive from a number of sources including the venoms of predatory/parasitoid animals (Gershburg et al., 1998; Volynski et al., 1999; Harrison and Bonning, 2000; Imai et al., 2000; Taniai et al., 2002), arthropod-pathogenic microbes including bacterial symbiotes of entomopathogenic nematodes (Fuxa, 1991; Beard et al., 2001), plant lectins, protease inhibitors (Slack et al., 1995; Cheng and Xue, 2003; Brunelle et al., 2005) or ribosome inactivating proteins (Sharma et al., 2004), arthropod hormones and neuropeptides (Menn and Borkovec, 1989; Borovsky et al., 1990, 1993; Ma et al., 1998; Altstein et al., 2000; Altstein, 2001, 2004; Borovsky, 2003a, b), biotin-binding proteins (Burgess et al., 2002), chitinases (Gopalakrishnan et al., 1993; Kramer and Muthukrishnan, 1997), enzymes controlling aromatic aldehyde synthesis (O'Callaghan et al., 2005), viral enhancins (Lepore et al., 1996; Granados et al., 2001; Cao et al., 2002), plant defensins (Lay and Anderson, 2005), and plant hormones (Dinan, 2001).

2.1. Predatory/parasitoid venom-derived toxins

One source of biopesticide leads is venom-derived peptides that have evolved in predator/parasitoid arthropods such as spiders (Tedford et al., 2004; Nicholson, 2006), scorpions (Froy et al., 2000), wasps (Gould and Jeanne, 1984; Dahlman et al., 2003), predacious mites (Tomalski et al., 1988), and cone snails (Olivera, 2002). Such venoms are composed of a mixture of salts, small molecules, proteins, and peptidic toxins specifically active against invertebrates, vertebrates, or both (Zlotkin et al., 1978, 1985; Zlotkin, 1991; Loret and Hammock, 1993; Gordon et al., 1998; Possani et al., 1999; Inceoglu et al., 2003). Venom-derived peptide toxins generally target voltage-gated Na⁺, K⁺, Ca²⁺, or Cl⁻ channels, although there are

several examples of peptides with unusual targets such as the intracellular calcium-activated ryanodine channel (Fajloun et al., 2000).

A subset of arthropod venoms, arachnid venoms are complex mixtures of highly evolved peptidic libraries with toxin activities that include antimicrobial (Moerman et al., 2002, 2003), pore forming (Corzo and Escoubas, 2003), and ion channel antagonists/agonists (Zlotkin et al., 1978; Zlotkin, 1991; Loret and Hammock, 1993; Gordon et al., 1998; Nicholson, 2006). Their utility has received some notice, providing pharmacological tools to understand the physiological role of ion channels, and as leads for therapeutic agents and novel insecticides; based on the number of total arachnid species and the average numbers of toxins observed in those animals studied, there are an estimated of 0.5 to 1.5 million arachnid derived insect-active peptidic toxins that may provide novel pest-control agents (Quistad and Skinner, 1994; Wang et al., 1999; Tedford et al., 2004).

In addition to peptide toxins of arachnid origin, insect-selective and highly potent toxins have been identified from other animals including lacewings, anemones, cone snails, and mites (Tomalski and Miller, 1991; Prikhod'ko et al., 1996; Olivera, 2002). For example, mites in the genus *Pyemotes* are predatory and possess venoms, which while non-specific for particular insects, cause mild to extreme toxicity in a wide variety of insect species (Tomalski et al., 1988, 1989, 1993). Considering the total number of species that produce insect-specific toxins and the variety of toxins within each venom type, the potential for the development and application of novel biopesticides from these sources appears virtually limitless—limitless, however, only if provided with suitable delivery systems. The lack of oral bioavailability of such peptidic toxins demands vectored delivery, as provided naturally by predator envenomation or as provided artificially by engineered pest-specific pathogenic microbes.

2.2. Arthropod neuropeptides and hormones

Endogenous regulators of insect development and physiology, insect hormones and neuropeptides have gained increasing consideration as possible platforms for bioinsecticidal control: antagonists disrupt and interfere with growth, development and behavior, thus potentially providing receptor-selective, insect-specific pesticides (Altstein et al., 2000; Altstein, 2001, 2004; Gade, 2004; Tedford et al.,

2004; Ben-Aziz et al., 2005; Gade and Hoffmann, 2005). The types, structure and functions of neuropeptides have been described in several reviews (Menn and Borkovec, 1989; Holman et al., 1990; Altstein, 2001; Gade and Goldsworthy, 2003; Gade, 2004; Gade and Hoffmann, 2005), and examples of both peptidomimetic and non-peptide mimics have been developed (Nachman et al., 1996; Altstein, 2004). Neuropeptide and hormone mimetics may take advantage of novel molecular targets and can be engineered for good oral bioavailability, but are often hindered by requirements of high concentration caused by multiple endogenous physiological regulatory systems within target insects: although many neuropeptides interact with their receptors at exceptionally low concentrations, endogenous systems which degrade peptides, target receptor isolation, and multiple homeostatic mechanisms often limit in vivo potency.

2.3. Pathogenic microbes and microbial toxins

A huge number of insect selective fungi and fungal-derived toxins—both peptidic and non-peptidic—are known. A great many fungi and yeast have been used as antimicrobial agents to manage crop diseases (Punja and Utkhede, 2003; Benitez et al., 2004), but there are also many fungi currently being utilized in insect control (Scholte et al., 2005). Examples include *Beauveria bassiana* for the control of numerous insects such as sandflies (Warburg, 1991), *Metarhizium anisopliae*, first used in 1888 (Taborsky, 1992) against *Clones punctiventris* and more recently against a variety of pests including the mosquito *Anopheles gambiae* (Scholte et al., 2005), *Lagenidium giganteum* against mosquitoes (Kerwin and Washino, 1987; Kerwin et al., 1994), and *Verticillium lecanii* as an aphid control (Ashouri et al., 2004; Kim et al., 2005). Other lesser known fungi have also been identified as pathogenic to and considered for biological control of mites like *Varroa destructor*, a honey bee ectoparasite (Hastings, 1994; Peng et al., 2002; Umina et al., 2004).

Like fungi, many bacterial species produce insecticidal toxins of tremendous biotechnological, agricultural, and economic importance. Although *Bt* currently accounts for at least 80% of the sprayable bioinsecticide market and is the only sanctioned source of insect-resistant genes for use within GM plants (see the review by Bravo et al. in this edition), the toxins of the bacteria *Serratia*

marcescens (Downing et al., 2000; Downing and Thomson, 2000; Inglis and Lawrence, 2001), *Photographus luminescens* and *Xenorhabdus nematophilus* may also provide useful alternatives (French-Constant and Bowen, 2000; Chattopadhyay et al., 2004; and see the review by French-Constant et al. in this edition).

Of the more than 20 known groups of insect pathogenic viruses, classified into 12 families, only a modest number of viruses have been explored for their insecticidal potential (Tanada and Kaya, 1993; Blissard et al., 2000). In addition to baculoviruses, insect parvoviruses have demonstrated insecticidal power (Tal and Attathom, 1993), and the introduction of *Oryctes* virus into outbreak areas of the rhinoceros beetle, which led to a dramatic reduction in palm damage in many areas of the Asia-Pacific region, was touted as a major success for viral biocontrol (Caltagirone, 1981; Jackson et al., 2005). Tetraviruses such as the cotton bollworm (*Helicoverpa armigera*) stunt virus, have been isolated from a number of pest species and may also find utility as direct control agents, by transgenic generation in plants or other organisms, or as gene delivery vehicles (Gordon et al., 1995; Hanzlik et al., 1995; Pringle et al., 2003; Bothner et al., 2005; Yi et al., 2005). However, among insect pathogenic viruses, members of the family Baculoviridae are the most commonly found, studied and used (Kamita et al., 2005). Due to inherent insecticidal activities, natural baculoviruses have been used as safe and effective biopesticides for the protection of field and orchard crops, and forests in the Americas, Europe, and Asia (Black et al., 1997; Hunter-Fujita et al., 1998; Moscardi, 1999; Vail et al., 1999; Copping and Menn, 2000; Lacey et al., 2001).

Unlike the proteinaceous toxins of bacteria or fungi, however, that can be extracted from fermentation cultures and used as pesticides, individual viral proteins generally do not have the same insecticidal puissance. Rather, viral toxicity often results from viral replication and release and/or the ability of the virus to suppress host-specific transcription and/or protein synthesis. Often viruses (such as members of Baculoviridae) are so well adapted to their hosts, that they enhance host feeding and change host behavior to aid in the production and distribution of progeny virus particles. Viral utility arises, therefore, primarily from their versatility as malleable delivery systems and as an additional barrier to the development to resistance.

2.4. Plant proteins

Plant insecticidal products such as lectins, defensins, protease inhibitors or ribosome inactivating proteins can be expressed in transgenic plants in a tissue or development-specific manner, or in response to environmental stimuli (Boulter, 1993; Sharma et al., 2004). Members of the plant defensin family of proteins, small molecular weight proteins that typically have antimicrobial activity, have also been identified with insecticidal activity (Jennings et al., 2001; Chen et al., 2002; Lay and Anderson, 2005). Diverse plant protease inhibitors (PIs) from numerous plant species have been isolated and have demonstrated a puissant defensive role against insects and pathogens, and the use of recombinant PIs to protect plants has already been incorporated within integrated pest management programs. Other plant-based approaches to crop protection include the modification of ecdysteroid levels and/or profiles in crop plants to enhance protection against insects; insertion and/or stacking of phytoecdysteroid genes may enhance host resistance to insect pests (Dinan, 2001). Plant-derived proteins have the advantage of good oral bioavailability in insect pests, but in general lack the potency of venom-derived toxins or microbial pathogens.

3. Biopesticide delivery through oral ingestion

A variety of techniques have been employed to deliver peptide/protein toxins to pest species, but these can be generalized into two routes of administration, either through direct ingestion of toxins—contained on or within food—or by vectored delivery through insect-specific microbes and/or symbiotes. In current practice, the primary route of peptide and protein-based biopesticide delivery has been through direct ingestion of toxins by arthropod pests, either as topical applications to plant surfaces or other foodstuffs, or more importantly as constituents contained within genetically enhanced plants or plant germplasm.

3.1. Genetically modified crops

There are many examples of GM crops using a variety of transgenes from plant, microbial and insect origins; transgenic resistance to insects has been demonstrated in plants expressing insecticidal genes such as δ -endotoxins from *Bt*, insect hormones and neuropeptides, arthropod-derived

toxins, PIs, enzymes, secondary plant metabolites, plant lectins, or as fusion protein combinations of these. A recent review lists 30 examples of GM plant species containing *Bt* toxins, 18 with serine PIs, three with cysteine PIs, eight with lectins, and another eight plant species modified to express some other type of insect-specific protein or peptide-based toxin (O’Callaghan et al., 2005).

The first products employing biopesticides were sprayable formulations of either bacteria or bacterially derived protein suspensions. Compared with such sprayable formulations, however, the genetic modification of crops to include *Bt* (or other) toxin genes has several advantages, including a continuous production of toxin within plant tissues that (i) eliminates the need for repeated pesticide applications, (ii) protects the toxin from UV (or other environmental) degradation, and (iii) provides the toxin at the first sign of pest encroachment (Federici, 2005).

These advantages, coupled with the highly specific and excellent activity of *Bt* endotoxins against some pest insects, as well as the conceptual simplicity in technological and intellectual property considerations, has made *Bt* genes the primary choice within commercial GM crops, despite the plethora of other transgene sources. Crops including rice, corn, and cotton utilize *Bt* δ -endotoxins (*cry1Ab*, *cry1Ac*, *cry2Ab*, and *cry9C*) more frequently than any other genetic modification other

than herbicide resistance genes (O’Callaghan et al., 2005).

First introduced in the mid-1990s, as of 2005, *Bt*-modified corn and cotton comprise 35% and 52%, respectively, of total crop area in the US alone (Fig. 1). The first GM rice plants to utilize *Bt* toxins were produced more than a decade ago; today there are several promising reports of successful applications in both laboratory and field tests (Bajaj and Mohanty, 2005). Chinese rice cultivars transformed with *Cry1Ab* reported a broad spectrum of resistance to several lepidopteran pest species, including economically important species; in China, Pakistan, the Mediterranean and India there are several examples of transgenic *Bt* hybrid rice found to be highly protected against pests (Bajaj and Mohanty, 2005). Besides offering a safer option of pest control, *Bt* rice may eliminate as much as 2–10% of the yield loss in Asia caused by lepidopteran pests (High et al., 2004).

The demonstrated pest-resistant efficacy of *Bt* within GM plants may also be applicable to the protection of grain storage. Pest-protection of stored grains, of equal importance to the protection of crops, has seen promising developments of GM technology (Bergvinson and Garcia-Lara, 2004). In general, genetic enhancement of storage-pest resistance must confer traits that deter pests, but that do not adversely affect grain processing or quality: preferred routes increase physical barriers or induce toxicity to pests.

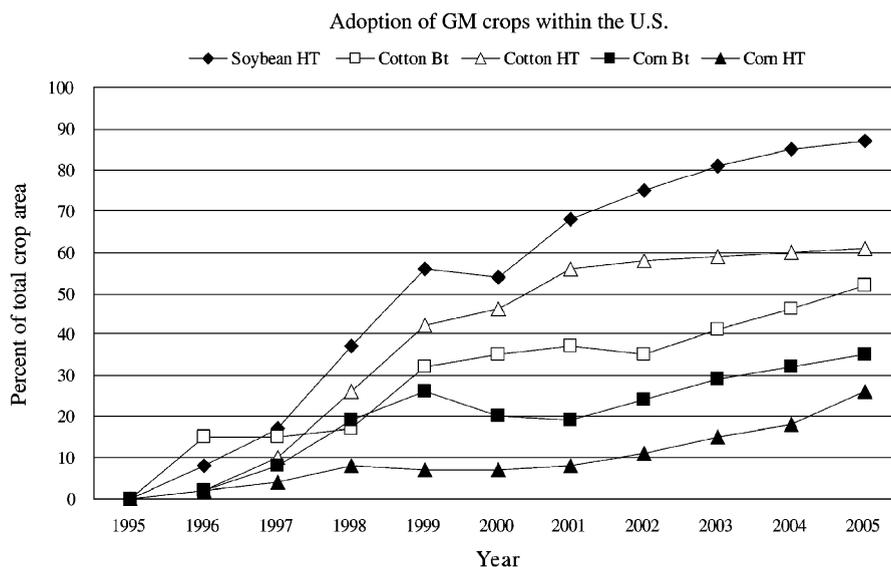


Fig. 1. Percentage of total crops within the US constituted by GM crops incorporating either herbicide resistance or *Bacillus thuringiensis* insect resistance genes. Data for each crop category include varieties stacked with both and *Bt* traits. Source USDA ERS website.

Exemplary transgenic proteins employed for this use include amylase and PIs, enzymes involved in wax production, peroxidase, avidin, and α -amylase (Bergvinson and Garcia-Lara, 2004).

Bt genes have enjoyed the most attention because many of the other transgenes do not confer the same degree of insecticidal activity; PI and lectin genes largely affect insect growth and development and, in most instances, do not result in insect mortality, requiring much higher effective concentrations of these proteins than required for the *Bt* toxin proteins. For example, transgenic tobacco plants carrying a baculovirus enhancin gene have been demonstrated to slow the development, and increase the mortality, of *Trichoplusia ni* larvae, but with comparatively less efficiency than *Bt* toxins (Hayakawa et al., 2000; Cao et al., 2002).

Other alternatives to *Bt* toxins, such as the plant ecdysteroids, may also be of value as GM plant inserts. Phytoecdysteroids—a family of about 200 plant steroids—may contribute significantly to the protection of the plants which contain them, and have been examined as possible biopesticides against a large number of arthropods (*Bt*, in comparison, controls a much more limited number of pests). Two types of phytoecdysteroids that mimic either ecdysone—the natural molting hormone—or juvenile hormone, have evolved separately within many plants as natural insect control agents. Although there are certain mono- and polyphagous insect pests unaffected by high concentrations of dietary ecdysteroids (Dinan, 2001)—limiting the general herbivore activity anticipated for the GM protection of crop species—because tolerant species appear to detoxify the ingested ecdysteroids following generalized routes, it may be feasible to generate plants which contain ecdysteroid analogues resistant to biochemical detoxification (Dinan, 2001).

While the complex synthetic pathways of phytoecdysteroids and juvenoids have deterred extensive work in this area, it may become more attractive to alter the production of steroid and terpene pest hormone mimics as our ability to work with multiple transgenes improves. Also, many of the steps in the biosynthesis of these products already occur in many plants, so only minor changes in biosynthetic pathways may be needed to gain economically useful levels of these materials. Commercial success of synthetic ecdysteroid receptor agonists has renewed interest in this area, and recent advances in molecular biology make it more

attractive to consider modifying pathways in addition to inserting single transgenes within plants.

Apart from the ecdysteroids, defensins have shown the most promise as plant-derived alternatives to *Bt* toxins, due to good oral bioavailability and potent insect toxicity. Although generally known as having antimicrobial properties, examples of insecticidal defensins also exist. Macrocyclic peptides known as cyclotides have been identified in several plant species and have demonstrated potent inhibitory effects on native budworm (*Helicoverpa punctigera*) larval growth and development (Jennings et al., 2001; and see the review by Gruber et al. in this edition). Another member of the defensins, the protein VrCRP was isolated from a bruchid-resistant mungbean variety and has exhibited in vitro insecticidal activity against the Southern Cowpea Weevil, *Callosobruchus chinensis* (Chen et al., 2002).

Used as a single transgene within GM plants, the narrow specificity of peptidic toxins like VrCRP may limit their crop protection efficacy. However, as seen from the success of multiple *Bt*-containing crops, it is not always necessary to control all pest species with the transgenic crop. If one or a few key pests are controlled—such as the *Heliothis*–*Helicoverpa* complex in cotton—without the use of pesticides that disrupt natural control systems, then pest management systems that employ the judicious use of pesticides and biological alternatives can more easily be designed.

Yet despite good success within some important crops, single transgene products (including *Bt*-modified crops), may not provide sustainable pest management within many standard cultivar systems because such single gene products often lack broad enough selectivity to work effectively against all major pest species within such systems (Sharma et al., 2004); fortunately, this limitation may be overcome by multiplexing techniques, such as genetic stacking in which multiple transgenes are expressed within a single GM crop species. Stacking of a *Bt* gene with the snowdrop (*Galanthus nivalis*) lectin gene, *gna* provides both an increase in pest-toxicity as well as an increase in the range of resistance against pest species, including non-lepidopteran sap-sucking insects (Maqbool et al., 2001; Ramesh et al., 2004), on which *Bt* products have no effect alone (Bernal et al., 2002). Gene stacking provides an attractive strategy for expanding tolerance to include multiple pest species, increasing efficacy of pest resistance, and coupling herbicidal with pest-resistance characteristics; in the

US and other countries gene stacking of *Bt* with herbicidal-resistance genes is increasingly finding usage within major crop species such as corn and cotton.

Engineered fusion proteins are elegant exemplifications of gene stacking. Transgenic rice and maize plants have been engineered to express a fusion protein that combines the δ -endotoxin Cry1Ac with the galactose-binding domain of the nontoxic ricin B-chain (RB). The fusion, termed BtRB, proved significantly more toxic in insect bioassays than *Bt* alone, and provided resistance to a wider range of insects, including important pests that are not normally susceptible to *Bt* toxins (Mehlo et al., 2005). While BtRB toxicity in non-pest species remains unstudied, multitargeting of thoughtfully chosen pest gut receptors may provide a formidable improvement of *Bt* toxin efficacy. Other fusion proteins have, for instance, improved upon the use of single PI transgenes, combining potent inhibitors of both aspartate and cysteine proteases, and thus may also provide new inroads to the development of more effective broad-spectrum biopesticides (Brunelle et al., 2005).

Another innovation has been in the ability to use insect neuropeptides gene constructs as biopesticides within GM plants. In general, neuropeptides are heavily regulated, undergoing rapid turnover within insect tissues, thus making them an unlikely tool for insect control. However, trypsin modulating oostatic factor (TMOF), a decapeptide hormone (YDPAPPPPPP) that stops 90% of trypsin biosynthesis in the mosquito gut at micromolar concentrations (Borovsky et al., 1990), has shown potential as a biopesticide. The peptide has demonstrated trans-species activity implying that it may work as a broad-spectrum pesticide: transgenic tobacco leaves containing a mosquito (*Ae. aegypti*) TMOF gene, when fed to budworm larvae (*Heliothis virescens*), inhibit trypsin biosynthesis, reducing larval growth-rate and increasing mortality by ~30% (Nauen et al., 2001; Tortiglione et al., 2002). The use of TMOF has also been applied to control of mosquitoes utilizing a variety of delivery methods (Borovsky, 2003a, b), and further refinement of gene constructs may provide greater control efficacy and allow wider application within GM plants.

3.2. Topical applications of peptide/protein-based pesticides

Direct topical application of TMOF peptides to crops or other insect comestibles may also work as a

broad-spectrum pesticide; genetic engineering and expression of TMOF in bacteria, yeast and algae is emerging as a potential larval control of mosquitoes in the field (Borovsky, 2003a, b), with techniques that show promise of applicability to a variety of other pests. TMOF or analogues fed to female mosquitoes with the blood meal, traverses the gut, enters the hemolymph, binds to gut epithelial receptor(s) and stops trypsin bio-synthesis and egg development (Borovsky and Mahmood, 1995); in Lepidoptera and fleshflies TMOF-like peptides also terminate trypsin biosynthesis in the gut after a blood or a protein meal (Borovsky et al., 1990; Bylemans et al., 1994; Nauen et al., 2001). TMOF peptide fused onto the tobacco mosaic virus (TMV) coat protein—incorporating a trypsin cleavage site to release TMOF in situ—adsorbed on yeast particles and fed to mosquito larvae caused inhibition of trypsin biosynthesis, starvation and larval mortality within 5 days. Cloning and expression of the hormone in *Saccharomyces cerevisiae* provided an effective oral larvicide readily consumed by mosquito larvae (Borovsky, 2003a, b). An initial 46% lethality to mosquito larvae—due to low production of TMOF in the fermented cells—was increased to 100% with improved fermentation yields (Borovsky, 2003a, b).

Peptide mimetics provide another means of utilizing neuropeptides as biopesticides. Benzethonium chloride (Bztc) was the first nonpeptidic agonist analog discovered for an insect neuropeptides (Nachman et al., 1996). Bztc mimics the physiological effects of the myosuppressin neuropeptide C-terminal pentapeptide, VFLRFamide (Yamamoto et al., 1988; Lange et al., 1995; Nachman et al., 1996), and acts as an agonist of dromyosuppressin in *P. regina*, inhibiting crop contractions (Richer et al., 2000). Bztc may thus interfere with normal food intake, storage, and crop-emptying (Haselton et al., 2004); although not immediately nor before sexual maturity, house flies fed various concentrations of Bztc in sugar solutions display a concentration-dependent mortality (Haselton et al., 2004).

Peptidomimetic antagonists of insect neuropeptides have been applied to the insect pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family as a model, which inhibited PBAN-mediated activities in moths in vivo (Altstein et al., 2000; Altstein, 2001, 2004). The PK/PBAN family (which currently comprises of over 30 peptides) is a multifunctional family of peptides

that stimulates cuticular melanization in moths, and mediates key functions associated with feeding (Nachman et al., 1986; Schoofs et al., 1991), development (Imai et al., 1991; Nachman et al., 1993) and mating behavior (Altstein, 2004) in a variety of insects. A D-Phe scan (sequential D-Phe replacement) library of linear peptides, synthesized on the basis of a slightly modified active sequence of PBAN produced partial melanotropic antagonists, selective pure melanotropic agonists, and pure pheromonotropic antagonists (Ben-Aziz et al., 2005). Such compounds show some promise as sprayable insecticides—since they are resistant to insect gut degradation—but may be limited in terms of their potency.

Topical applications of protein-based insecticides may become a more viable technique through the application of fusion protein constructs. Fusion of the baculoviral polyhedrin protein (Polh)—derived from the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV)—with a truncated *Bt* Cry1Ac provides approximately equal lethality to commercial *Bt* toxin-based insecticides, but is produced with a 3.6-fold expression increase over the Cry1Ac protein alone (Brunelle et al., 2005; Seo et al., 2005); in vivo, the Polh–Cry1Ac fusion protein has demonstrated high insecticidal activity against the pest, diamondback moth, *Plutella xylostella*. Expression of fusion protein constructs employing Polh in *Escherichia coli* shows almost the same characteristics as native baculoviral Polh when ingested by susceptible insects—rapid solubilization and proteolytic digestion within the midgut, forming easily isolatable inclusion bodies. Because this novel bio-insecticide employs *E. coli* as the host, mass production at a low cost should be possible; its protein-based design also avoids living modified organism issues such as environmental and ecological safety (Seo et al., 2005). Of course, currently available sprayable formulations of microbe-derived protein suspensions continue to have broad appeal and applicability. (A discussion of the use of intact microbe suspensions as sprayable pesticides will follow in the next section).

There are dozens of *Bt* proteins and over 100 *Bt* microbial insecticides registered in the US. These protein products derive from four subspecies of *Bt*, and are, in general, only toxic to particular insect orders: *Bt kustaki* and *Bt aizawai* against caterpillars, *Bt tenebrionis* (also called *Bt* San Diego) against beetle larvae, and *Bt israelensis* against fly larvae (including fungus gnats, blackflies, and

mosquitoes), noting that not all species of caterpillars, beetles or flies are susceptible. Sprayable formulations of proteinaceous pesticides require thorough plant coverage—since they must be ingested by insect pests—and also require early pest detection and application—since young larvae are generally more susceptible. Although some additives promote adherence to leaf surfaces and/or protect *Bt* from photo degradation, the spray deposit may remain viable only a few days necessitating repeated applications.

4. Biopesticidal delivery via insect-specific infectious agents or symbiotes

A number of infectious and symbiotic agents have been used in pest control. These agents generally exhibit narrow species specificity, preventing toxicity in non-target organisms, and rapid environmental inactivation compared to chemical pesticides, reducing the possibility of target-insect resistance development. The reduced potential for pest resistance development is a major advantage, resulting not only from their rapid environmental inactivation, but also because pathogenicity of infectious agents results from the concerted effect of multiple factors involved with microbial replication rather than from the toxicity of a single pesticidal compound. Their limitations, on the other hand, include comparatively slower speed of kill, lowered toxicity, inability to broadly target all pest species involved in a particular crop system, and the necessity for more frequent application than their chemical counterparts.

4.1. Bacterial, fungal and nematodal delivery

A variety of infectious bacterial, fungal and nematode strains have been utilized as sprayable biopest controls, the production and formulation of which has been thoroughly described (Taborsky, 1992). Of these, *Bt* has proven the most widely adopted and applicable (Federici, 2005). The first *Bt* products, including many still available today, were made from naturally occurring wild-type species of *Bt* (e.g. DiPel[®], Javelin[®], and XenTari[®]). Newer strains of *Bt* have been created through a process called conjugation or transconjugation, in which two or more subspecies of *Bt* transfer, via plasmid, quantities of DNA from one another in a way that facilitates the formation of new strains with

desirable qualities from both parents. Exemplary products include Condor[®] and Cutlass[®].

Recombinant forms of *Bt* have been designed which provide wider coverage of, and increased toxicity to pest species by incorporating the Cry1C gene from one bacterial strain within the host bacterial genome of a second strain, thus providing complementarity to the host's endogenous Cry1A protein (Yue et al., 2005). As previously reviewed, (Gill et al., 1992; Schnepf et al., 1998; Aronson and Shai, 2001) *Bt* produces cytolytic toxins and δ -endotoxins—encoded by the *cyt* and *cry* genes, respectively—that are solubilized in the alkaline conditions of the insect midgut and proteolyzed into active toxins. These active toxins bind to specific receptors found on the insect's midgut epithelial cells and aggregate to form ion channels, leading to osmotic cell lysis (Gill et al., 1992; Schnepf et al., 1998; Aronson and Shai, 2001).

As alternatives to *Bt*, the bacteria *Photorhabdus luminescens* and *Xenorhabdus nematophilus* may find use in nematodal formulations (French-Constant and Bowen, 2000; Chattopadhyay et al., 2004). Both symbiotes of entomopathogenic nematodes, these bacteria release toxins into an insect upon invasion by the nematode, establishing the insect cadaver as a monocultural breeding ground for both bacteria and nematodes.

Genetic manipulation of bacterial symbiotes has allowed paratransgenic control of the triatomine vectors of Chagas disease (Beard et al., 2001). As obligate hematophagous insects, feeding on vertebrate blood throughout their entire developmental cycle, triatomines harbor populations of bacterial symbiotes within their intestinal tract, which provide the required nutrients lacking from their diet. Genetic transformation of symbiote cultures from various triatomine species—inserting either the insect immune peptide cecropin A or an active single chain antibody fragment—and reintroduction of the bacteria into their original host species produced stable paratransgenic insects refractory for infection with *Trypanosoma cruzii*. Utilizing the coprophagic behavior of these insects, this approach allows introduction of GM bacterial symbiotes into natural populations of Chagas disease vectors (Beard et al., 2001).

Infectious fungi have also been utilized against a variety of insect pests. *Metarhizium anisopliae* has been applied to rice pests, the sugarcane spittle bug *Mahanarva postica*, and against Colorado beetle *Leptinotarsa decemlineata* (Taborsky, 1992). Among

other pests, *B. bassiana* has been used against insects of the Coleoptera, Lepidoptera, and Diptera orders. *Verticillium lecanii* has been shown to be effective against aphids. As mosquito control agents, *L. giganteum*, *B. bassiana* and *M. anisopliae* have shown promise. Exposure to the fungus *B. bassiana* caused higher mortality rates in malaria-infected mosquitoes, reduced the proportion of surviving mosquitoes carrying sporozoites in their salivary glands, and diminished the likelihood for infected mosquitoes to take subsequent bloodmeals (Blanford et al., 2005). Similarly effective, the entomopathogenic fungus *Metarhizium anisopliae* reduced the degree of malaria transmission by 75% in independent field experiments (Scholte et al., 2005).

4.2. Viral delivery

As with bacteria and fungi, viruses have been used for biological control purposes for nearly a century, but like most bioinsecticides, are slow to act and require killing efficiency that exceeds their native level in order to achieve commercial competitiveness with chemical pesticides. Utilizing well-described methodologies (Summers and Smith, 1987; O'Reilly et al., 1992; Richardson, 1995; Merrington et al., 1999), several innovative and successful approaches have been taken to improve the speed of kill of baculoviruses through genetic modification (reviewed in Kamita et al., 2005). These approaches include insertion of a foreign gene into the baculovirus genome, deletion of an endogenous gene from the baculovirus genome, and incorporation of active toxin into the occluded virus. Combinations of these approaches have been successful at increasing inhibition of insect feeding and lethality.

Most examples of GM viruses utilize viruses of the Baculoviridae family and include exogenous gene insertions such as those encoding insect-specific toxins (Merryweather et al., 1990; Maeda et al., 1991; Gershburg et al., 1998; Harrison and Bonning, 2000; Regev et al., 2003), hormones (Eldridge et al., 1991, 1992a, b), neuropeptides (Ma et al., 1998) and enzymes (Bonning et al., 1992; Gopalakrishnan et al., 1993; Harrison and Bonning, 2001). Baculoviruses are not, by any means, the only or the best vectors for delivery of insect control agents. However, a variety of factors including relative ease of production, technological history, and ease of genetic manipulation suggest

them as the first vector targets (Inceoglu et al., 2001).

4.2.1. Insect hormones, neuropeptides and enzymes

Insertion of an insect neurohormone gene to increase the insecticidal activity of the baculovirus was first conceived in the late 1980s as a way of turning “an infected cell into a neuropeptide factory within the insect” (Keeley and Hayes, 1987; Menn and Borkovec, 1989). A recombinant baculovirus expressing a diuretic hormone gene was created that disrupted the normal physiology of silkworm (*B. mori*) larvae, increasing the speed of kill roughly 20% over that produced by the wild-type virus (Maeda, 1989). Subsequently, at least four biologically active peptide hormones have been expressed using recombinant baculoviruses: ecdysis hormone (Eldridge et al., 1991), prothoracicotrophic hormone (O’Reilly et al., 1995), pheromone biosynthesis activating neuropeptide (Vakharia et al., 1995; Ma et al., 1998), and neuroparsin (Girardie et al., 2001), unfortunately each with little success in improving the insecticidal activity of the baculovirus.

Second generation neurohormone systems have not yet been reported, but obvious targets are neurohormones stabilized to key degradation pathways, synergistic combinations of neurohormones, and more judicious selection of the transgene based on an intimate understanding of the biology involved.

Another approach, aimed at altering the normal hormonal levels found in the insect, was the insertion of a gene encoding the enzyme, juvenile hormone esterase (JHE), into the baculovirus AcMNPV that degraded JH, a key hormone in insect development (Hammock et al., 1990a, b). Anticipating that the recombinant virus would be ingested by early larval instars and produce JHE at a developmentally inappropriate time, it was discovered that specific uptake and degradation mechanisms for JHE limited *in vivo* efficacy: the virus reduced feeding and weight gain, killing infected larvae only slightly more quickly than the wild-type virus (Hammock et al., 1990a, b; Eldridge et al., 1992a, b). An elegant design, yet to date none of the JHEs tested have enhanced the speed of viral kill as well as scorpion toxins or proteases.

In practice, viral delivery and over-expression of an insect hormone or hormone-regulating enzyme have not been dramatically effective at improving viral speed of kill. Since critical events in the insect’s

physiology and life cycle are often controlled by redundant regulatory systems and protected by sequestration, this paucity of success is not completely unexpected (Kamita et al., 2005). With increasing knowledge of endocrine regulation in insects, new neuropeptide targets may be found or protease-resistant peptides may be designed, providing for viral gene constructs that can effectively exploit the pest insect’s neurophysiology as a control measure.

4.2.2. Degrading enzymes and proteases

Enhancins are baculovirus-encoded lipoproteins that can enhance the oral infectivity of a heterologous or homologous baculovirus in lepidopteran larvae (Tanada, 1959; Yamamoto and Tanada, 1978a, b). These proteins function by degrading proteins of the insect gut peritrophic matrix and/or by enhancing fusion of the virion with the insect gut epithelium (Kamita et al., 2005). Enhancin genes have been expressed by recombinant baculoviruses in order to improve the ability of the virus to gain access to the midgut epithelium cells, producing an 8% enhancement in the speed of kill (Popham et al., 2001; Li et al., 2003).

Harrison and Bonning have constructed recombinant baculoviruses expressing three different proteases—rat stromelysin-1, human gelatinase A, and flesh fly (*Sarcophaga peregrine*) cathepsin L—that digest basement membrane proteins and increase the rate of viral infectivity (Harrison and Bonning, 2001). The construct expressing cathepsin L generated a 51% faster speed of kill of *H. virescens* neonate larvae than wild-type virus, with infected larvae consuming ~27- and 5-fold less lettuce in comparison to mock or wild-type-infected second instars.

Chitinases are enzymes that can degrade chitin—an insoluble structural polysaccharide of the insect exoskeleton and gut linings—into low molecular weight oligosaccharides (Cohen, 1987; Kramer and Muthukrishnan, 1997). Baculovirus-encoded chitinases and proteases degrade chitinous and proteinaceous components of the host cadaver in order to induce liquefaction (O’Reilly, 1997; Hom and Volkman, 2000). A recombinant AcMNPV that expresses the chitinase gene of *M. sexta*, accelerated larval death by nearly 1 day over those infected with the wild-type virus (Gopalakrishnan et al., 1995). Systems such as these that could compromise the peritrophic matrix or a barrier to infection could be very useful as biopesticides, and if used in tandem

with other methods could provide dramatic pest control efficacy.

4.2.3. *Venom-derived insect-specific toxins*

Scorpion toxins—classified on the basis of size and pharmacological target site into long- and short-chain neurotoxins—provide a rich source of toxins with selective activity against insects (Zlotkin et al., 1978; Loret and Hammock, 1993). Long-chain neurotoxins mainly target voltage-gated sodium and calcium channels (Zlotkin, 1991; Loret and Hammock, 1993; Gordon et al., 1998), while short-chain neurotoxins primarily target potassium and chloride channels (Loret and Hammock, 1993). Among long-chain neurotoxins are insect-selective toxins— α -insect toxins, excitatory toxins, and depressant toxins—each targeting different molecular sites on the voltage-gated Na^+ (Na_v) channel and displaying unique symptoms when injected into larvae of the blowfly *Sarcophaga falculata* (Zlotkin et al., 1995; Gordon et al., 1998; Cestèle and Catterall, 2000; Inceoglu et al., 2001; and see review by Gordon et al. in this edition). Members of the short-chain family of scorpion toxins also include insect-specific toxins—including Peptide I of *Mesobuthus tamulus sindicus*, neurotoxin P2 of *Androctonus mauretanicus mauretanicus*, Lqh-8/6 and chlorotoxin of *Leiurus quinquestriatus hebraeus*, insectotoxins I5, I5A and II of *Mesobuthus eupeus*, and the *Mesobuthus tamulus* lepidopteran-selective toxin ButaIT—which cause flaccid paralysis (Wudayagiri et al., 2001; and see review by Gurevitz et al. this edition).

The first to attempt to express biologically active scorpion toxin, insectotoxin-I of *Buthus eupeus* (*BeIt*), met with limited success; constructs were expressed, but toxin-specific biological activity was not observed in larvae of *T. ni*, *Galleria mellonella*, or *Sarcophaga* (Carbonell et al., 1988). The insect-selective neurotoxin *Androctonus australis* insect toxin I (AaIT) was the first scorpion toxin to be expressed by recombinant baculoviruses that showed biological activity (Maeda et al., 1991; McCutchen et al., 1991; Stewart et al., 1991). AaIT, highly specific for the Na_v channel of insects (Zlotkin et al., 2000), induces a neurological response similar to that evoked by the pyrethroid insecticides, but apparently acts at a different site within the Na_v channel. Numerous other baculovirus vectors have been modified with AaIT gene insertions, in general producing viruses with 30–40% improvements in the speed of kill (Darbon

et al., 1982; Maeda et al., 1991; McCutchen et al., 1991; Stewart et al., 1991; Harrison and Bonning, 1999, 2000; Chen et al., 2000; Treacy et al., 2000; Sun et al., 2002, 2004). Infected larvae are typically paralyzed, stop feeding and fall off of the plant approximately 5–11 h prior to death, reducing the amount of leaf area consumed by up to 62% and 72% over that consumed by the wild-type infected and uninfected larvae, respectively (Cory et al., 1994; Sun et al., 2004). As this knock-off effect implies, median survival time is not necessarily the best predictor of viral efficacy (Hoover et al., 1995).

AaIT-expressing, recombinant baculovirus efficacy results from its ability to continuously provide toxin to the insect central nervous system (Zlotkin et al., 2000). Lepidopterous larvae infected with an AaIT-expressing baculovirus display symptoms of paralysis identical to those induced by injection of the native toxin, but possess an ~50-fold lower hemolymph toxin concentration than insects paralyzed by the native toxin. This observation has been attributed to the constant production of toxin by virally infected cells lining the insect's tracheal epithelia, which introduce the expressed toxin to the insect central nervous system and provide it with critical target sites inaccessible to the native toxin (Elazar et al., 2001).

Another well-studied series of scorpion-derived insecticidal toxins that have been used to produce recombinant baculoviruses come from the venom of yellow Israeli scorpions, *Leiurus quinquestriatus hebraeus* and *L. quinquestriatus quinquestriatus*. Both excitatory and depressant insect selective toxins have been isolated from these scorpions (Zlotkin et al., 1985, 1993; Kopeyan et al., 1990; Zlotkin, 1991; Moskowitz et al., 1998), and recombinant viruses have been generated that express either the excitatory LqhIT1 or depressant LqhIT2 toxin (Gershburg et al., 1998). These toxins are excreted from the cell, producing median effective times (ET_{50} s) for paralysis and/or death roughly 24% and 32% faster, respectively, than the wild-type virus. At least two other examples exist in which recombinant baculovirus expressing LqhIT2 fused to a bombyxin signal sequence were generated (Harrison and Bonning, 2000; Imai et al., 2000). When expressed under the *polh* gene promoter, the recombinant virus improved the median time to effectively paralyze or kill (ET_{50}) roughly 35% over that of the wild-type; in neonate larvae of the European corn borer the median survival time (ST_{50}) decreased by as much as 41% from that

witnessed from wild-type viral infection. The ST_{50} s of LqhIT2-infected neonate *H. zea* and *H. virescens* were also significantly lower than control neonates infected with a recombinant expressing AaIT.

The *L. quinquestriatus hebraeus*-derived α -toxin gene, Lqh α IT has also been tested within a recombinant AcMNPV (Chejanovsky et al., 1995), decreasing the LT_{50} by 35% over the wild-type virus in cotton bollworm (*H. armigera*) larvae. While some have suggested that since the Lqh α IT toxin binds at a different site on the insect Na_v channel from that of the excitatory toxins, a baculovirus expressing both alpha and excitatory toxins may yield a synergistic interaction between the toxins (Zlotkin et al., 1978; Cestèle and Catterall, 2000), it's important to note that anti-mammalian α -toxins are not insect-specific, also having toxicity in mammals, and therefore will not find commercial applicability as biopesticides. However, other examples of the expression of insect-selective toxins from *L. quinquestriatus hebraeus* abound—constructs varying in viral vector, gene promoter, secretion signal, and/or insertion locus of the toxin—and it may be that judicious selection and combination of toxins from two or more of these will substantially increase pesticidal efficacy (Kamita et al., 2005).

In addition to peptide toxins of scorpion origin, insect-selective and highly potent toxins have been identified from other organisms including lacewings, spiders, sea anemones, mites and bacteria (see reviews by King et al., Nicholson, Rohou et al., Bosmans & Tytgat and ffrench-Constant et al. in this edition). The paralytic neurotoxin TxP-I of the insect-predatory straw itch mite *Pyemotes tritici*, induces rapid, muscle-contracting paralysis in larvae of the greater wax moth *G. mellonella* (Tomalski et al., 1988, 1989; Tomalski and Miller, 1991). A recombinant, occlusion-negative AcMNPV expressing toxin under a modified polyhedrin promoter was shown to paralyze or kill fifth instar cabbage looper (*T. ni*) larvae by 2 days post-injection; control larvae injected with wild-type AcMNPV never showed symptoms of paralysis (Tomalski and Miller, 1991). Variations of the viral promoter used to drive expression of the toxin have been examined, in one case resulting in earlier and higher level of toxin expression, reducing the ET_{50} of in neonate larvae of fall armyworm *S. frugiperda* and *T. ni* by ~56% and 58%, respectively, in comparison to wild-type AcMNPV (Tomalski and Miller, 1991, 1992; Lu et al., 1996; Burden et al., 2000).

Recombinant baculoviruses containing insect-selective toxins from the spider *Agelenopsis aperta* and sea anemones *Anemonia sulcata* and *Stichodactyla helianthus* reduced ET_{50} s by 17–38% (Prikhod'ko et al., 1996). Viruses expressing insect-specific toxins from the spiders *Diguetia canities* and *Tegenaria agrestis*, stopped infected larvae from feeding ~17–42% more quickly than larvae infected with wild-type virus, but interestingly, the speed of kill was not directly correlated with cessation of feeding (Hughes et al., 1997). The lack of correlation between these two criteria again emphasize that enhanced speed of kill is not necessarily a reliable indicator of viral effectiveness (Hughes et al., 1997); reduction in crop damage is, of course, a key criterion, and even small changes in herbivore coordination can lead it to fall from the host plant, an event that in the field is tantamount to death.

4.2.4. Microbial toxins

Several studies have examined *Bt* toxin expression in baculovirus, investigating either the full-length protoxins or active forms of *Bt* toxin gene-products (e.g. *cryIAb*, *cryIAc*, etc.) (Martens et al., 1990, 1995; Merryweather et al., 1990; Ribeiro and Crook, 1993, 1998; Woo et al., 1998). In all cases, *Bt* toxin was highly expressed by the baculovirus, processed into the biologically active form, but did not improve the virulence or effectiveness of the virus. These findings are understandable considering that the site of action of *Bt* toxins is the extracellular surface of midgut epithelium, whereas the baculovirus-expressed *Bt* protoxin is produced intracellularly within the insect body. Consequentially, the expressed protoxin may not be processed to the active form because of the lack of appropriate proteases within the cytoplasm, may be poorly secreted, or may be cytotoxic to the cell (Martens et al., 1995).

In an attempt to improve toxin secretion, several *Bt* toxin gene fusion constructs were made that incorporated a N-terminal signal sequence from *H. virescens* JHE (Martens et al., 1995). The toxin-fusions were translocated across the endoplasmic reticulum (ER) membrane, but remained sequestered within cellular compartments, suggesting that the expression of the *Bt* toxin gene will have little or no effect in improving insecticidal activity once the virus crosses the midgut.

In order to deliver the *Bt* toxin directly to the insect midgut epithelial cells, a recombinant baculovirus

that occludes the toxin within its polyhedra was tested (Chang et al., 2003). The baculovirus, termed ColorBtrus, was constructed so that it would co-express both native polyhedrin and a polyhedrin-Cry1Ac-green fluorescent protein (GFP) fusion (Chang et al., 2003). Although the Cry1Ac toxin was fused at both the N- and C-termini, trypsin proteolysis resulted in a product functionally identical to authentic Cry1Ac toxin. With an estimated 10 ng of Cry1Ac per 1.5×10^6 viral polyhedra, the recombinant virus led to a dramatic reduction in both the median lethal dose and median survival time. Such enhancement of both viral virulence and killing efficiency is remarkable, since in general, viral recombinants show little influence on the median lethal dose. Furthermore, because of GFP fluorescence under UV, infected insects can be rapidly detected in the field (Chao et al., 1996; Chang et al., 2003).

Constructs such as these, which stack multiple pest-control components within a single delivery vehicle, have obvious advantages. Thoughtful choice of gene inserts provides additive and in some instances synergistic pesticidal effect, increasing the range of targeted pest species while also increasing lethality. With viral constructs such as ColorBtrus, the choice of gene products also allows efficient *in vivo* production of viral pesticides in non-susceptible insect species; ColorBtrus, for example, is not effective against *Spodoptera* species such as the beet armyworm *S. exigua* (Bai et al., 1993), which therefore could be used for industrial production of the virus. In addition, the common complaint of poor environmental longevity with regards to biopesticides, may become an asset with stacked gene products, since again, low environmental persistence may reduce the likelihood of pest resistance. In addition, it has been observed that traits that confer resistance to toxins delivered within GM plants often, but not always, detrimentally impact competitive fitness and provide for rapid loss of those genetic traits within the larger population (Ferre and Van Rie, 2002); similar results may also be observed for virally delivered toxins.

The concept of stacking genes for resistance management in transgenic baculoviruses may, on the other hand, have more perceived than real value in the field, since one should anticipate resistance development to viral-induced mortality, not to any transgenically delivered toxins. Resistance of insect pests to baculovirus and other viruses already exists, and can be anticipated to increase if their use and

efficacy increase. Although gene stacking within plants appears to reduce the occurrence of resistance, in considering engineered viral vectors, resistance development will most likely have less to do with the gene insert than with the vector itself. Regardless of the presence of the transgene, a baculovirus-infected larva will die: transgenes are simply quick kill genes, converting an excellent natural control agent into a poor one due to reduced environmental recycling. Thus, although virally delivered transgenes can serve as effective ‘green’ pesticides for augmented biological control, they should not be viewed as bulletproof pesticides.

The limitations of viral delivery systems are many, but with careful design of stacked gene inserts, choice of promoter, many of those limitations are being overcome. GM baculoviruses can easily become an integral part of pest insect control, especially in developing countries and for the control of insects that have become resistant to synthetic chemical pesticides (Hammock et al., 1993; McCutchen and Hammock, 1994; Miller, 1995; Wood, 1995; Bonning and Hammock, 1996; Inceoglu et al., 2001; Bonning et al., 2002). In fact, many baculoviral vectors have been successfully registered for use as microbial pesticides by commercial companies and governmental agencies: viruses of the velvet bean caterpillar *Anticarsia gemmatilis* (AgMNPV) and *Helicoverpa armigera* (HaSNPV) are being used with particular success for the protection of soybean in Brazil (Moscardi, 1999) and cotton in China (Sun et al., 2002), respectively.

5. Conclusions and future directions

During the past decade a number of products have emerged from the effort to develop alternative biopesticidal technologies. These products include microorganisms and microbial toxins, insect-derived compounds—mating disruption pheromones, hormones and enzymes—and phytotoxins. Many of these have been expressed within GM plants or microbial vectors with excellent results, but limitations—both real and perceived—have, however, retarded their mainstream acceptance and commercial adoption. Despite predictions by the Organization for Economic Cooperation and Development that biopesticide sales may expand to 20% of the world’s pesticide market by 2020, currently, biopesticides represent only about 1% of the world

pesticide market, with *Bt* products constituting nearly 80% of this amount (Whalon and Wingerd, 2003).

Several drawbacks have daunted many of these technologies and products, primary among which is their high production cost and limited applicability. In comparison to chemical counterparts, biopesticides are often slow acting, and have narrow host-specificity and poor longevity in the field, although many of these limitations have beneficial as well as detrimental aspects. For instance, rapid environmental degradation of biopesticides may necessitate more frequent application, but also reduces selection pressure and the resultant chance of pest-resistance development. The typically narrow pest-specificity of biopesticides often requires application of multiple types of pesticides to account for all major pest species involved in a particular agricultural setting, but also eliminates harm infliction on non-target organisms. With these issues in mind, through careful engineering, it is possible that biopesticide limitations may be ameliorated, while their benefits retained.

Apparent disadvantages of biopesticides must also be weighed against the high environmental and human health cost of other insect control methods, particularly in developing countries where sophisticated IPM systems are difficult to implement. The negative environmental impact not only of pesticides but even of technologies like cultivation could be reduced with the careful use of such green pesticides.

Much of the work of the last decade has sought to overcome the limitations of slow-action and narrow host specificity, and to increase toxicity and rate of delivery of the biopesticide. Many of these problems have been demonstrably improved through a thoughtful combination of multiple toxins within single delivery systems.

Constructions of GM plants containing stacked genes for herbicide and pest resistance have demonstrated good pest control without substantial development of pest resistance. Plants modified to contain *Bt* toxins, for example, have only a single example of a pest with significant resistance development (Ferre and Van Rie, 2002; Gunning et al., 2005). Similar constructs which stack several pest-resistance genes that act in unrelated ways—for example a combination of fused *Bt*-Polh toxin, lectin, and TMOF inhibition of trypsin synthesis—could further increase the range of pest-specificity and pest-toxicity, while also dramatically reducing

or alleviating both pest resistance development and impact upon non-pest species. Of course, gene stacking must also be used in tandem with IPM measures—as are already in place in some countries—to further minimize selection pressure and control resistance development (Macdonald and Yarrow, 2003). The obstacles, then, with production of such GM plants lie more within the effects that such gene insertions may have on the quality of foodstuffs produced, on regulatory issues, and on the public perception of this type of food engineering.

Public perception, whether soundly based or not, is an important criterion for policy making. Thus alternatives, such as engineered insect viruses, may or may not provide a viable alternative to GM plants, regardless of technical improvements. Engineered viruses have been proven effective pest-control means and, as this review shows, a number of promising developments in the types of gene inserts, gene promoters and viral vectors have been examined in recent years. But many of the same concerns expressed by the public with regards to GM crops will undoubtedly come into play if large-scale commercialization of viral-based insecticides occurs. For example, broadcast on a variety of internet websites, are calls for a moratorium on GM foods, based on incendiary claims that GM foods cause inflammatory diseases, lymphoma and may induce pediatric immunological disorders. Likewise, some of the public may wrongly associate the transmutability of avian influenza viruses with all virus types, and assume that insect viruses such as baculoviruses may become human vectors for disease. Such considerations must be made if research is to provide real solutions to pest control that can be put into production by agrichemical companies.

Although baculoviruses are generally regarded as safe and selective bioinsecticides, and have been used worldwide against many insect pests, their application as microbial pesticides has not met their potential. Strategies to counteract some of the limitations of baculoviruses, especially their slow killing activity, have been validated a number of times, and yet with few exceptions—for example viral control of the soybean caterpillar (*A. gemmatalis*) in Brazil (Moscardi, 1999)—such viral pesticides have not been broadly applied, for what some have called the “psychological effects of seemingly unsuccessful commercialization” (Kamita et al., 2005).

Biopesticides currently constitute ~2–3% of the insecticides market, however for biopesticides to take a larger role in the pesticide market, they must meet all of several criteria. They must: (i) be cheap to produce, (ii) have broad pest-species specificity, (iii) have low toxicity in non-target organisms, (iv) be easy to formulate and deliver, (v) remain in the environment long enough to be effective, but not so long as to induce resistance development within pest species, (vi) be publicly perceived as innocuous; and (vii) be readily accessible to both small farmers as well as large agribusinesses. These goals have not yet been realized, but there have been significant technological improvements and the possibility for future competitive biopesticidal products exists. Future research will undoubtedly continue to facilitate the meeting of those criteria, as well as bring other disparate technologies together in innovative ways to provide new formulation and biopesticide technologies.

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