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APPLICATION OF BIOTECHNOLOGY IN PLANT PROTECTION

Saniya Tyagi

Department of Entomology and Agricultural Zoology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India.

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Corresponding author:
Saniya Tyagi
saniyatyagi2311@gmail.com

ABSTRACT

Biotechnology offers many opportunities for agriculture and provides the means to address many of the productivity constraints. With the rapid development of biotechnology, plant breeding is drastically improved by the introduction of recombinant DNA technology. The most well known example of this technology is the introduction of gene coding for the production of delta endotoxin of *Bacillus thuringiensis*. Other strategies for plant protection against insects include the use of their genetically engineered natural enemies, recombinant Baculoviruses, plant-derived genes (enzyme inhibitors, lectins, secondary metabolites), and from animal sources, including insects (biotin-binding proteins, neurohormones, enzyme inhibitors), are currently being developed to control insect pests. In this review we will consider many such applications of biotechnology in the context of insect pest management.

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INTRODUCTION

Each year, in agriculture, billions of dollars are spent worldwide in controlling insect pests (Krattiger, 1996). But in spite of this expenditure, up to 40% of a crop is lost due to field and storage insects, particularly in developing countries (Oerke, 2006). Though insecticides are effective in solving the immediate problem of insect attack on crops, they are generally found to be harmful to beneficial organisms and non-target organisms, many of which play key roles in suppressing the build up of insect populations (Hassan *et al*., 1994). These problems have made the researchers think for a solution in a different way so as to develop different insect control strategies that are more environmentally friendly. One such approach has been the use of transgenic plants through biotechnology and genetic engineering, expressing plant defence molecules. The concept of utilizing a transgenic approach to host plant resistance was first seen in the year 1987 by developing a transgenic tobacco plant that expressed an insecticidal gene. This transgenic tobacco plant produced cowpea trypsin inhibitor against the lepidopteran pest *Heliothis virescens* (Harsulkar**,** 1999). In the mid 1990s commercial introduction of transgenic potato, maize, and cotton plants expressing genes encoding the entomocidal δ-endotoxin from *Bacillus thuringiensis* (*Bt*) started a new pest-control era.

Genetic modification through biotechnology can potentially offer a much larger range of novel insecticidal genes that are otherwise beyond the scope of conventional breeding. Genetic engineering is the process of using recombinant DNA (rDNA) technology to alter the genetic makeup of an organism with desirable gene(s) by whatever means into virus, bacterial plasmid or other vector systems so as to allow their integration into the host in which they do not naturally occur but are capable of continued propagation (Smith, 1996). Since the commercialisation of biotech crops in 1996, farmers have adopted the technology at such a dramatic rate, that in 2011, planting of Bt cotton in India surpassed the historical milestone of 10 million hectare for the first time and occupied 88% of the recorded 12.1 million hectare cotton crops (Gautam et al., 2013). In India, Bt-cotton has increased cotton yields by 60%, and has reduced insecticide sprays by around half.

The aim of modern biotechnological strategies is to improve the performance of an engineered product over its wild type by increasing their host range, speed of action, delivery to the pest, and improving their persistence. In this chapter we review the potential of biotechnology in biological control agents that are receiving interest as alternatives to chemical insecticides and in enhancing the efficacy of bio-insecticides and transgenics for sustainable agricultural productivity.

INSECT NATURAL ENEMIES AS BIOCONTROL AGENTS

Biological control of insect pests or other harmful organisms of crops is their suppression by using their natural enemies (parasites, predators and pathogens). It involves the deliberate attempt to use natural enemies, either by introducing new species or by increasing the effectiveness of those present already in the environment (Sankaran, 1986).
Genetic improvement projects with natural enemies of insects could provide solutions to a number of basic and applied problems that limit their use as biological control agents eg. mass rearing, improved climatic tolerances, host finding ability, synchronization with the host, changes in host preference, and insecticide resistance.

New DNA-based methods, for monitoring genetic variations of natural enemies, are now available such as: mitochondrial DNA analysis, DNA sequencing, restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD)-PCR and ribosomal DNA analysis. Many of these methods are also of potentially valuable identifying and monitoring establishment and dispersal of specific biotypes of insect natural enemies (Tipvadee, 2002). Genetic manipulation of natural enemies is a potential approach to increase their efficacy of insect control. Transgenic techniques provide the opportunity to reduce frequent mass releases by introducing and expressing alien genes and/or disrupt existing gene functions so that the desirable characteristics may be inherited by subsequent progenies.

Introduction of DNA into insect germ cells can be achieved by using physical or biological means. Microinjection, biolistics and electroporation are some physical gene transfer methods. The use of gene vectors in the form of several transposable elements, or viruses e.g. Sindbis viruses and retrovirus are some examples of biological means (Atkinson *et al.*, 2001). Micro-injecting DNA carried in P-element vectors has been used for gene transfer in numerous insect species (McGrane *et al.*, 1988, Morris *et al.*, 1989). A technique called maternal microinjection is developed for certain species, in which the exogenous DNA is microinjected through the cuticle of gravid females without the aid of any transposable-element vector, for example, *M. occidentalis* transformants (Jeyaprakash *et al.*, 1998). Thus, genetic enginnering provides opportunities for the development of natural enemies of insects conferring many beneficial traits, such as pesticide resistance, cold hardiness and sex ratio alteration.

RECOMBINANT BACULOVIRUSES FOR-INSECT CONTROL

Entomopathogenic viruses have been employed as bioinsecticides for many agricultural systems. Baculoviruses, mainly the nucleo-polyhedral viruses (NPVs) are the most commonly used for development as microbial bio-insecticides largely for the control of lepidopteran insects pests on crops. NPVs are easy to apply as they are formulated as sprays, similar to chemical insecticide and *Bt* strains. However, not much success has been achieved due to several key limitations, like a relatively slow speed of kill, less persistence in the field, and a narrow spectrum of activity. There is a lack of a cost-effective system for their mass production *in vitro* (Gould, 1998).

Approaches to engineer improved NPVs as biological insecticide include deletion of undesirable genes for prolonging host survival, and insertion of desirable genes for expressing an insecticidal protein during viral replication. Deletion of the ecdysteroid UDP-glucosyltransferase (EGT) gene of *Autographa californica* NPV (AcNPV) in fall armyworm, *Spodoptera frugiperda* larvae led to less feeding and death about 30% sooner than those infected with wild-type AcNPV (O' Reilly and Miller, 1991).
A number of insect-specific baculoviruses (NPVs) have been modified to contain resistance genes which, when expressed in the host insect, produce resistance effects for chemical insecticides, for instance, an acetylcholinesterase gene from *D. melanogaster* and *Anopheles stephensi;* a parathion hydrolase gene from *Pseudomonas diminuta* and Flavobacterium, the amplification core and esterase B1 gene isolated from Culex mosquito, imparting resistance to organophosphorus insecticide.

There are several insect hormones that play an imperative role in the regulation of insect morphogenesis and reproduction. They are now looked upon for engineering into baculoviruses.

Some of them are; prothoracicotropic hormone (PTTH), which is involved in triggering the molting process; eclosion hormone that initiates ecdysis, allatostatins and allatotropins, which regulate the release of juvenile hormone. Juvenile hormone esterase (JHE), another interesting candidate for genetic manipulation of baculovirus, is an enzyme gene that causes reduction in JH level. A reduction in the JH titer early in the last instar initiates metamorphosis and leads to cessation of feeding.

Thus integrating the gene coding for proteinaceous insect toxins (trypsin inhibitor, scorpion toxin, mite toxin), hormones (juvenile hormone, eclosion hormone, diuretic hormone), and metabolic enzymes (juvenile hormone esterase) into nucleopolyhedrovirus (NPVs) and granulosis virus (GV) genomes are some of the approaches by which virulence and host specificity can be significantly enhanced (Tipvadee, 2002).

BACILLUS THURINGIENSIS AND TRANSGENIC INSECT RESISTANT PLANTS

Bacillus thuringiensis (*Bt*) is a spore-forming, rod-shaped, Gram-positive, soil dwelling bacterium of major agronomic and scientific interest. This bacterium colonizes and kills a large variety of host insects by producing one or more proteins that crystallize intracellularly during the sporulation stage. The strain of each subspecies of this bacterium tends to be highly specific. Toxins for insects in the orders Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils), and Hymenoptera (wasps and bees) have been identified (Maagd, 2001), that are non toxic to mammals and most other non-target organisms.

The most well-established technology for producing transgenic plants is by using genes encoding endotoxins from *Bacillus thuringiensis* with enhanced resistance to the larvae of lepidopteran insect pests (Duke, 2011). Regarding mechanism of bacterial toxicity, when the insect larvae feed on transgenic plant parts, Bt crystals and spores are ingested into the midgut of the insect, where they are solubilized and proteolytically cleaved to remove the C-terminal region, thus generating an “activated” 65–70 kDa toxin, since the pH is alkaline. The toxins (also referred to as d-endotoxins; Cry proteins) form lytic pores in the cell membrane of the insect gut and lead to septicaemia.

Bt cotton was first released in 1996 for commercial production in the USA and consequently grown in several countries including Australia, Argentina, China, Indonesia, Colombia, South Africa, Mexico, and India (James, 2011). Since then many other transgenic crop species with *Bt* toxins have been commercialized including tomato, maize, brinjal and potato. The multifaceted benefits of these crops including reduced insecticide use, lower production costs and higher yields lead to rapid adoption of *Bt* crop varieties by farmers (Brookes and Barfoot 2005).

TRANSGENIC PLANTS EXPRESSING INHIBITORS OF INSECT

This in turn has led to a search for new insecticidal proteins and their encoding genes that have commercial potential for insect pest control (Haq *et al*., 2004). They include a second class of protein that is effective against certain insects such as alpha amylase inhibitors (Carlini *et al*., 2002; and Franco *et al*., 2002), vegetative insecticidal protein (VIP) (Fang *et al*., 2007), alpha-endotoxin, chitinases (Kabir *et al*., 2006)

and protease inhibitors (Ferry *et al*., 2005; and Maheswaran *et al*., 2007), as well as several other proteins directed to target the insect gut and a variety of secondary metabolites (Baum *et al*., 1999) (Table 1). These proteins may play a major role in improving efficacy, cost-effectiveness and in expanding the markets for the bio insecticides. (Tipvadee, 2002).

Several single gene products of plant origin have been proven to be resistant to insect damage as a part of their natural defence system and have been transferred to another plant species. One famous example is lectin and lectin-like proteins which are carbohydrate binding molecules that are abundant in seeds and storage tissue of plants. The role for lectin as plant defensive protein is well documented mainly for homopterans such as aphids, leafhoppers and planthoppers, which routinely feed on phloem tissues (Powell *et al*., 1993). Genes encoding the pea lectin (P-Lec) and the snowdrop lectin (GNA) have been introduced into transgenic plants resulting in significant reduction of insect damage.

It is evident that transgenic plants resistant to insect pests will be a major element of future pest-management systems in agriculture. One of the major constraints for the utilization of Bt crops is the risk of developing resistance of insects to *Bt* transgenic plants. However, strategies have been developed to delay the resistance by involving refuge crop, high dose expression in engineered plants, pyramiding traits and good agronomic practices (Gould, 1998).

ROLE OF BIOTECHNOLOGY IN INSECT MANAGEMENT

The conceptual framework and technical approaches of molecular biology and genetic engineering provides the means to develop - many insect related commercial processes and products. In the insect research field, biotechnological tools have been applied for a variety of issues such as insect identification, insect control and insect genetic relationships.

RNA interference (RNAi) has emerged as a powerful technique for down-regulating gene expression in insects caused by exogenous injection of double-stranded RNA (dsRNA). This method was used to explore the functions of proteins, such as metalloproteinase inhibitors, metalloproteinases, and heat shock proteins, in development and immunity of the model beetle *Tribolium castaneum*. This technology enables engineering of a new generation of insect-resistant transgenic crops (Knorr and Vilcinskas, 2011).

The concept of DNA fingerprinting is comparatively new in the insect field. Based on the understanding that the chemical structure of everyone's DNA is the same and the only difference between organisms (or any insects) is the order of the base pairs, DNA fingerprinting can be used to identify insects and to study their phylogeny. This technique enables us to determine whether two DNA samples are from the same insect, related insects, or non-related insects (Tipvadee, 2002).

For the expression of transgenes in plant cells, suitable promoter sequences have been introduced alongside the gene to ensure efficient transcription of mRNA, such as Cauliflower Mosaic Virus (CaMV35S) promoter that has been used in the majority of insect-resistant transgenic plants. Pi gene was transferred to tobacco plants for resistance against *Heliothis zea, Spodoptera litura* and *Manduca sexta* (Srinivasan, 2006).

Optimization of entomopathogenic fungi by genetic engineering for insect control have been studied in a few such as, *M. anisopliae* which -

Table 1: Use of transgene and their mode of action (source: Talukdar, 2013)

Transgene	Source and mode of action	Example of use
Bacillus thuringiensis (*Bt*) endotoxin	The *Bacillus thuringiensis* endotoxin	The *Bacillus thuringiensis* endotoxin
Vegetative insecticidal protein (VIP)	VIPs are produced by *Bacillus cereus* and *Bacillus thuringiensis*. They have similar activity to endotoxins from *Bt*. Vip1/Vip2 are toxic to coleopteran insects and Vip3 is toxic to lepidopteran insects	Highly toxic to *Agrotis* and *Spodoptera* species. VIP induced gut paralysis, complete lysis of the gut epithelial cells and resulted in larval mortality. *VIP3Ac1* has insecticidal activity against larvae of *S. frugiperda*, *Helicoverpa zea* and *Trichoplusia ni*
Chitinase (enzyme)	Chitinase catalyzes the hydrolysis of chitin, which is one of the vital components of the lining of the digestive tract in insects and is not present in plants and higher animals.	Transgenic rapeseed (Brassica napus) expressing *M. sexta* chitinase and scorpion insect toxin increased mortality and reduced growth of *Plutella maculipenis*
Cholesterol oxidase (enzyme)	Cholesterol oxidase is a bacterial enzyme that catalyzes the oxidation of cholesterol and other 3-hydroxysterols, resulting in production of the corresponding 3-hydroxysterols and hydrogen peroxide. Functions by damaging midgut membranes	Cholesterol oxidase from Streptomyces caused stunting of *H. virescens*, *H. zea* and *Pectinophora gossypiella* when incorporated into an artificial diet.
Lipoxygenases (enzyme)	Dioxygenase enzymes are widely distributed in plants and catalyze the hydroperoxidation of cis-pentadiene moieties in unsaturated fatty acids. Functions by damaging midgut membranes	Lipoxygenase from soybean retards the growth of *Manduca sexta* when incorporated into artificial diet
Alpha-amylase inhibitors	Alpha-amylase inhibitors block starch digestion.	Development of pea weevil larvae (*Bruchus pisorum*; Coleoptera) was blocked at an early stage after ingestion of transgenic peas, expressing an alpha amylase inhibitor from the common bean (*Phaseolus vulgaris*)
Trypsin modulating Ostatic factor (TMOF)	A peptide that blocks trypsin biosynthesis in mosquitoes (*Aedes aegypti*; Diptera [Aea-TMOF]) and flesh flies (*Sarcophaga*; Diptera)	Injection or oral ingestion of Aea-TMOF caused inhibition of trypsin biosynthesis and larval growth in *H. virescens*. Mortality of *H. virescens* increased when fed transgenic tobacco plants expressing Aea-TMOF

causes green muscardine diseases. Various genes have been cloned from M. anisopliae related to formation of the appressorium (a specialized structure involved in host cuticle penetration by the fungus), virulence, and nutritional stress. The larvae infected with *M. anisopliae* recombinant strains died 25% sooner and feeding damage was reduced by 40% (St. Leger *et al.*, 1996).

Despite several insecticidal proteins produced by entomopathogenic fungi, fungal genes have played little part in agricultural biotechnology to date. Vertical resistance wherein resistance is based on a single gene can achieve high levels of resistance is convenient and compatible with breeding schemes used for enhancing crop yield and quality. As a result today most resistance

breeding methods have resulted in vertical resistance. However, sometimes due to its gene-for-gene nature, there is a breakdown of resistance through the evolution of virulence genes in insect pests, as in the case of brown plant hopper on rice. One solution to this problem is the deployment of horizontal resistance or other forms of resistance like partial resistance which is effective and sustainable as it depends on the quantitative effect of many genes. Unfortunately, the ongoing plant breeding and now biotechnology strategies for pest resistance favours vertical resistance, despite its limitations. The solutions suggested to resistance problems involve alternative strategies of gene deployment, such as, "gene stacking" that has recently been used for the use of fusion proteins in transgenic plants. Fusion proteins lead to increasing durability, targeting efficacy of insecticidal molecules, including peptides. It thus addresses the potential limitations in conventional transgenic insect pest control. For example, expression of the fusion protein resulted in the insect becoming sensitive to Bt by enhancing toxin binding capabilities and thus delaying resistance.

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