THE USE OF TRANSGENIC BIOLOGICAL CONTROL AGENTS TO IMPROVE THEIR PERFORMANCE IN THE MANAGEMENT OF PESTS

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ABSTRACT

Many wild-type microorganisms have been successfully exploited for the control of a range of insect pest species. Our current knowledge on their limitations as control agents gives the molecular biologist an opportunity to improve strains and provide genetically modified microorganisms for future markets. This review briefly introduces the types of microorganisms used and the approaches that have been adopted for modifying strains.

INTRODUCTION

Global losses in crops due to insect damage can account for between 20-30% of total production, even with current management and control methods. (Estruch *et al.*, 1997). Other insects are important vectors of human and animal diseases and their control is also important. In this paper we have presented a brief outline of the current microbial products that are used for the control of insects. However, it is not our intention to offer a comprehensive list and the details of all products used, but to set the scene on some of the advances that have been made, and our view on the future direction of research within this field.

BACULOVIRUSES

It is recognised that virus diseases play an important role in the natural regulation of insect populations. The baculoviruses in particular cause disease in insects principally belonging to the butterfly and moth family, many of which are pests of agricultural and horticultural crops. Their potential as biocontrol agents was investigated in field trials as early as 1950, after their host specificity and virulence were appreciated.

Baculoviruses are currently being produced in several countries worldwide for the control of a variety of lepidopteran pests e.g. Brazil, Japan, China, Europe and North America. Although resulting in very high mortalities, their speed of action, taking a few days to cause death, is slower than chemicals. The use of naturally occurring isolates of baculoviruses may provide acceptable control particularly against foliar feeders in crops where a certain level of foliage damage is acceptable e.g. in forestry or some agricultural crops. They have an important role as a component in integrated pest management programmes and in organic farming. However, there is scope for producing improved strains of these viruses by genetic engineering and several laboratories, both commercial and academic, are studying the molecular biology of baculoviruses with a view to producing faster acting viruses. Until

recently, most of this work concentrated on one genus of these viruses, the nucleopolyhedroviruses (NPVs). This is partly because they are very good control agents against certain forestry pests (e.g. Douglas fir tussock moth, gypsy moth, spruce budworm) and agricultural pests (e.g. velvet bean looper, tobacco budworm), but principally because some NPVs can be grown successfully in cell culture.

By far the most extensively studied baculovirus is *Autographa californica* NPV (AcMNPV), since this was the first baculovirus to be cultured more than 25 years ago. Genetically engineered strains of this virus are currently being developed for pest control. Two strategies have been used. One is the expression of foreign genes such as insect-specific toxins, in particular neurotoxins (e.g. TxP-I toxin from the straw itch mite *Pyomotes tritici* and AaIT from the North African scorpion *Androctonus australis*) or insect genes (e.g. diuretic hormone, prothoracicotropic hormone and juvenile hormone esterase). The second strategy is the deletion of viral genes that might prolong the life of the infected host, resulting in extended crop damage, such as the ecdysteroid UDP-glucosyl transferase gene (*egt*). Expression of foreign genes in AcMNPV has made use almost entirely of promoters from two very late highly expressed genes, polyhedrin (the occlusion body protein) and *p10*, although promoters from earlier genes and synthetic promoters have been shown to be potentially useful. When susceptible larvae are infected with an AaIT-expressing recombinant baculovirus e.g. AcMNPV they gradually become paralysed and fall off the plants and die, resulting in about a 50% reduction in feeding damage (Stewart *et al.*, 1991). A faster acting recombinant AcMNPV, expressing the AaIT gene under the control of an early promoter, was assessed in field trials in 1995. Other EPA approved field trials on recombinant AcMNPV expressing other neurotoxins e.g. the depressant insect toxin LqhIT2 isolated from the scorpion *Leiurus quinquestriatus hebreus* were initiated by DuPont in 1996. Many insect-specific neurotoxins are available with the potential to increase the speed of kill of AcMNPV as well as for other NPVs that can be grown in cell culture. The efficacy of these recombinants can be further optimised using various promoters and signal sequences with the resulting recombinant viruses appearing to have no affect on the intrinsic infectivity of the virus for the permissive and semi-permissive hosts or on its natural host range.

Deletion of the viral ecdysteroid UDP-glucosyltransferase (*egt*) gene has been shown to increase the speed of kill in AcMNPV (0'Reilly & Miller, 1991). American Cyanamid carried out greenhouse and small scale field tests in 1993 and 1994 to compare the efficacy of the wild type AcMNPV and *egt* minus AcMNPV and two commercial *Bacillus thuringiensis* bioinsecticides. Improved plant protection was observed with the *egt* minus AcMNPV compared to the wild type AcMNPV against cabbage loopers (*Trichoplusia ni*) and tobacco budworms (*Heliothis virescens*), primarily on cotton and leafy vegetables. However, in field tests this improvement was less significant when compared to that observed in the laboratory and greenhouse. Although the *egt* minus AcMNPV was less effective than the *B. thuringiensis* standard in many of the tests the differences between the two treatments were frequently not significant. However, although the speed of kill is increased it is still slower than that achieved by chemical insecticides. Initially, a recombinant virus where genetic material has been removed may be more acceptable to the customer than one in which an insect-specific toxin gene has been inserted. Even so, viruses deficient in *egt* and/or expressing a toxin gene should provide a safe bioinsecticide with efficacies comparable to chemical insecticides.

Baculoviruses have been traditionally produced *in vivo* but the production of modified baculoviruses that kill in only 2-3 days compared to the normal 7-9 days inevitably results in poor virus yields. Therefore, production of these fast killing baculoviruses requires their *in vitro* production and is dependent on the availability of permissive cell lines. As a result, there is a major effort to produce permissive cell lines for the improvement and production of other baculoviruses to control other major pests. In addition to AcMNPV, several other NPVs have been completely sequenced which is facilitating their genetic improvement.

In contrast to the situation in forestry and agriculture, many of the potentially useful viruses for control of pests of horticultural crops belong to the second baculovirus genus, the granuloviruses (GVs). Examples include GVs for codling moth (*Cydia pomonella* GV, CpGV), small white butterfly (*Artogeia rapae* GV), cutworm (*Agrotis segetum* GV), diamondback moth (*Plutella xylostella* GV), tomato moth (*Lacanobia oleracea* GV) and summer fruit tortrix (*Adoxophes orana* GV). At HRI studies have been undertaken with several of the horticulturally important GVs and in the course of this work it was shown that CpGV was a particularly effective control agent for codling moth, a key pest on apples, and to a lesser extent on pears, both in the UK and worldwide. Although current chemical control of codling moth is effective, there are several reasons for needing to develop alternative control strategies including elimination of pesticide residues from unprocessed crops; increased pest target specificity; development of pesticide resistance in pest populations and minimizing chemical inputs into the environment. Because of the importance of the pest and the effectiveness of the virus, attention, in recent years, has focused particularly on CpGV.

The major problem obstructing studies on the molecular biology of GVs has been the inability to obtain cell lines which fully support the replication of these viruses. Two previous attempts, both screening around 200 cell lines, had at best obtained cultures with about 25% of cells susceptible but even this level of susceptibility was gradually lost on passage (Miltenburger *et al.*, 1984). Furthermore, the titre of virus obtained from cells was too low to allow it to be passaged. Because of this, less is known about the molecular biology of this group of baculoviruses and it was not possible to produce genetically engineered strains. However, in 1989 cell lines were selected from *Cydia pomonella* (codling moth) embryos that supported the replication of *C. pomonella* granulovirus (Winstanley & Crook, 1993). These have been used to produce a recombinant virus lacking the *egt* gene which shows similar improvements in efficacy to the *egt* minus AcMNPV in bioassays on fifth instar larvae but this virus has yet to be assessed in the field. In the case of codling moth the neonate is the target and the timing of application as well as the speed of kill or inhibition of feeding is critical if penetration of the developing fruit is to be prevented. The wild type CpGV kills neonates in approximately four days during which time they continue to feed resulting in sting damage to the fruit. However, deep entry damage caused by older larvae inside the fruit is reduced to that achieved using broad-spectrum chemical insecticides. Therefore, a modified CpGV which can inhibit feeding and or kill faster should reduce both sting and deep entry damage.

Research in the GV area is now accelerating and the complete genomes of at least four GVs will be available in the near future and several laboratories are now actively involved in the production of GV permissive cell lines. Soon it may be possible to improve the efficacy of the most pathogenic GVs such as *P. xylostella* (the diamond back moth) granulovirus and *C. pomonella* granulovirus to provide better bioinsecticides to satisfy the requirement for the

control of insecticide and *Bacillus thuringiensis* resistant pests, and/or reduce inputs on unprocessed food crops.

Two major agrochemical companies, American Cyanamid and DuPont, have invested large amounts of money in the genetic improvement and *in vitro* production of AcMNPV and are now very close to producing commercial products. Public and grower awareness and education will be essential if these recombinant virus products are to be successful. For a viral bioinsecticide to be commercially viable, whether wild type or modified, it must have a large potential market such as AcMNPV which has a relatively broad host range. However, GVs and even other NPVs are highly host specific and therefore their development and commercialisation will be dependent on the size and value of the affected crop and the presence of a predominant pest species e.g. *P. xylostella* on brassica or *Anticarsia gemmatalis* on soya bean. *In vivo* production of a range of baculoviruses is still the most realistic approach when dealing with a wide range of pests for niche markets where registration costs will be a major stumbling block. In some countries however, where the pest treatment regime to reduce chemical inputs is prescriptive and subsidised e.g. the use of *A. orana* GV to control *Adoxophyes sp* on tea plantations in Japan, the real benefits of viral bioinsecticides will be assessed.

BACILLUS THURINGIENSIS

Bacillus thuringiensis currently accounts for over 90% of all biological pest control products used, and it is one of the most successful biocontrol agents on the market. However, *B. thuringiensis* based products still only account for less than 1% of world pesticide sales. The low market share for *B. thuringiensis* is due to a range of factors including lack of persistence on the crop, limited host range and inability to control pests which feed internally. To overcome the limitations in the use of sprayable *B. thuringiensis* products, genetic methods have been used to improve the activity and host range of strains.

To date, a number of different strategies have been used to try and develop more active *B. thuringiensis* products. The most widely used approach has exploited the fact that most toxin genes are encoded on large self-transmissible plasmids (Gonzalez *et al.*, 1982). Using a combination of plasmid curing and plasmid transfer it has been possible to construct strains of *B. thuringiensis* containing new more potent combinations of toxin genes (Jarrett & Burges, 1986, Carlton, 1993). This approach has resulted in a number of new *B. thuringiensis* products which have been brought onto the market for the control of lepidopterous pest species. The fact that these strains have not been genetically manipulated, and could arise in nature by the exact system used in the laboratory, has meant that these strains avoid the regulations concerning the release of genetically modified microorganisms in to the field.

The advantages gained by using transconjugant strains include an improvement in activity and host range, and that commercial products can be protected by patenting. For example, the product, Agree® controls a wider range of lepidopteran insects, or insects from different orders, such as lepidopteran and coleopteran insects. With the large diversity of *B. thuringiensis* strains available in culture collections there is great potential for constructing new combinations of toxins. The process is essentially limited by the transfer frequency of

the plasmids (since very low transfer rates would preclude the detection and identification of transconjugant colonies), plasmid incompatibility, which would prevent the stable maintenance of two similar plasmids, and surface exclusion or the lack of a suitable plasmid transfer system.

The use of molecular techniques has enabled the cloning and expression of *B. thuringiensis* toxin genes in other microorganisms. This approach has also allowed the expression of genes in different *B. thuringiensis* strains (Baum *et al.,* 1996). This can be accomplished with the removal of foreign DNA that was acquired during gene cloning. Using this approach the construction of new toxin combinations can be achieved, and the strains could be considered safe, or at least closer to the wild type strains originally used in their construction. However, the main scope of this work has been aimed at the expression of *B. thuringiensis* toxins in other host organisms. The best known example is Mycogens' Cell Cap system where toxin genes are expressed in *Pseudomonas* strains. The products are killed before application to overcome the problems associated with the regulations concerning the release of GMMs. The advantage of this product is improved foliar persistence. Toxin genes can also be introduced into organisms that persist, or thrive in the environment where insect control is required, but where *B. thuringiensis* would not be suitable. For example, toxin genes from *B. thuringiensis* var. *israelensis* have been inserted into blue green algae for the control of mosquitoes (Chungjatupornchai, 1990).

Improvements in the activity of specific genes can be achieved by manipulation of the DNA sequences. The use of site directed mutagenesis, or the exchange of the toxin domains can be used to alter insect activity (Wolfersberger *et al.*, 1996). However, a detailed knowledge of the mode of action of the toxins is required to make use of these powerful techniques. A great deal of progress has been made in the understanding of the structure and functional domains of a few *B. thuringiensis* toxins. However, they are highly complex and activity is affected by a number of factors including activation in the insect gut, binding of the toxin to specific gut receptors and pore formation in insect midgut cells.

In recent years, a few problems associated with the use of *B. thuringiensis* have become apparent. Insect resistance is probably of most concern. *Plutella* spp. have developed resistance to a number of *B. thuringiensis* products in many countries around the world (Tabashnik, 1994; Liu & Tabashnick, 1997). The potential for resistance to occur in a wide range of lepidopteran pests has also been investigated. In laboratory stocks high levels of resistance have been induced in species such as *Heliothis virescens* (Lee *et al.*, 1995; Gould *et al.*, 1995). Secondly, *B. thuringiensis* is not active against all pest species, therefore there is a great need for new and novel toxins with new modes of action and wider host range. It is now essential to find new toxins to manage and slow down the development of resistance.

POTENTIAL NEW TOXINS

The search for new insecticidal proteins has intensified in the last few years and a number of microbial proteins have already been identified and characterised. A new group of toxins, produced during vegetative growth have been isolated from *B. thuringiensis* and related species. These toxins have been termed the vegetative insecticidal proteins (VIPs) and have a

relatively simple binary structure (Estrich *et al.*, 1996). A more complex series of toxins have been identified in bacteria associated with insect parasitic nematodes. These toxins have activity against a variety of insects including those in the order Lepidoptera, Coleoptera and Diptera. Bowen *et al.* (1998) described the toxins identified from *Photorhabdus luminescens* (Ensign *et al.*, 1997), and Jarrett *et al.* (1997) described them in *Xenorhabdus nematophilus* and related species. The toxins have a particulate structure composed of a range of over six proteins from 280kDa to 20kDa. These proteins are proteolytically cleaved and hence produce a complex array of peptides, some of which have insecticidal activity. Currently the active toxin within these proteins has not been identified although the activity from *X. nematophilus* has been cloned and expressed in *E. coli*.

FUNGI

Fungi capable of controlling insects have been isolated and used in the laboratory for many years. The pathogenicity factors associated with infection are numerous and complex. It has only been in the last few years that molecular techniques have been used to try to improve the pathogenicity of entomopathogenic fungi. These studies have been rapidly advanced by the identification of a number of putative pathogenicity determinants from the fungus *Metarhizium anisopliae* (St Leger & Roberts, 1997). Manipulation of one of these pathogenicity determinants by increasing the expression of a specific fungal protease led to an increase in the speed of kill and a reduction in food consumption by the infected larvae. (St Leger *et al.*, 1996). These experiments have shown that it is possible to improve the virulence of fungi although it may prove difficult to make significant improvements in pathogenicity due to its complex nature. This may allow some of the major constraints such as the requirements for high humidities to allow infection to be overcome. One area of great potential is the identification of fungal proteins with insecticidal activity. Since some fungi have evolved to kill insects they may provide a rich and potential new source of insecticidal toxins.

CONCLUSIONS

Microorganisms provide a useful alternative to chemicals for the control of insects. Their environmentally friendly image provides a useful platform for their exploitation in future years. In addition, they have great potential for providing a rich new source of insecticidal protein toxins. Novel or rapid screening methods for detecting these proteins will be important for selecting and identifying the range of microorganisms of interest.

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