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Biotechnology Advances 24 (2006) 143–160
www.elsevier.com/locate/biotechadv

Research review paper

Baculoviruses—re-emerging biopesticides

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Received 17 April 2005; received in revised form 19 August 2005; accepted 9 September 2005
Available online 27 October 2005

Abstract

Biological control of agricultural pests has gained importance in recent years due to increased pressure to reduce the use of agrochemicals and their residues in the environment and food. Viruses of a few families are known to infect insects but only those belonging to the highly specialized family *Baculoviridae* have been used as biopesticides. They are safe to people and wildlife, their specificity is very narrow. Their application as bioinsecticides was limited until recently because of their slow killing action and technical difficulties for in vitro commercial production. Two approaches for the wider application of baculoviruses as biopesticides will be implemented in future. In countries where use of genetically modified organisms is restricted, the improvements will be mainly at the level of diagnostics, in vitro production and changes in biopesticide formulations. In the second approach, the killing activity of baculoviruses may be augmented by genetic modifications of the baculovirus genome with genes of another natural pathogen. It is expected that the baculoviruses improved by genetic modifications will be gradually introduced in countries which have fewer concerns towards genetically modified organisms.

Keywords: Baculovirus; Biopesticide; Nucleopolyhedrovirus; Expression vector; Detection; Genetic modification

Contents

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doi:10.1016/j.biotechadv.2005.09.001

1. Introduction

1.1. Agents used for bioregulation of pests

Biological regulation can be defined as a regulation of a species which has reached the level of a pest by another living organism added to the environment to suppress pest densities. So it is ecologically based management of pests by naturally occurring, or genetically modified enemies. Natural enemy choice will vary greatly depending on the target species. Apart from predators, insects have many natural pathogens; these include bacteria, fungi, nematodes and viruses. These pathogens when applied artificially as microbial pesticides may effectively suppress pests. The population of microbial pathogens and/or predators increases because pests are used as a nutritional source and this, in turn, leads to the gradual decline of pest population. Biological control can be potentially permanent because the natural enemies supplied from the outside will establish themselves in the pest population and are likely to exert long-term protection against the target pest species.

The ways to implement biological regulation can be roughly divided into three major groups: importation, conservation/augmentation of natural enemies (which includes predators, parasitoids and causal agents of diseases), and application of microbial pesticides. Importation can be defined as the introduction of a foreign agent for biological regulation of populations while conservation and augmentation comprise the actions which preserve a native agent and provide the conditions for a native agent to increase its number and opportunities to act as homeostatic factor against a pest. Out of these methods, the importation of foreign enemies poses the greatest risk of changing the ecological equilibrium (Myers et al., 2000). On the other hand, one of the most successful attempts to introduce a bioregulatory agent was achieved by introduction of a foreign species. As early as the end of XIXth century, Australian species of a ladybug, *Rodalia cardinalis*, was introduced into California orange orchards to control cottony cushion scale, *Icerya purchasi*. Within few years, ladybugs reduced the pest to marginal numbers. The remaining methods are widely used and are relatively safe for the environment. Overall, the benefits from the successes in using biopesticides outweigh by far the money lost on failures which occasionally occur. Economic benefits, though important, are not the only advantages of biocontrol. Animal life, farmers and their families also greatly benefit, as successful biocontrol reduces the exposure to harmful chemicals.

Microbial pesticides are probably the most widely used and cheaper than the other methods of pest bioregulation. Insects can be infected with many species of bacteria but the species belonging to the genus *Bacillus* are the most widely used pesticides. Of these *Bacillus thuringiensis* is the most successful. It is a gram-positive, spore-forming bacterium with parasporal crystals. *B. thuringiensis* has developed an array of molecular mechanisms to produce pesticidal toxins, most of them coded by several cry genes (Agaisse and Lereclus, 1995). There are about 200 registered *B. thuringiensis* products in the USA and at the end of the last century worldwide sales amounted to about 100 million dollars (about 2% of the total global insecticide market). Though the resistance to Cry proteins may develop after prolonged usage (Schnepf et al., 1998), safety considerations favour the future development of these toxins and their share in pesticide market steadily increases.

An extensive number of fungi are associated with a variety of insects and other arthropods, establishing diverse interactions, including the pathogenicity. Approximately 750 species, belonging to 100 genera, of entomopathogenic fungi have been reported, but only about ten of them have been or are now being developed for insect control (Hajek and St. Lager, 1994). According to Benjamin et al. (2004), the entomopathogenic fungi can be classified into two main groups:

Biotrophic fungi. These fungi require living cells of their hosts, some of them are commensals that obtain nutrients from the digestive tract of the insect. This class of fungi is wide-spread in many regions, but they are not broadly used for pest control since they are either asymptomatic in insects or the changes caused by pathogens are difficult to observe (Lacey and Kaya, 2000).

Necrotrophic fungi. These are the fungi that live at the expense of dead cells; they have to kill their hosts before consuming it. Fungi belonging to this group are very effective in their attack, for this reason many of them are potential agents of biological control of insects. They can attack insects of the orders Coleoptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera, Homoptera, Diptera; the attack can occur in several stages of their life cycles (Goettel and Johnson, 1995). Several toxic compounds have been isolated from some species of fungi belonging to the genera *Beauveria*, *Metarhizium*, *Nomuraea*, *Aspergillus*, *Verticillium*, *Paecilomyces*, *Isaria*, *Fusarium*, *Cordyceps*, *Entomophthora*. One of the substances well studied is the beauverin, a peptide isolated from *B. bassiana*, with activity against mosquito larvae

(Uribe and Khachatourians, 2004). Some species of entomopathogenic fungi possess specificity against their hosts but usually they have a wide spectrum of action. The choice of the host is based on the biochemical affinity to the substrate and not on its morphological characters. This situation has been observed for *B. bassiana*, *N. rileyi* and *M. anisopliae* which can infect about 100 species of insects of a wide variety of orders of insects (Bidochka et al., 2001; Devi, 1994; Fang et al., 2005). Fungi belonging to the genera *Beauveria* and *Metarhizium* has been found to be effective against many insect pests including African locust (Langewald et al., 1999). These fungi are formulated as commercial products and are good alternatives to organophosphate and organochlorinated pesticides, mainly because some of them are exerting long-term effect and persistence in the environment. New generation fungi pesticides are often engineered to produce substances significantly improving biocontrol effectiveness (St. Lager et al., 1996).

Some nematodes, mostly those belonging to families *Steinerrnematidae* and *Heterorhabditidae*, are successfully used to control insects. In the strict sense of the word nematodes are multicellular eukaryotic organisms which do not belong to the microbial world. However, a close look at the mechanism of action of nematodes on insects justifies the classification of nematodes as microbial pesticides. Entomopathogenic nematodes penetrate insect hosts through natural openings and then defecate symbiotic bacteria belonging to genera *Xenorhabdus* or *Photorhabdus*. The bacteria quickly kill the hosts with toxins (Blackburn et al., 1998; Brown et al., 2004) and nematodes then reproduce in decaying host tissues. Nematodes are sensitive to desiccation, so their use is limited to control of pests in moist habitats.

Viruses of fifteen families are known to infect insects but only those belonging to the highly specialized family *Baculoviridae* have been considered as potential pesticides. They are very safe to people and wildlife. Their specificity is very narrow, often it is limited to only one species. They have been used worldwide but their application as bioinsecticides was limited (a few millions of dollars annually for the end of last century). Expansion of baculoviruses as commercial insecticides was hampered by their slow killing action and technical difficulties for in vitro commercial production. Due to the slow killing action of baculoviruses, primary users (used to fast-killing chemical insecticides) regarded them as ineffective. This attitude changes with time and baculovirus protection becomes a method of choice for long term protection of crops. Up to date the most successful project was implemented in Brazil where over two million hectares of soybean are controlled by baculovirus AgMNPV (Moscardi, 1999; Moscardi and Santos, 2005). The success of Brazilian project revived the interest in baculovirus as a biopesticide and gradually many countries have begun to increase the area of fields and forests to be experimentally protected by baculovirus pesticides.

2. Molecular biology of baculoviruses

Baculoviruses are the major group of arthropod viruses well known due to their potential as agents of biological control of pests in agriculture and forestry. They are also widely used as expression vectors in biotechnology. The family *Baculoviridae* comprises two genera: the *Nucleopolyhedrovirus* (NPVs) and the *Granulovirus* (GVs) (Van Regenmortel et al., 2000). NPVs can be phylogenetically subdivided into group I and group II. These viruses produce a large number of occlusion bodies in infected cells (polyhedra and granules, typical polyhedra are shown in Fig. 1) which allow virus to survive in the environment and to transmit the disease from one insect to another.

Baculoviruses infect arthropods and they do not replicate in vertebrates, plants and microorganisms. However, though they do not replicate, they may, under special conditions, enter animal cells. This unexpected property made them a valuable tool in the last few years for studies of transient expression of foreign genes under vertebrate promoters introduced into baculovirus genome (Boyce and Bucher, 1996; Kost et al., 2005). Baculoviruses are a large group of double-stranded

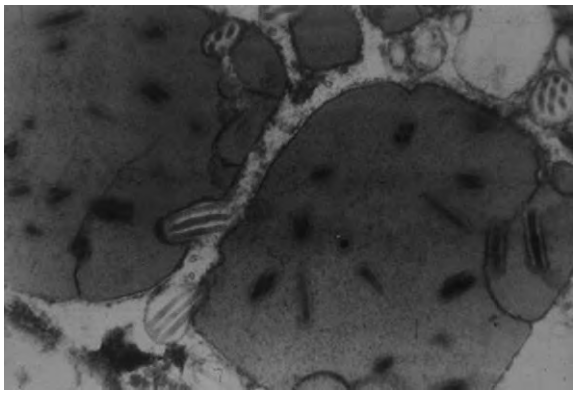

Fig. 1. Electron micrograph illustrating *Anticarsia gemmatalis* nucleopolyhedrovirus occlusion body formation. The large polyhedral occlusion body contains many virions which may have more than one nucleocapsid. The paracrystalline structure is surrounded by a carbohydrate-rich layer known as the outer polyhedron membrane.

DNA viruses (over 600 species have been described); the majority have been isolated from a few insect orders: Lepidoptera, Diptera, Hymenoptera and Coleoptera. Individual baculoviruses usually have a narrow host range limited to a few closely related species. Virions consist of one (SNPV) or more (MNPV) nucleocapsids embedded in a membranous envelope. Viral genome ranges in size from 80 to 200 kb in length. The most widely studied baculovirus is the *Autographa californica* nuclepolyhedrovirus (AcMNPV). Early work on AcMNPV was directed towards the development of viral pesticides and construction of baculovirus-based expression vectors (reviewed by Wood and Granados (1991)).

The circular DNA genome of AcMNPV is surrounded by a small basic protein (p 6.9) of molecular mass around 7 kD which neutralizes the negative charge of the DNA. This structure is protected by proteins forming a nucleocapsid. The genomic circular DNA is infectious in the naked form. Two morphologically distinct forms of the virus are produced at different times post-infection. Budded virus particles (BV) serve for the transmission of the virus to other tissues of the caterpillar body. BVs of type I nucleopolyhedroviruses contain GP64, a low-pH-dependent membrane fusion protein required for virus entry and cell-to-cell transmission (Monsma, 1996; Blissard, 1996; Hefferon et al., 1999; Kingsley et al., 1999). BVs of group II nucleopolyhedroviruses and granuloviruses lack a homolog of GP64. In this case, the low-pH-dependent membrane fusion during viral entry is triggered by F protein (Ijkel et al., 2000; Pearson et al., 2000). Whereas GP64 is unique for type I nucleopolyhedroviruses, homologs of F proteins are found as envelope proteins not only in insects and insect viruses, but also in some vertebrate viruses (Westenberg et al., 2004). It was found recently that GP64 is obligatory for transduction of mammalian cells; viruses producing F protein do not possess this property (Liang et al., 2005). Occlusion bodies (OBs) are responsible for the survival of the virus in the environment and the spread of the virus from insect to insect. The occlusion bodies (polyhedra) contain many nucleocapsids surrounded by a matrix composed mainly of polyhedrin, a major structural protein (Miller, 1997; Braunagel et al., 2003). Polyhedra are stable and the protected virions can survive in the environment for more than twenty years. The morphological changes and viral morphogenesis during infection have been reviewed by Williams and Faulkner (1997).

The natural cycle of infection by AcMNPV in insect larvae is summarized in Fig. 2. Caterpillars ingest polyhedra as contaminants of their food. The paracrystalline polyhedrin matrix is solubilized in the alkaline environment of the midgut of the larvae and the released virions (occlusion derived virus—ODVs) enter midgut cells after fusion with membranes of the microvilli. The virions are uncoated and enter the nucleus where viral genes are expressed in strictly controlled manner (reviewed by O'Reilly et al., 1992). Four phases of transcription are recognized: immediate early, delayed early, late and very late. Immediate early genes are transactivated by host transcription factors and viral proteins are not necessary at this stage. Transcription of delayed early genes requires activation by products of immediate early genes. Initiation of transcription occurs within highly conserved promoter elements: CGT for early genes and TAAG for late genes which are usually located 30–90 bp upstream from ATG start codon (Nissen and Friesen, 1989; Dickson and Friesen, 1991). The delayed early phase is followed by the synthesis of DNA and the late gene products of the virus (Hefferon and Miller, 2002). The late phase of infection is defined as those events that occur following the initiation of viral DNA replication. Late AcMNPV genes are transcribed primarily between 6 and 24 h p.i., whereas very late genes are transcribed in a rapid burst beginning around 18 h p.i. and continuing through 72 h p.i. (Lu and Miller, 1997). In the late phase nucleocapsid structural proteins are synthesized, including glycoprotein GP64 playing a crucial role in the horizontal infection by budded virus which is released from membranes 10–24 h post-infection (Whitford et al., 1989). During the very late phase the production of infectious BV is greatly reduced. Nucleocapsids interact with nuclear membranes and eventually become enveloped usually in groups of a few particles. Envelopment of the nucleocapsids appears to be an essential primary step in the process of occlusion of nucleocapsids by the very late protein—polyhedrin. The occlusion continues until eventually nucleus becomes filled with occlusion bodies. Typically more than 30 polyhedra are seen within the nucleus of AcMNPV. More than 10^{10} polyhedra are produced per late instar larvae before death which may account for over 30% of the dry weight of a larvae (Miller et al., 1983). As occlusion proceeds, the fibrillar structures begin to accumulate in the nucleus (sometimes also in the cytoplasm). These structures are composed mostly of a single polypeptide p10 which is a very late protein (Van Der Wilk et al., 1987). The function of fibrillar structures is not clear but they may play role in the controlled cellular disintegration in caterpillars (Williams et al., 1989; Van Oers et al., 1994). In the terminal stages of infection two viral proteins, chitinase and cathepsin, act together to facilitate host cuticle breakdown (Hawtin et al., 1997). After death the caterpillar liquefies and releases polyhedra which can infect other insects.

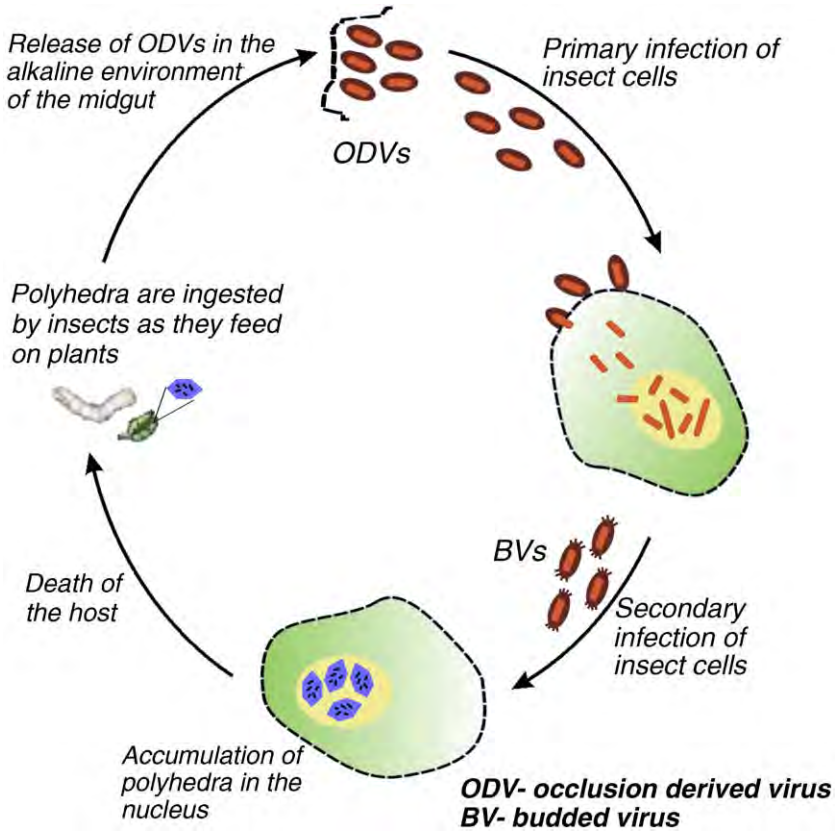

Fig. 2. Natural life cycle of baculovirus AcMNPV. Polyhedra are taken orally by the larvae with plant material and are dissolved in the alkaline environment of the midgut. ODVs are liberated and infect epithelial midgut cells. Virus replicates and budded viruses (BVs) are produced. They infect other tissues. After secondary infection, polyhedra are accumulated. Finally, the larval body disintegrates and millions of new polyhedra are released to the environment. BV — budded form of the virus. ODV — occlusion derived form of the virus.

The maintenance in insect population requires transmission of baculovirus from infected to uninfected larvae. Horizontal transmission plays the most important role. It is primarily through larvae ingesting occlusion bodies present on plants. The occlusion bodies can be further distributed by excrements of infected larvae and predators (Entwistle et al., 1993; Vasconselos, 1996). Vertical transmission may also play a role. It usually occurs through surface contamination of eggs or virus entering inside the egg (Kukan, 1999; Fuxa et al., 2002).

Our current knowledge about the biology of AcMNPV is to a large extent due to the developments of baculovirus-based expression vectors. Baculovirus system of expression of foreign genes has many advantages over other systems. Very high levels of foreign genes are usually achieved compared to other eukaryotic expression systems and it is possible to express more than one foreign gene (French et al., 1990; Brown and van Lent, 1991; Roy, 1992). Baculovirus genome can accommodate large pieces (around 20 kbp) of foreign DNA. Insertion of specific signal sequences in front of a foreign gene leads very often to the export of the gene product outside of the infected cell.

Baculovirus genome is large, so the insertion of a foreign gene by direct cloning into a defined locus is very difficult. Recombinant baculoviruses are, therefore, usually constructed in two steps. Initially, a heterologous gene is introduced into a baculovirus transfer vector. It consists of a bacterial replicon of a multicopy plasmid, a selection marker gene, promoter and terminator regions along with flanking baculovirus sequences from a non-essential locus, and a multiple cloning site (or a single unique restriction site) downstream from a viral promoter. Usually the promoters and the flanking DNA originate from one of the late genes: polyhedrin or p10 gene. The baculovirus transfer vector containing foreign DNA, and genomic viral DNA are then introduced into insect cells where they recombine yielding recombinant virus with an integrated heterologous gene. Polyhedrin and p10 promoters are very strong promoters, so high level of protein synthesis in

insect cells is to be expected. Basic protein promoter is weaker but sometimes it may be preferred because it is an early promoter.

A classical method of Summers and Smith (1987) for isolation of recombinant viruses made an advantage of different morphology of occlusion-negative and occlusion-positive baculovirus plaques. Plaques originating from viruses which have insertions in the polyhedrin gene appear less refractile than the polyhedrin-positive plaques and can be distinguished under a good quality microscope. It requires, however, considerable experience to become confident of locating the recombinant plaques. Therefore, it is not surprising that many modifications of this procedure were described. A major breakthrough in the selection of recombinants was the construction of transfer vectors which allowed for the introduction of a reporter gene along with a cloned gene into baculovirus genome. The reporter gene, most frequently *lacZ* gene, is usually inserted in the opposite direction to the cloned gene and it is under control of one of weak baculovirus promoters (Vialard et al., 1990; Zuidema et al., 1990). The reporter gene is introduced into baculovirus along with the foreign gene, therefore the addition of a chromogenic substrate for β-galactosidase (e.g. X-gal) to the selection plates yields coloured plaques (blue in case of using X-gal) which originate only from recombinant viruses.

Linearization of baculovirus genome at one or more locations simplifies the construction of recombinant baculoviruses. Linear baculovirus DNA exhibits a greatly reduced infectivity compared to preparations of circular DNA. When a unique Bsu36I restriction site was introduced into AcMNPV genome which allows for linearization in the vicinity of the polyhedrin gene, then recombinant viruses were obtained at a frequency of about 30% (Kitts et al., 1990). It should be pointed out that recombination between linear genomic DNA and a transfer vector results in circulation of the genome. So, even though the titer of recombinants per transfection is similar to this of the normal cotransfections with circular genomic DNA, the percentage of recombinants is greatly increased because the background of non-recombinants originating from linear DNA is greatly reduced. A development of the above method increased the percentage of recombinant viruses to almost 100% (Kitts and Possee, 1993). The modified virus contains three Bsu36I restriction sites near the polyhedrin locus. One is in a *lacZ* gene under the control of the polyhedrin promoter, the second is in a non-essential gene upstream from the polyhedrin promoter, the third is in an essential gene ORF1629 downstream from the *lacZ* gene. The majority of the viable progeny (approaching 100%) have ORF1629 restored by homologous recombination between the transfer vector and the large Bsu36I fragment which contains the remainder of the viral genome.

Recombinant baculoviruses are usually propagated in insect cell lines. Around 400 insect cell lines are available but only a few support the growth of AcMNPV. These lines were obtained from two parental organisms: *Spodoptera frugiperda* and *Trichoplusia ni*. The most widely used line is Sf9 which grows well in suspension (Vaughn et al., 1977). Currently the cell line BTI-Tn5B1-4 derived from *T. ni*, known as High Five cells, has been also largely used for viral growth (Granados et al., 1994). Cell lines which support the growth of *Lymantria dispar* nucleopolyhedrosis virus (LdMNPV), *Heliothis zea* nucleopolyhedrosis virus (HzSNPV), *Bombyx mori* nucleopolyhedrovirus (BmSNPV), *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) and a few other baculoviruses are also available.

Recombinant baculoviruses can be used to infect caterpillars by ingestion of occlusion bodies or by injecting the budded virus into the hemocoel (reviewed by O'Reilly et al., 1992). The first method can be used for recombinants with healthy polyhedrin gene, so in the past the foreign gene was usually introduced into p10 locus for driving a high-level of foreign gene expression in baculovirus infected cells. The second method can be employed for recombinants with foreign genes in polyhedrin locus. Recombinant occlusion-negative viruses can be also packaged into polyhedra by cells infected with a second, occlusion-positive virus (e.g. wild-type virus) (Wood et al., 1993). An alternative to replacement of a viral gene with a foreign gene is the duplication of a viral promoter (Roy, 1992). In this case none of the viral genes is lost. This allows for the expression under different promoters, e.g. basic protein gene promoter (Bonning et al., 1994). Lu et al. (1996) found out that the production of toxin Tox34 of straw itch mite *Pyemotes tritici* was the most effective when its gene was expressed from the 6.9 DNA binding protein (p6.9) gene promoter. *Rachiplusia ou* MNPV (RoMNPV) expressing a gene coding for scorpion *Androctonus australis* toxin (AaIT) or *Leirus quinquestriatus hebraeus* toxin (LqhIT2) killed larvae of corn borer *Ostrinia nubilalis* most effectively when the gene was cloned behind p6.9 promoter. When p10 promoter was used, in some cases the reduced level of polyhedra was produced and virions were not occluded efficiently (Harrison and Bonning, 2000). Recombinant AcMNPV expressing cathepsin L of the flesh fly through ie-1 promoter killed *Helicoverpa virescens* larvae only slightly faster than wild-type

AcMNPV. On the other hand, when the gene was expressed from the p6.9 promoter, the recombinant virus killed the host about 50% faster than the wild-type baculovirus (Harrison and Bonning, 2001). Recently, Tuan et al. (2005) showed that the early p-PCm promoter was better than the very late p10 for controlling insect pests when LqhIT2 scorpion depressant toxin gene was introduced into AcMNPV genome. These facts are consistent with higher susceptibility of earlier instars of larvae to baculovirus infection. It should be pointed out that though AcMNPV, which infects many lepidopteran species, is by far the most frequently used baculovirus for genetic modifications, other baculoviruses, including HzSNPV, *Helicoverpa armigera* SNPV, RoMNPV, are also employed for this purpose (Li et al., 1999; Treacey et al., 2000; Sun et al., 2004b).

3. Baculovirus pesticides—past and present

The attempts to use baculoviruses for the protection of European forests date back to 19th century but the first introduction of baculovirus into the environment which resulted in successful regulation of the pest in a large area occurred accidentally in 1930 s (reviewed by Moscardi, 1999). A parasitoid was imported from Scandinavia to Canada to control spruce sawfly *Diprion hercyniae*. Along with a parasitoid, an NPV specific for spruce sawfly was introduced which established itself in Canada (Bird and Burk, 1961). Since then no control measures have been required against *D. hercyniae*. This is an example of "introduction–establishment" approach—baculovirus becomes a permanent part of an ecosystem in which it was not previously present. When using baculovirus in pest management two other strategies are much more common: short-term insecticide approach when the infested areas are sprayed with highly concentrated baculovirus to quickly suppress the pest, and the seasonal colonization approach in which baculovirus release results in viral replication and establishment in pest population for more than one generation (Fuxa, 2004).

Although baculoviruses are regarded as safe and selective bioinsecticides, restricted to invertebrates, their application has not matched their potential to control pests in crops, forests and pastures. One notable exception is the *A. gemmatalis* nucleopolyhedrovirus (AgMNPV) used to control the velvet bean caterpillar in soybean (Moscardi, 1999; Moscardi et al., 2002). This program was implemented in Brazil in the early eighties, and currently over 2,000,000 ha of soybean are treated annually with the virus (Moscardi and Santos, 2005). Although the use of this virus in Brazil is the most impressive example of bioregulation with viral pesticide worldwide, the virus is still obtained by in vivo production mainly by infection of larvae in soybean farms. The details of the production, implementation and evolution of the use of AgMNPV in Brazil was presented by Moscardi et al. (2002). The demand for virus production has increased tremendously reaching currently the need for protection of four million hectares of soybean annually. This high demand for AgMNPV calls for the studies aiming at the sustained inexpensive in vitro production of the virus because large scale in vivo production of baculoviruses encounters many difficulties. However, a major problem in producing the virus, particularly relevant in in vitro serial passages, is the generation of mutants which may lead to the loss of virulence.

In the beginning of the 1980's, a pilot program for AgMNPV use in soybean fields was initiated by a Unit of the Brazilian Agricultural Research Corporation (Embrapa) located in Londrina, State of Parana. The implementation of the program began in 1982 when ca. 2000 ha of soybean was treated with AgMNPV. Initially frozen killed larvae were distributed for treatment of demonstration plots and virus production in the field. This provided inoculum for treatment of other areas in the same season or for subsequent seasons. In the early to mid 1990's, AgMNPV use approached 1,000,000 ha. An important step of the program was the development of wettable powder formulation of the virus in 1986. The transfer of technology from Embrapa to private companies started in 1990. Field production of the virus became a profitable business and large quantities of virus-killed larvae were produced at low cost. The bioinsecticide cost was 20–30% lower than the average cost of chemical insecticides, and the virus provided control of the pest with only one application per season, compared to an average of two applications for chemical insecticides. The production from one collection site may reach up to 600 kg of AgMNPV-killed larvae in a single day, enough for treatment of ca. 30,000 ha with the virus (Moscardi et al., 2002). However, since 1999 field production of the virus has not been sufficient to fulfill the rapidly growing demand for this bioinsecticide. Therefore, much effort has been devoted to the elaboration of methods for the commercial production of AgMNPV in the laboratory. In November 2004, the Research Station of the Farmers' Cooperatives for the State of Paraná (COODETEC) inaugurated a "Biofabric" for large-scale production of the virus (COOPERVIRUS PM). This laboratory is increasing its virus production with the aim to inoculate 600.000 larvae/day, which may be sufficient for spraying the virus over 1.3–1.5 million

of hectares of soybean per year. Recently, a "Pilot Plant" was built at Embrapa Soja, Londrina, for improvement of the laboratory process and also for training people in virus production. This laboratory will have the capacity to inoculate 25.000–30.000 larvae per day (Moscardi and Santos, 2005).

The use of AgMNPV in Brazil brought about many economical, ecological and social benefits. At the soybean grower level, the financial savings from the use of the virus may reach up to ca. U$7/ha/season, including product cost and application cost. The current annual savings at the grower level, in the total area sprayed with the virus is over U$11,000.000. Since the beginning of the program more than 17 million liters of chemical insecticides were not sprayed in the environment, resulting in considerable environmental benefits (Moscardi et al., 2002).

The occurrence of genomic alterations in wild-type isolates of baculovirus populations is a well-documented phenomenon. Genomic variability has been described for many wild-type virus including *A. californica* MNPV (Lee and Miller, 1978), *S. frugiperda* MNPV (Knell and Summers, 1981), *Spodoptera litura* MNPV (Maeda et al., 1990), *Panolis flammea* MNPV (Weitzman et al., 1992) and *Mamestra configurata* NPV (Li et al., 2005). Genotypic variants are easily recognized by the presence of submolar fragments in the electrophoretic patterns of restriction endonuclease digestion products. Plaque purification of wild-type isolates confirmed that they contain a mixture of these variants. Genotypic variation in baculovirus genomes can include point mutations, both small and large deletions and insertions (Krell, 1996). All regions of the genome appear capable of variation but the presence of some hot spots is postulated for certain genomic alterations such as insertions due to transposable elements or deletions in the hypervariable DA26 gene region (O'Reilly et al., 1990; Kamita et al., 2003). AgMNPV genomic variability has been also carefully studied because the selection pressure due to the application of AgMNPV in the field during subsequent years could lead to alterations in virus stability. The method of choice was the technique of restriction endonuclease analysis (REN). It was used to monitor the genetic stability of this virus, by comparing the DNA profiles of eleven different seasonal isolates. Viral DNA were initially purified from diseased larvae collected during several crop seasons and compared to AgMNPV-79, a wild-type virus that was used originally and subsequently in this program (Souza et al., 2001). In general, when a change was introduced in the viral population, such as a new cleavage site, it persisted in subsequent years. The most important conclusion inferred from the analysis of the virulence of these isolates by bioassays was the fact of retaining the pathogenicity of the virus throughout the years of its application. These results indicated that the virus maintains considerable stability, even with the existence of some genetic changes shown in the DNA restriction profiles.

The relative stability of the AgMNPV in in vivo conditions may not be exactly the same as in continuous passages in cell cultures. The major problems with the passage effect on baculovirus replication and expression are the loss of virulence of the polyhedra occlusion bodies. The most common mutations that occur in cell culture are DIPs (defective interfering particles) and FP (few polyhedra) mutants (reviewed by Krell (1996)). When the virus has gone through a long period of multiple infection cycles, the ability to infect insect cells decreases due to formation of defective interfering particles. These virions are lacking part of their genome, which include genes for polyhedrin and genes involved in DNA replication. The DIPs, which replicate faster (as they are smaller), need an intact virus as helper, and inhibit the replication of standard virus (Taticek et al., 1995). More of these DIPs are present in higher passage numbers, and when higher multiplicities of infection are used, their number quickly increases. In order to avoid DIP formation in virus stocks, insect cells should be infected at low multiplicity of infection, thereby minimizing the probability of a DIP entering a cell along with an intact virion (Wickham et al., 1991). It was found that non-homologous origin of DNA replication (non-*hr ori*) is involved in the mechanism of DIPs generation, and the removal of non-*hr ori* strongly enhanced stability of virus upon serial passage in insect cells (Pijlman et al., 2002). Generation of DIPs is highly cell-line specific which may reflect tissue related differences (Piljman et al., 2003). These findings may help to solve problems connected with large-scale production in insect–cell bioreactors (Piljman et al., 2004).

"Few polyhedra" (FP) mutants are often generated by serial passage of nucleopolyhedrovirus in established cell lines. The genetic defect is usually attributed to loss of a 25-kDa protein (Fraser et al., 1983). FP mutants frequently acquire host genome fragments (often containing a transposon) and/or lack a portion of the viral genome, and it has been demonstrated that the FP phenotype is an indirect result of disruption of the viral 25 K gene (Harrison and Summers, 1995). Recently it was shown that the gene product is also involved in host degradation process caused by virus infection (Katsuma et al., 1999). The studies of morphological changes of AgMNPV due to the serial passages in cell culture as well as an analysis of the susceptibility of different

lepidopteran cell lines to AgMNPV are currently being carried out in Brazilian institutes in charge of AgMNPV programme (Castro et al., 1997, 1999, 2002).

Despite relatively slow increase in share of pesticide market, the number of registered pesticides based on baculovirus increase steadily. The exact list of commercially available baculovirus pesticides is difficult to assess but it may exceed fifty formulations, some of them being the same baculovirus preparations distributed under different trade names in different countries (e.g. there are at least five commercial products consisting of *Cydia pomonella* granulosis virus). Both NPVs and GVs are employed as pesticides but the former group is much larger. Baculoviruses have been used for the protection of agricultural annual crops, fruit orchards and forests. Apart from the successful bioprotection of soybean fields by AgMNPV, many other baculoviruses were field-tested in the past.

Historically, the first viral insecticide Elcar™ was introduced by Sandoz Inc. in 1975 (Ignoffo and Couch, 1981). It was a preparation of *H. zea* NPV. HzSNPV infects many species belonging to genera *Helicoverpa* and *Heliothis* which include many important pests attacking a wide range of crops (Chakraborty et al., 1999). HzSNPV provided control, of not only cotton bollworm, but also of pests belonging to these genera attacking soybean, sorghum, maize, tomato and beans. In 1982 Sandoz Inc. decided to stop the production probably because more profitable new chemical pesticides became available. With the advent of increased ecological awareness in nineties and resistance to many chemical insecticides including pyrethroids, the same virus was registered under the name GemStar™ and was marketed by ThermoTrilogy Company. The virus spray produced in the United States is now marketed also in Australia by Aventis CropScience where it is a preferred product for protection of crops against *H. armigera* (Mettenmeyer, 2002). *H. armigera* is probably the most infamous of heliothine moths attacking nearly 200 crops including cotton, soybean, pigeon pea, chickpea, sunflower, tobacco, tomato, pepper, maize and many others. It is not surprising that many countries, including India and China try to introduce their own programs aiming at the reduction of this pest by biological means. In the state of Maharashtra, West Central India, *H. armigera* was traditionally removed by shaking pigeon pea plants (one of the main high protein food in vegetarian India) until the larvae fell from the plants onto blankets. Today this technique is used to obtain caterpillars which are fed on nuclepolydrovirus-infected chickpea seeds. The caterpillar carcasses are then used by farmers to prepare a bioinsecticide spray applied on pigeon pea fields. HaSNPV, which is almost identical to HzSNPV (Chen et al., 2002), has been adopted for large scale production as a viral pesticide in China and has been extensively used on cotton fields (Zhang et al., 1995). Since 1993 it was registered in China as a pesticide. In the past ten years enough biopesticide was produced to treat 100,000 ha of cotton with the virus.

Several species of the genus *Spodoptera* are important pests of annual crops. *S. frugiperda* NPV has been used in Brazil to control the larvae on maize; the area protected by the virus did not exceed 20,000 ha annually (Moscardi, 1999). Here additional problem was encountered; *Spodoptera* larvae are cannibalistic, therefore they must be reared in separate containers and this greatly increases the price of the insecticide. Besides, they easily liquefy in the final stages of the infection which makes more difficult the collection of their bodies and consequently it leads to the loss of many polyhedra. Currently, at least two commercial products based on *Spodoptera* NPV are available in the USA and Europe: SPOD-X™ containing *Spodopera exigua* NPV to control insects on vegetable crops and cut flowers in greenhouses, and Spodopterin™ containing *Spodoptera littolaris* NPV which is used to protect cotton, corn and tomatoes. Many other species belonging to the Noctuidae family are economically important pests of annual crops such as sugarcane, legume, rice and others. The genera *Autographa* and *Trichoplusia* played a major role in the development of baculovirus expression system but, on the other hand, NPVs which attack members of this genera were also extensively studied as potential biopesticides (Copping and Menn, 2000). *A. californica* and *Anagrapha falcifera* NPVs were registered in the USA and field-tested at a limited scale. These two NPVs have relatively broad host spectrum and can be used on a variety of crops infested with *S. exigua*, *T. ni*, *H. zea* and many other pests (Cunningham, 1995; Hostetter and Putler, 1991; Vail et al., 1993). Even codling moth, *C. pomonella*, a common pest of orchards in temperate zone is susceptible to these two broad range baculoviruses (Lacey et al., 2002) though the susceptibility is much lower than to the granulovirus CpGV of *C. pomonella*, its natural host. CpGV is the active component of at least five products commonly used for protection of orchards in Europe against codling moth (Carpovirusine™ in France, Madex™ and Granupom™ in Switzerland, Granusal™ in Germany, Virin-CyAP in Russia). The importance of *C. pomonella* is worldwide, hence the use of CpGV gradually increases and may reach 100,000 ha annually sprayed with this virus. Another application of baculovirus pesticides is connected with

protection of cassava plantations (Bellotti, 1999). *Erinnyis ello* (cassava hornworm) granulosis virus is now commercially available in some South American countries after it was found to be very efficient in suppressing and controlling hornworm populations.

Forests of temperate regions are subjected to the temporary defoliation occurring usually once every 10–20 years which is caused by relatively few lepidopteran pests. European and North American forests are often attacked by Lymantridae (*L. dispar*, *Lymantria monacha* and *Orgiya pseudotsugata*) and Noctuidae (mainly *P. flammea*). A few formulations of *L. dispar* NPV (trade names: Gypchek, Disparivirus, Virin-ENSH) and *O. pseudotsugata* NPV (trade names: TM BioControl-1 and Virtuss) are available. Their use is still limited but the environmental concern stimulates forest authorities to increase the area protected with baculoviruses (Cunningham, 1995; Reardon et al., 1996).

4. Future prospects

In the preceding chapter many naturally occurring baculoviruses were described which have been used as pesticides in a number of cropping systems worldwide. The most spectacular success—protection of over 2 million hectares of soybean fields against velvet bean caterpillar was a breakthrough which proved that the baculovirus protection is feasible and can be done at relatively low cost. Therefore, we can expect that after a lag period lasting for many years, more and more attempts will be made to reevaluate the use of baculovirus pesticides in the changing world which puts much more stress on environmental protection.

We expect that two parallel approaches will be pursued in the development of baculovirus pesticides:

- The first approach is based on the assumption that in some countries use of genetically modified organisms is restricted or even banned and only naturally occurring baculovirus without any genetic modifications may be used as a biological pesticide. The improvements in the application of the biopesticide in this case will be at the level of diagnostics of infection, development of the in vitro cultures and changes in the formulations of the biopesticide. Better diagnostics allows for much faster detection of the virus and hence for the successful prognosis of the progress of infection. The in vitro cultures prevent contamination with other microorganisms from the field and may greatly reduce the price of biopesticides, while improved formulations increase the stability of the biopesticide in the field.
- The second approach is applicable for countries with more enlightened attitude towards genetically modified organisms. This approach takes into account slow action of baculoviruses and aims at the acceleration of the killing activity of these organisms. The faster action can be achieved by genetic modifications of the baculovirus genome (e.g. with genes of another pathogen).

4.1. First approach—improvement of diagnosis, development of in vitro cultures and improvements in formulations of the biopesticide

One of the major problems in using baculoviruses as pesticides is their slow action and lack of morphological changes in larvae in first stages of baculovirus development. Effective natural infection with baculovirus may escape attention of forest or agricultural services if a massive infestation of a pest is observed and larvae contain baculovirus in the early phase of development. Lack of noticeable symptoms of infection by natural biological control agents is a signal for respective service to use chemical means of protection which, from the ecological point of view, is redundant and could be avoided if a quantitative, or even semi-quantitative assay for the progress of infection has been available. The same reasoning applies to cases when poorly trained services apply baculovirus as a pesticide. Long lag period can be prematurely taken as a sign of the resistance of the pest resulting in the lack of effectiveness of a biopesticide. Again a decision to spray the area with chemical pesticides may follow.

Therefore, it is crucial in pest management with baculovirus to have a sensitive method to assess the effectiveness of infection. Fast and sensitive methods used in diagnostics can be roughly divided into immunological methods based on protein composition and content, and genome detection methods usually based on PCR techniques. The application of methods based on protein detection is of little use in fast baculovirus diagnostics. As mentioned in the chapter on the biology of baculoviruses two proteins produced in large amounts, polyhedrin and p10, which could be used in ELISA or other immunochemical tests are very late proteins. So, they are not correct indicators of early stages of infections. Out of other proteins, probably glycoprotein GP64, specific for BV forms (Blissard, 1996), is a good candidate but no reports on the use of this protein for baculovirus quantitation are available. Analysis of baculovirus genomes provides us with much greater opportunities. Polyhedrin gene is one of the most conserved genes in the viral world. Baculoviruses of

evolutionarily very distant pests like *Neodiprion sertifer* (order Hymenoptera) nucleopolyhedrovirus (NdNPV) and *L. dispar* (order Lepidoptera) nuclepolyhedrovirus (LdMNPV) differ significantly in the size of the genome (LdMNPV is almost twice bigger than NdNPV) as well as in the gene alignment and sequence (Smith et al., 1988; Garcia-Maruniak et al., 2004) but the sequence of polyhedrin gene is similar in both viruses. Some sequences within polyhedrin gene are almost identical in all baculoviruses suggesting that it is possible to construct degenerate oligonucleotide primers which will detect any baculovirus in the sample. Therefore, these oligonucleotides can be used as universal primers for baculoviruses. Using these primers different samples may be analysed by polymerase chain reaction; these will include not only target larvae but also vectors for baculovirus transfer—predating invertebrates and birds. In terms of their sizes, NPVs have polyhedral-shaped occlusion bodies ranging from 0.5 to 15 μm in diameter while GVs produce much smaller ovicylindrical occlusions from 0.3 to 0.5 μm (Williams and Faulkner, 1997). So, while NPVs can be visualised by light microscopy it is very difficult to confirm the presence of GVs by this type of microscopy. Therefore the use of PCR to detect GVs in field samples can be used as a valuable diagnosis method because granulin is highly conserved among granuloviruses.

One of the powerful methods for characterization of positive samples is single-strand conformational polymorphism. This technique allows for determining not only viral species but also viral strains usually without additional DNA sequencing. Classical single-strand conformational polymorphism analysis is based on the observation that single-stranded DNA fragments attain a number of conformational forms which may be separated by electrophoresis in a native polyacrylamide gel. For a fragment of a specific nucleotide sequence usually more than one conformational isoform is thermodynamically stable and we observe a characteristic pattern of electrophoretic bands for a particular DNA sequence. Even minute sequence changes (e.g. a point mutation) may have significant effect on electrophoretic pattern of single-stranded DNA fragment. This method proved to be a very valuable tool in genotyping of many diseases in humans and animals and should find wider use in bioprotection. Changes of gel temperature during SSCP electrophoresis increase the sensitivity of mutation detection in PCR products and significantly reduce the overall time of analysis. This modified technique was named MSSCP—multitemperature single-strand conformational polymorphism analysis (Kaczanowski et al., 2001).

Polymerase chain reaction and single-strand conformational polymorphism are relatively simple analytical methods giving wealth of information about occurrence and spread of the virus. Most of analytical laboratories associated with pest management are able to buy this equipment and train technicians to perform routine analysis. Strictly quantitative assay will require quantitative PCR methods. Here, real-time PCR is a method of choice. Though the equipment–light cyclers are still very expensive now, their price gradually decreases and it is very likely that within three to five years from now they will become routine equipment, also in laboratories which are responsible for regional monitoring of pests.

In the preceding chapter we have discussed the present developments and future prospects for AgMNPV propagation in cell culture. The use of other baculoviruses requires similar measures if they have to be produced at large scale in in vitro conditions. In the next 5–10 years the research efforts towards producing these biopesticides in cell culture will have to be greatly intensified to simplify commercial production of baculoviruses and to reduce the cost of large-scale production so that they become competitive with chemical pesticides.

Occluded baculoviruses persist unaffected for years when stored under normal environmental conditions. Viral survival can be influenced by temperature, pH, humidity or presence of additives. Alkaline pH is a physiological factor for dissolution of polyhedra in larval midgut on ingestion so the effect of alkaline pH on viral survival is not a surprise (Ignoffo and Garcia, 1966; Kawarakata et al., 1980). The most detrimental effect on virus activity is attributed to UV light. Under field conditions very little activity is left when the virus is exposed to the sunlight and is not shaded by plant canopy. Much effort has been devoted to the development of UV protectants. Many formulations have been tried but the most promising are at present stilbene fluorescent brighteners which are marketed under many trade names. Shapiro (1992) demonstrated the protective power of a number of optical brighteners against LdMNPV. The brighteners marketed under names Phorwite AR, Blankophor or Tinopal LPW were very effective in protecting LmNPV, HzNPV or SfNPV against sunlight exposure (Shapiro et al., 1994; Zou and Young, 1994, 1996). The developments in the field of brighteners greatly increased the interest in baculovirus formulation technology which may lead to lowering of the price of baculovirus production and make it competitive with chemical pesticides in the near future. Some other UV-protection agents like lig-

nosulfates, gelatin and titanium dioxide are also exploited in the formulations to protect polyhedra from solar irradiation (Black et al., 1997). Another approach to protect baculoviruses from UV radiation was proposed by Petrik et al. (2003). A recombinant AcMNPV was constructed which produces algal pyrimidine dimer specific glycosylase involved in the first steps of repair of UV-damaged DNA. The recombinant virus was three times more resistant to UV inactivation. DNA photolyase genes were also found in baculovirus, *Chrysodeictis chalcites* NPV (Van Oers et al., 2004, 2005); potentially the genes may be introduced into other baculoviruses to improve their UV resistance. Inactivation of baculoviruses may be also caused by plant metabolites. One of such possibilities is inactivation by plant peroxidases which generate free radicals (Hoover et al., 1998; Sun et al., 2004a). The inactivation can be reduced by addition of free radical scavengers such as mannitol or enzyme superoxide dismutase (Zhou et al., 2004).

4.2. Second approach—genetic modification of existing baculoviruses

Slow action of baculoviruses often hampers its practical application and many attempts have been made to improve the timing of the killing action by baculoviruses. Two broad strategies have been pursued at the laboratory scale (in some cases in greenhouse conditions) to achieve this goal: interference with host physiology and introduction of an insect-specific toxin (Bonning and Hammock, 1996; Mishra, 1998; Inceoglu et al., 2001).

The first strategy has been used by introducing genes coding for some insect hormones or hormone-modifying enzymes into baculovirus genome, or by deletion of the baculovirus-encoded ecdysteroid glucosyltransferase (*egt*) gene. Maeda (1989) was first to introduce a diuretic hormone gene into *B. mori* baculovirus genome to cause insect to lose water. Modified BmNPV killed larvae about 20% faster than wild-type BmNPV. Two other insect hormone genes (eclosion hormone and prothoracicotropic hormone genes) were also tried as potential factors for modification of baculovirus but no significant improvement over wild-type virus was observed (Eldridge et al., 1991; O'Reilly et al., 1995). Another strategy was based on the control of juvenile hormone which in lepidopteran larvae regulates the onset of metamorphosis at the final molt. Juvenile hormone is regulated by juvenile hormone esterase which overexpressed decreases the concentration of the hormone. This in turn is a signal to stop feeding and pupate. This elegant theoretical strategy for improvement of baculovirus action encountered many difficulties in practice but it is being pursued in order to make it more efficient in the natural conditions (Hammock et al., 1990; Ichinose et al., 1992; Inceoglu et al., 2001). Another approach was used by O'Reilly and Miller (1991); they deleted the baculovirus-encoded ecdysteroid glucosyltransferase gene. The product of the *egt* gene normally prevents larval moulting during infection and indirectly increases feeding activity of infected caterpillar. The infection with recombinant virus resulted in 30% faster killing of larvae and significant reduction in food consumption. The egt enzyme is responsible for transfer of sugar molecules to the hormone ecdysone rendering it inactive. The inactivation of ecdysone results in a prolongation of larval stage and in an increased plant consumption. When larvae are infected with an *egt*-minus virus, molting proceeds normally and consequently larvae eat less food. The *egt* gene is not essential for viral replication and can be replaced with an exogenous gene, thus enhancing the insecticidal activity of the recombinant virus (Arif, 1997; Popham et al., 1997; Sun et al., 2004b).

Modification of baculovirus genome by introduction of a specific toxin gene was much widely exploited. Historically, introduction of *cry* toxin gene of *B. thuringiensis* was one of the first attempts (Merryweather et al., 1990). However, most of reported research was focused on arthropod toxin genes isolated from mites, spiders or scorpions (reviewed by: Bonning and Hammock, 1996; Inceoglu et al., 2001). This line of research proved to be highly successful but the reluctant attitude of European (but also some non-European) societies to genetically engineered products hampered their introduction or even promoted press and other mass media attacks against field trials. The first reports on successful introduction of insect specific toxin genes into baculovirus genome were published around 15 years ago (Carbonell et al., 1988; Maeda et al., 1991; Stewart et al., 1991; Tomalski and Miller, 1991). The most promising insect-specific toxin gene used for construction of baculovirus recombinants is probably the gene coding for AaIT toxin originating from scorpion *A. australis*. The reported speed of kill by this baculovirus recombinant was increased by about 40% and the feeding damage was reduced by about 60% (Inceoglu et al., 2001). Toxin genes isolated from other scorpions, e.g. *Leiurus quinquestriatus hebraeus* (Chejanovsky et al., 1995; Gershburg et al., 1998; Froy et al., 2000), straw itch mite *P. tritici* (Lu et al., 1996; Burden et al., 2000) or spiders *Diguetia canities* and *Tegenaria agrestis* (Hughes et al., 1997), introduced into baculovirus genomes were highly active against insect pests and are also under intensive studies as potential biopesticides.

Most of these toxins attack insect sodium channels, which means they have very similar effect to the chemical pesticides belonging to the pyrethroid group (Bloomquist, 1996). However their specific site of action within sodium channels is different, so they may have synergistic effect when used together (McCutchen et al., 1997). Another promising approach for improvement of baculovirus insecticidal efficacy was demonstrated by Regev et al. (2003). Cooperative insecticidal effect was observed when a recombinant AcMNPV expressing toxin pairs (a combination of excitory and depressant scorpion toxins) was used against *H. virescens*, *H. armigera* and *S. littoralis* larvae. The recombinant producing excitory toxin LqhIT1 and depressant toxin LqhIT2 from *Leiurus quinquestriatus hebraeus* provided an improvement of 40% in effective time to paralysis when compared to wild-type AcMNPV and an improvement around 20% when compared to recombinants producing each of toxins separately. Chang et al. (2003) have explored still another method for improvement of recombinant baculoviruses which may find application in future biopesticide constructs. They generated a baculovirus that produced occlusion bodies incorporating Bt toxin. The recombinant baculovirus genome codes for native polyhedrin and a fusion protein in which polyhedrin is fused to the Bt toxin. The speed of action and pathogenicity of the recombinant were greatly enhanced compared to wild-type virus thus yielding a biopesticide which combines the positive properties of the virus and the bacterial toxin.

The level of recombinant gene expression in the baculovirus system is promoter-dependent. In the chapter Molecular Biology of Baculoviruses we have discussed the use of some baculoviral promoters in the construction of recombinants. In order to further enhance the insecticidal efficacy of recombinants, new promoters will be searched for early expression of insecticidal genes. Recently, Sun et al. (2004b) constructed a chimeric promoter by insertion of the p6.9 promoter downstream of the polyhedrin promoter and used this dual promoter in the expression of AaIT scorpion toxin gene in *egt* locus of HaSNPV. This HaSNPV-AaIT recombinant was found out to be much more effective biocontrol agent than the wild-type virus or *egt*-deleted virus. Speed of action of genetically modified baculoviruses can be also enhanced by signal sequences in front of cloned genes. Van Beek et al. (2003) constructed a series of AcMNPV recombinants expressing LqhIT2 scorpion toxin gene with different signal sequences, including signal sequences of AcMNPV GP64, cuticle protein II of *Drosophila melanogaster*, bombyxin of *B. mori*, dipteran chymotrypsin and some scorpion toxins. Bombyxin signal sequence proved to be the most effective for enhancing insecticidal efficacy. Further search for more effective signal sequences in transporting a toxin outside of expressing cell is to be expected in the large array of natural and synthetic signal sequences which are known to date.

Biosafety of genetically modified baculoviruses is an important problem which requires special consideration. A number of studies indicated that baculoviruses pose no hazard to other animals than their hosts. Recombinant HaSNPV expressing AaIT scorpion toxin gene was not pathogenic to bees, birds, fish and other vertebrates (Sun et al., 2002). Genetically modified AcMNPV did not effect aquatic microbial community in any respect (Kreutzweiser et al., 2001). Natural enemies of larvae such as parasitoids and predators were not adversely affected by preying upon larvae infected with recombinant viruses (Li et al., 1999; Smith et al., 2000; Boughton et al., 2003). Another concern of biosafety is the potential of the cloned gene to jump from the donor recombinant baculovirus to the recipient organism. This is in theory possible (Inceoglu et al., 2001) but it has not been proven until now. On the basis of these reports it can be concluded that there is no evidence that recombinant baculoviruses pose greater threats to animal world and environment than the parental baculoviruses. It is generally believed in the scientific world that benefits from genetically modified baculoviruses outweigh the undefined risks from their use. In spite of that, the attempts to introduce recombinant baculoviruses expressing toxin genes raised massive public protests in Western Europe. These irrational protests were triggered by the choice of toxins used in baculovirus recombinants. Australian spider venom, African scorpion toxin—these exotic names by their mere sound are threatening for a man-in-the-street. It is also not surprising that these protests occurred in rich Western democratic countries where “green” ecological movements strongly oppose to genetic modification of organisms. Though irrational, these voices have to be taken into account by scientists and alternative less “exotic” toxins, e.g. originating from naturally occurring parasitoid wasps, should be examined as potential chemical compounds which may augment natural killing action of baculoviruses.

Hopefully, the negative influence of social perception of genetically modified organisms on biotechnological research will gradually decrease as it is already evident in the less stringent attitude of law-makers in many countries towards this problem. This can be observed in two most highly populated countries of the world—China and India. So it is highly probable that genetically

modified biopesticides will gradually increase their share in pesticide market. To make it happen faster, the public should have thorough information on risks and benefits of chemical and biological pesticides. The knowledge of baculovirus biology does not leave any doubt that biopesticides based on baculovirus formulations pose much lower risk to the environment than classical chemical pesticides.

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