



E-ISSN: 2320-7078

P-ISSN: 2349-6800

JEZS 2018; 6(1): 646-651

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Received: 29-11-2017

Accepted: 30-12-2017

Hari Sankar SS

Teaching Assistant,

Department of Agricultural

Entomology, College of

Agriculture, Vellayani, Kerala

Agricultural University, Kerala,

India

Reji Rani OP

Assistant Professor,

Department of Agricultural

Entomology, College of

Agriculture, Vellayani, Kerala

Agricultural University, Kerala,

India

Genetic improvement of entomopathogenic microbes: A retrospection

Hari Sankar SS and Reji Rani OP

Abstract

Entomopathogenic microbes are one among the three pillars upon which, the practical applicability of biological control as a pest management strategy has built upon. Their usage as effective pesticidal compounds started long before the advent of first chemical insecticides. The most prominent one, *Bacillus thuringiensis* (Bt) has been in use since 1939. In spite of their eco-friendliness and safety parameters, their wide scale acceptance is still hindered by and large due to various factors. Narrow host range, slow kill nature, and expensive mass production protocols are few lacunae worth quoting. With the advent of biotechnology, genetic manipulation rose to be the single best solution to augment microbial pathogens of insects to emerge as suitable contenders for the less preferred chemical pesticides. On one side there is a vast pool of potent genes including arthropod toxin genes and on the other, there is an ocean of opportunities facilitated by modern biotechnology. The rising genetic manipulation also calls for addressing the ethical issues associated with it.

Keywords: Genetic engineering, ATMT, ethics in genetic manipulation, protoplast fusion

Introduction

Biological control employs living entities or their products in pest management. In this era of pesticide free agriculture and integrated approaches to tackle agricultural pests, entomopathogenic microbes like fungi bacteria and viruses debilitate and annihilate such pests, providing a better alternative to the persistent and polluting pesticides. Bt, Nuclear polyhedrosis virus (NPV), *Metarhizium* and *Beauveria* are some examples of microbes successfully used worldwide in pest management. Due to their systemic mode of action, bacteria and viruses have to be consumed by pests in order to bring about mortality, hence not effective against sucking pests. Moreover, prolonged time of kill, narrow host range, and higher costs of mass production, owing to requirement of living hosts in case of viruses are major drawbacks for bacterial and viral pathogens. With a contact mode of action, wider host range and mass production using cheap naturally available resources. Entomopathogenic fungi scores well above the other insecticidal microbes. Virulence and pathogenicity are an outcome of gene-enzyme consonance. Choice of the right gene, inserted at the right spot, in an appropriate host is the essence of creating a cost effective transgenic pathogen^[10].

Genetic engineering in entomopathogenic viruses (EPV's).

Strategies for improving EPV's were aimed at reducing their time to kill, increase virulence or most importantly expansion of host range. Gene encoding diuretic hormone isolated from *Manduca sexta* (tobacco hornworm) when inserted into Silk worm, *Bombyx mori* NPV, increased the susceptibility of *B. mori* to NPV by 20 fold^[15]. Hybrid genes sourced from AcNPV (*Autographa californica* Nucleo Polyhedro Virus) and BmNPV expanded host range of AcNPV to *Bombyx mori*^[3]. Recombinant strains of AcNPV with the deletion of gene encoding the enzyme EGT (ecdysosteroid UDP glucosyl transferase) which plays a significant role in moulting, enabled a decrease in time to kill of beet armyworm *Spodoptera exigua*^[24].

Genetic engineering in entomopathogenic bacteria

Numerous strains of species specific *Bt* have been isolated and identified. The specificity is attributed to a cocktail of toxins coded by specific Cry genes. Potential genes from one strain can be used to create recombinant strains of another *Bt* to improve its virulence and environmental persistence. Environmental persistence of cry proteins has been enhanced by incorporating Cry genes into other bacteria like *Pseudomonas fluorescens*, so as to extend its

Correspondence

Hari Sankar SS

Teaching Assistant,

Department of Agricultural

Entomology, College of

Agriculture, Vellayani, Kerala

Agricultural University, Kerala,

India

control over root feeders [16]. *B. thuringiensis* ssp. *jegathesan* posses Cry 11b gene that codes for toxins that are more potent than the cry genes in *B. t* ssp. *israelensis*. Unfortunately *B. thuringiensis* ssp. *jegathesan* cannot be employed in mosquito control. Cry 11b genes were transferred to *B. thuringiensis* ssp. *israelensis* to improve its virulence [5]. Mosquitocidal proteins in *B. sphaericus*, when engineered into *B. thuringiensis* ssp. *israelensis* showed 10 times more infectivity to mosquitoes [17].

GE in entomopathogenic fungi (EPF)

Fungal bio agents have a tectonic role among microbials due to their contact mode of action. This renders them infective to sucking pests as well. Although sensitivity to low humidity is a major setback for entomopathogenic fungi, the possibility is ruled out in Indian conditions. Field persistence and virulence are the major concern in application of fungal pathogens. Though remarkable improvement in field persistence can be achieved by befitting formulations, evolutionary development of defense mechanism in insects may reduce their efficiency. Genetic engineering to improve their virulence focuses on reducing lethal conidial dosage and lethal time, which are the factors governing their virulence and pathogenicity. These two traits are an outcome of gene enzyme consonance. The various tools employed in the genetic improvement of entomopathogenic fungi are protoplast fusion, electroporation, biolistics/ gene gun, and vector mediated transformation.

1) Protoplast fusion

Protoplast fusion is a physical phenomenon. Protoplasm is cell inclusions except cell wall. They possess genetic material. During fusion, two or more protoplasts come in contact and adhere to one another either spontaneously or in the presence of fusion inducing chemicals. After adhesion, membranes of protoplasts fuse in some localized areas and, eventually, the cytoplasm of the two protoplasts intermingle. Fusion of compatible protoplasts can yield recombinants with desirable hybrid characters. It was developed as a method to transform plant cells. A real beginning in protoplast research was made in 1977 by Cocking [1] who used an enzymatic method for the removal of the cell wall. Rapid progress occurred after 1980 in protoplast fusion to improve the plant genetic material, and the development of transgenic plants. Eventually the technology was adopted to transform Entomopathogenic fungi as well. Fungal protoplast fusion has been established as a means to transfer genetic material and it provides an effective method for genetic manipulation and strain improvement [21]. Highly virulent hybrid strains of entomopathogenic fungi were obtained by certain researchers [2]. Successful fusion of protoplasts in *Trichoderma* and *Aspergillus* has been well demonstrated [21].

During protoplast fusion the fungal cells are treated with cell wall degrading enzymes rich in chitinase, chitosanase, to yield protoplasts. Cell membrane is selectively impermeable to substances. There are various membrane bound channels that helps maintaining the selectivity These are then treated in combination with other protoplasts in a suitable media. After a successful fusion, the development of a cell wall around the membrane is facilitated. This is followed by the cell division that gives rise to a small colony. With suitable manipulations of nutritional and physiological conditions, the cell colonies may be grown to full fledged fungal colonies.

A major step in fungal protoplast fusion is the isolation of protoplasts. During the cell wall degradation process, some

protoplasts may fuse together in the media itself. This is a rare and non beneficial phenomenon as parental cells are fused, and no hybrid character can be expected. Such fusions are called spontaneous fusions, while, a fusion of freely isolated protoplasts from different sources with the help of fusion inducing conditions is known as induced fusion. Normally, isolated protoplasts do not fuse with each other because the surface of the isolated protoplast carries a negative charge (-10 to -30 mV) around the plasma membrane and thus, there is a strong tendency for protoplasts to repel one another. So this type of fusion needs a fusion inducing system which actually reduces the electronegativity of the isolated protoplasts and allow them to fuse with each other. Actually, induced fusion is a highly important and a valuable technique because the protoplast from widely different and sexually incompatible fungi can be fused by this procedure. The fundamental objectives of somatic hybridization are mainly based on induced protoplast fusion i.e. Artificially induce the fusion by overcoming the surface polarity of the protoplasts. The electronegativity can be overcome by any of the three ways;

a. Mechanical fusion: In this process, the isolated protoplasts are brought into intimate physical contact mechanically under microscope using micromanipulator and perfusion micropipette. This micropipette is partially blocked within 1 mm of the tip by a sealed glass rod. In this way the protoplasts are retained and compressed by the flow of liquid. By this technique occasional fusion of protoplast has been observed. Due to low recombinant frequencies, its not a widely accepted protocol for fungal transformation.

b. Chemofusion: Several chemicals have been used to induce somatic protoplast fusion. Sodium nitrate, PEG (Poly Ethylene Glycol), Calcium sulfate are common chemical fusogens. Chemical fusogens cause the isolated protoplasts to adhere to one another and leads to tight agglutination followed by fusion of protoplast. The adhesion of isolated protoplast takes place either due to the reduction of negative charges of protoplast or due to attraction of protoplast by electrostatic forces caused by chemical fusogens. In 1974, Kao and Michayluk¹⁴ from Canada discovered a fusion inducing chemical which is the most effective agent discovered so far. Many fusion experiments are performed by PEG. Recently, high pH/Ca²⁺ and PEG method have been combined. By this method, the agglutination of protoplasts can be brought about using sufficient quantities (0.1-5 ml) of protoplast in a centrifuge tube or micro densities (150 µ l) of protoplast on a coverslip. The PEG method has been modified slightly for different species of fungi.

c. Electrofusion: In this method the membrane bound polarity is reversed by applying an electric current in the medium containing protoplasts. The spontaneous reversal of polarity facilitates induction of protoplasts. This method is not so common in fungal transformation.

Pioneer research works that employed protoplast fusion as a means to create GM entomopathogenic fungi

Insect cuticle are essentially composed of chitin microfibrils. Fungal infection initiates with penetration of cuticle. Cuticle degrading enzymes such as proteases and chitinases aid at this juncture. Enhanced production of proteases/ chitinases by overexpression of a related gene, can bring down the time of infection process. Protease hyper producing recombinant strains were produced by inter-generic protoplast fusion of

entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*. Fungal protoplasts were isolated using lytic enzymes rich in chitinase. The isolated protoplasts were incubated with 20% PEG + 0.01M CaCl₂ + 0.05M glycine for 30 min for facilitating fusion. The recombinants were regenerated in Czapekdox agar. Pr1 and Pr2 (protease enzymes) specific activities were found to be increased by two-fold in recombinant strains than the non-fusants and the parental strains [20].

Beauveria sulfurescens is non pathogenic to neither European corn borer *Ostrinia nubilalis* nor Colorado potato beetle *Leptinotarsa decimlineata*. Hypo virulent strain of *Beauveria bassiana* were made hyper virulent by protoplasmic fusion of *B. bassiana* with *B. sulfurescens*. The fusion product was highly pathogenic to *O. nubilalis* and *L. decimlineata* with 39% and 73% reduction in LT₅₀ respectively, when compared to the parent *B. b* strain [2]. The toxic gene of *B. sulfurescens* was acquired by *B. Bassiana* and the recombinant strain retained its virulence after an infection cycle.

Mutants of *Neurospora crassa* having phenomenal resistance to the fungicide benomyl have been identified. The resistance is attributed to a β -tubulin gene. The field persistence of entomopathogenic fungi are drastically affected when fungicide treatments are done. Recombinant epf's with enhanced fungicide resistance can be tailor fit into integrated management systems. *Metarhizium anisopliae*, *Paecilomyces fumosoroseus* and *P. lilacinus* were transformed with β -tubulin genes from *Neurospora crassa* to confer benomyl resistance. The recombinant strains were exceptionally tolerant to high doses of benomyl in the media, even upto 30 $\mu\text{g mL}^{-1}$ [12].

Although it has been a conventional and widely accepted method, with an advantage of using any cellular organelle, owing to its severe drawbacks, protoplasm fusion has been replaced by more novel techniques. Some prominent drawbacks are

1. Difficulty in isolation of protoplasts: Different fungal strains possess different cell wall composition. This calls for separate cell wall degrading enzymes, that has to be standardized for each fungi. For experiments involving intergeneric fusions, the standardization of lysis enzymes itself is a herculean task
2. Batch to batch variation in quality of degeneration enzymes affect quality of extraction: in case of large scale fusions or multiple fusion experiments, slight change in the composition or quality of degradation enzymes can drastically affect the results.
3. Lower yields in terms of number of recombinants per fusion.

2) Electroporation

Natural membrane polarity prevents foreign DNA entry into a cell as a defense mechanism. In electroporation method, instantaneous reversal of membrane polarity is brought about by an electric field, thereby permitting uptake of recombinant DNA in the media by cells. Transformed cells are identified and cultured.

Pioneer research works that adopted electroporation for transforming EPF

Arthropod predators like scorpions and spiders are a warehouse of toxins. Some of these are insect specific. AaIT1, a sodium channel blocker of scorpion origin, renowned for its potency against insects was introduced into a strain of *M. anisopliae* [25]. The recombinant strain was 22 times toxic to

M. sexta (Tobacco hornworm) than wild type. Caterpillars infected with AaIT recombinant demonstrated severe prelethal effects including muscle contractions that decreased body length. Infection by the recombinant, reduced cumulative fecal production by 50% compared to wild type, indicating a significant reduction in feeding. Effect of recombinant strain on *Aedes aegypti* were also studied [25]. Mosquitoes infected with recombinant fungi also demonstrated prelethal effects including spasmodic leg and wing movements several hours before death. They invariably died with their wings extended, indicative of muscle contraction caused by AaIT. When exposed to a bare human hand across wire mesh, 50% of treated female flies failed to illicit feeding response (i.e. fly towards the exposed human arm) which was 1.5 days ahead of the same response being elicited in wild type treated adults

Although wide variety of filamentous fungi can be transformed by this method, a few shortfalls like low recombinant frequency and irreversible electroporation (Electric field permanently disables/disrupts the selective impermeability of cells, leading to cell homeostasis disruption. If the cell homeostasis is jeopardized, the resultant recombinants would be unstable) restricted the wide scale adoption of this method.

3) Biolistic transformation

Heavy metal (Tungsten/ Gold) micro beads are coated with DNA, and these particles are accelerated and collided with fungal cells at a very high velocity, using sophisticated equipment. The coated DNA are instantaneously incorporated into the genome just like a bullet fired by a gun gets embedded in its target.

This method is ideal for those fungi in which protoplast yield by cell wall degradation is too low and for thick walled fungi. The major advantage is its suitability with any host strain, as the genes are literally fired into the host genome the host strain or compatibility does not hinder transformations. In spite of such huge benefits, the adaptability of biolistics in fungal transformation is limited owing to requirement for sophisticated equipment and procedures and lower success rate for transforming entomopathogenic fungi

Pioneer research works that employed biolistics to create GM entomopathogenic fungi.

The field application of locust specific epf, *M. acridium* (Green muscle[®]) is delimited by the usage of herbicide glufosinate ammonium (Basta[®]). Bar gene from *Streptomyces hygroscopicus* that confers resistance to the herbicide was used to transform wild *M. acridium* via biolistic transformation. The recombinant strains were exceptionally tolerant to glufosinate ammonium, even to the tune of 100 $\mu\text{g mL}^{-1}$ [12].

According to Utermark, Karlowsky [23] electroporation and biolistics were rarely used in fungal transformation owing to their lower yields of transformants and relatively less stability of recombinants.

4) Vector mediated transformation:

In this method plasmids are used as gene carriers. These are extra nuclear cyclic DNA with specific genes coding for a variety of proteins, mostly involved in host infection. These plasmids can be lab designed to create gene cassettes. Specific gene of interest is inserted into plasmids and allowed to infect host cells so that the gene gets incorporated into its genome of all the various types of vector mediated transformation, *Agrobacterium tumefaciens* mediated transformation (ATMT)

is the most widely used. *A. tumefaciens* is a soil borne bacteria with a Ti plasmid that incorporate T-DNA into the host plant genome, subsequently causing tumours. In ATMT the T-DNA is replaced with gene of interest tagged with a suitable marker and let to attack host cells so that the target gene gets incorporated into host genome. This was widely used to transform plant cells, until de Groot *et al.*,^[4] proved this method to be successful in transforming fungi as well. In his experiment using hygromycin resistance as a marker, de Groot observed that ATMT was 600 times more efficient than conventional protoplast fusion in transforming *Aspergillus awamori*. Apart from this other bacterial plasmid vectors like pAN52-1, pAN52-1N, pAN52-4 from *E. coli* and numerous other sources including synthetic plasmids are used nowadays, thanks to advances in biotechnology.

The advent of vector mediated transformation has alchemized the fungal transformation scenario with novel gene sources having huge potential, like fungal adhesins that facilitate binding of spore to insect cuticle, being incorporated into entomopathogenic fungi. Both protoplast fusion and vector mediated transformation are equally desirable in terms of their utility. There are no benchmark criteria to compare them as the efficiency of each are expressed in different units altogether. (no. of transformants per μg of DNA used for protoplasm fusion and no. of transformants per plate/experiment in case of vector mediated transformation)

Methodology

A cocktail of restriction enzymes available helps to cut certain portions from plasmid and a gene of interest is inserted into that gap. Required gene is attached to a suitable promoter and incorporated into a plasmid vector. Suitable marker genes are also used to identify the recombinants. Promoters play a crucial role as they ensure that the foreign genome is translated in the host. Therefore genes coding for normal physiological processes like glucose metabolism are used as promoters, and the gene of interest is attached along with. Availability of suitable vectors including several synthetic ones, and identification of the key promoters in entomopathogenic fungi has eased the process of transformation. Incidentally vector mediated transformations are most widely adopted than protoplast fusion.

Pioneer research works that employed vector mediated transformation to create GM entomopathogenic fungi.

The first ever recombinant entomopathogenic fungi was developed by St Leger *et al.*^[22], by cloning multiple copies of chitin degrading- protease gene Pr1 into *M. anisopliae*. The recombinant strains when treated to *Manduca sexta* (Tobacco hornworm) reduced time of kill by 25% as compared to wild strain. Feeding deterrent action was also observed for the recombinant strain. *Bbchit* is an endogenous chitinase gene in *B. bassiana*. It has only a single copy of the gene, and shares little similarity with other epf's endochitinases. Recombinant plasmid vector was created to clone *Bbchit1* gene and the gene cassette thus developed was used to transform wild strains of *B. bassiana* via *Agrobacterium tumefaciens*. The recombinant strains tested against *Myzus persicae* had 19.8% lower LT_{50} than wild type. This reduction in LT_{50} is attributed to decreased time of pathogen entry due to increased levels of chitin degradation^[8]. A recombinant *B. bassiana* was developed with chitin degrading hybrid genes from both *B. bassiana* and *M. anisopliae* (*BbChit: CDEP1*) incorporated into its genome. The genes expressed synergistic action of both genes in recombinant than donor strains, with an

enhanced chitinase activity^[7]. This was achieved by using an *E. coli* vector pAN52-1.

Trehalose is the dominant sugar in insect haemolymph. For successful pathogenesis, fungal pathogens need to efficiently utilize trehalose for their growth within insect system. The more competitively the pathogen utilizes trehalose, more virulence it possess. *Metarhizium sp.* Specific to locusts, *M. acridium* has inherent trehalase gene. *M. acridium* strains were genetically engineered to over express trehalase gene ATM1, using pBarEx vector, the recombinant strains recorded 8.3fold reduction in LD_{50} as compared to wild strains against locusts^[18].

A critical factor that determines the field persistence of entomopathogenic fungi are their UV tolerance. UV in sunlight disintegrates fungal cell wall and can cause mutations, rendering them non- pathogenic. Incidentally various UV protectants are integral components of commercial fungal formulations^[9]. Melanization of fungal cell wall can enhance their UV-tolerance. In fungi, tyrosinases are functionally important in the formation, browning and pigmentation of spores as well as in defense and virulence mechanisms^[11]. Tyrosinase gene was incorporated to *Beauveria bassiana* using ATMT. The recombinants were tolerant to UV and had improved virulence to the mealworm *Tenebrio molitor* with a 16% reduction in LT_{50} when compared to wild type^[19].

Recombinant strains of *Lecanicillium lecanii* transformed with carbendazim resistant (mrt) gene from *Botrytis cinerea* showed 380 fold resistance to the fungicide^[27]. *L. lecanii* was transformed with toxin gene BmKit-12 from scorpion *Buthus martensi*, showed 7 fold reduction in LC_{50} and 26.5% reduction in LT_{50} as compared to wild type. a scorpion toxin gene (*BmKit*) from *Buthus martensi* was cloned and transferred into *L. lecanii* and then its resulting activity against cotton aphids (*Aphis gossypii*) when assessed showed that the engineered strain BmKit recombinant grew significantly quicker than WT in host insects. The median lethal concentration (LC_{50}) for BmKit recombinant was 7.1-fold lower than that for WT (Wild Type), and the median survival time (LT_{50}) for BmKit recombinant was reduced by 26.5% compared with that for WT^[26].

Advantages

- Simple and comparatively less expensive protocol
- High transformation efficiency
- No deleterious effect on conidiation and virulence of recombinant strains.
- 58 sp of ascomycetes, 15 species basidiomycetes, 4 sp. zygomycetes and 3 sp oomycetes have been transformed using ATMT^[23].
- Relatively large length chromosomal segment can be transferred with little arrangement

Disadvantage

Not an universal- fool proof transformation method.

Future prospects of vector mediated transformation

Undoubtedly VMT has alchemized the fungal transformation scenario. With the possibility of large scale mutagenesis and location specific inserts using this method, immense opportunities have been created. There is an infinitively large gene pool, unexploited yet potential that can be inserted to transform microbial pesticides. Photoreactivation system^[6], cold sensitivity, dominant lethality are some facets of great potential, worth exploring.

Ethical issues in microbial transformation

As with other transgenics like *Bt* plants, concerns exist for microbial transformation as well. Addressing these issues are as important as developing improved strains. Designing them with minimal environmental persistence and zero reproductive potential ensure safety. In this way they can serve as safe substitutes to highly persistent chemical insecticides. Photoreactivation system [6], cold sensitivity, dominant lethality are some facets of great potential, worth exploring.

Conclusions

Genetic improvement of microbes can overcome the delimitations of microbial pesticides. Biotechnology has endowed us with unique gene sources and tools to incorporate them. The notion of cross species genetics has opened gates to unlimited opportunity. Time awaits to witness future perfect pathogens capable of addressing all pests in a crop by a single microbe. Deliberate yet cautious steps are to be taken to achieve this goal.

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