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MINI-REVIEW

Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests

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Abstract Molecular biology methods have elucidated pathogenic processes in several fungal biocontrol agents including two of the most commonly applied entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. In this review, we describe how a combination of molecular techniques has: (1) identified and characterized genes involved in infection; (2) manipulated the genes of the pathogen to improve biocontrol performance; and (3) allowed expression of a neurotoxin from the scorpion *Androctonus australis*. The complete sequencing of four exemplar species of entomopathogenic fungi including *B. bassiana* and *M. anisopliae* will be completed in 2010. Coverage of these genomes will help determine the identity, origin, and evolution of traits needed for diverse lifestyles and host switching. Such knowledge combined with the precision and malleability of molecular techniques will allow design of multiple pathogens with different strategies to be used for different ecosystems and avoid the possibility of the host developing resistance.

Keywords Entomopathogenic fungi · *Metarhizium anisopliae* · *Beauveria bassiana* · Virulence gene · Genetic engineering

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Introduction

Viruses and microorganisms that cause disease in insects have been under evaluation as insecticides for more than a century, but few biopesticide products have been widely used in spite of their potential. The reasons given for failure to adopt biocontrol strategies have included slow kill, failure to identify strains active at low doses, and inconsistent results compared to the chemicals with which they compete (Gressel 2001). The development of recombinant DNA techniques, however, has made it possible to significantly improve the insecticidal efficacy of viruses, bacteria and fungi (Inceoglu et al. 2006; Wang and St. Leger 2007a, b, c). These advances have been achieved by combining new knowledge derived from basic studies of the molecular biology and genomics of these pathogens with technical developments that enable increases in gene expression and the use of genes from other organisms that encode insecticidal proteins to improve efficacy. With increasing public concern over the continued use of synthetic chemical insecticides, these new types of biological insecticides offer a range of environmentally friendly options for cost-effective control of insect pests (Federici et al. 2008). Given the increasing public acceptance of genetically modified (GM) crops expressing *Bacillus thuringiensis* toxins, field application of GM insecticidal microbes should have a bright future if care is taken to ensure social acceptance through rigorous risk benefit analysis.

While many of the approximately 1,000 known species of entomopathogenic fungi have narrow host ranges, collectively, they target most if not all known insect species including sucking insects and many coleopteran and orthopteran pests, etc., which have few known viral or bacterial diseases (Lomer et al. 2001). Fungi can target

sucking insects such as mosquitoes because unlike bacteria and viruses, they do not require ingestion by the host. Instead, these fungi infect by direct penetration of the cuticle. Following penetration, the fungus propagates in the insect hemocoel (Fig. 1). Upon the death of the insect host, hyphae reemerge to cover the cadaver and produce massive numbers of conidia to infect new hosts. Some isolates of *Metarhizium anisopliae* are also rhizosphere competent and can establish a symbiotic relationship with plant roots (Hu and St. Leger 2002; St. Leger 2008). To date, 13 species or subspecies of insect pathogenic fungi have been formulated and registered as mycoinsecticides or mycoacaricides, with most of these formulations consisting of the ascomycetes *M. anisopliae* and *Beauveria bassiana* (de Faria and Wraight 2007). In the USA, *M. anisopliae* formulations have been developed commercially for control of various beetles, termites, flies, gnats, thrips, and ticks. In other countries, several strains of *M. anisopliae* have been developed as biological control agents for grasshoppers, locusts, cockchafers, spittlebugs, grubs, and borers and released on a large scale (Milner et al. 2002; Shah and Pell 2003; Maniania et al. 2003; Blanford et al. 2005). Although zygomycete insect pathogens, e.g., *Entomophaga maimaiga*, *Erynia neoaphidis*, etc. cause frequent epizootics in insect pest populations (Hajek 1999), they are hard to mass produce. This review is thus mainly focused on recent studies on insect pathogenic ascomycetes.

Exploitation of wild-type entomopathogenic fungi

The successful use of *M. anisopliae* to control locusts has established that it is possible to develop effective biopesticide products based on entomopathogenic fungi. More generally, the devastating plagues of locusts in Africa in the mid-1980s provided a sense of urgency to use of *M. anisopliae* and a requirement for standardized products that advanced our knowledge in formulation, quality control, storage,

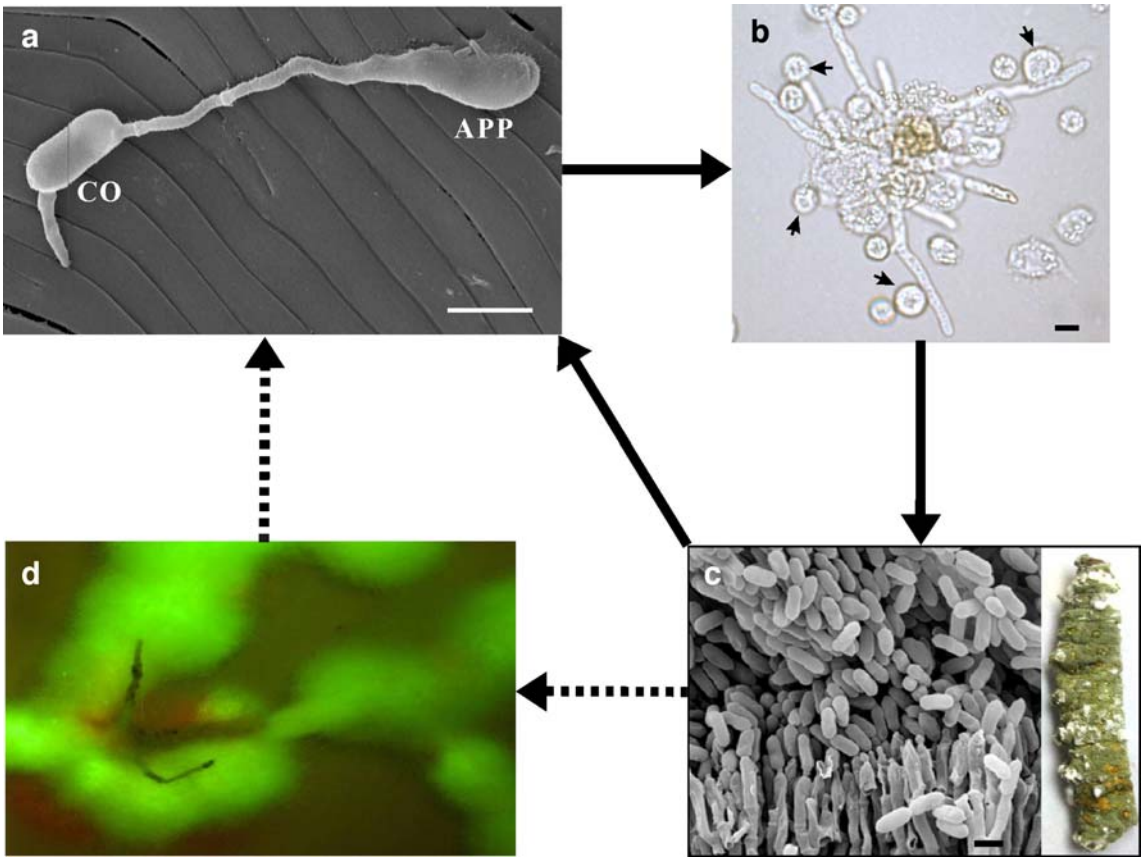

Fig. 1 Infection cycle of the insect pathogenic fungus *M. anisopliae*. **a** Infection is initiated by a conidium (*CO*) that adheres to the insect cuticle using an adhesin protein (MAD1, Wang and St. Leger 2007c). Germination results in a germ tube and appressorium (*APP*; penetration structure). The cuticle is penetrated by a combination of mechanical pressure (MPL1, Wang and St. Leger 2007b) and the action of cuticle-degrading enzymes (Bagga et al. 2004); **b** the fungus grows in the hemolymph and evades recognition by hemocytes (pointed by *arrows*) by masking itself with a collagen-like protein MCL1 (Wang and St. Leger 2006); **c** external conidia (*left*) are massively produced on the mycosed insect cadaver (*right*) and infect another insect (showed by *solid arrowed line*); **d** conidia may colonize a plant root (*dash arrowed line*) as shown by green fluorescent protein-labeled *M. anisopliae* growing out of a bean root (Hu and St. Leger 2002). *Bar* 5 μm

application, environmental impact, safety testing, and host-pathogen ecology (Thomas and Read 2007a). Industrial production of *M. anisopliae* is now highly automated (Langewald and Kooyman 2007). New manufacturing plants for production of *M. anisopliae* var. *acridum* are being built in South Africa and Senegal. The first large-scale application of Green Muscle™ (an *M. anisopliae* var. *acridum*-derived product) took place in Tanzania in 2009 when spraying over 10,000 ha has contained an outbreak of the red locust. The UN Food and Agriculture Organization had feared that infestations of red locusts would turn into a full-scale invasion, endangering the food security of millions, but the biopesticide contained the outbreak. A similar product, Green Guard™, registered in Australia in 2005, forms an integral part of locust-control operations, with a steadily increasing market share as the technology has become established. However, because of relatively slow kill, *Metarhizium*-based products are more appropriate for prevention than controlling outbreaks, and this requires accurate surveying and forecasting to detect juvenile locusts (hoppers) in time (Langewald and Kooyman 2007). A company in China has developed an oil-based formulation of *M. anisopliae* var. *acridum* that is stable for at least a year, and the product was applied to 200,000 ha in China in 2007, but only as a preventative against grasshoppers (Yuxian Xia, Personal communications). The current cost of commercialized *M. anisopliae* var. *acridum* is approximately US$20/ha for 50 g/ha, which is 1.5–2 times the price of conventional chemical insecticides (Langewald and Kooyman 2007). The number of *M. anisopliae* var. *acridum* spores required to kill a locust may be as low as 100, but this is uncertain (Matthew Thomas, personnel communication). If transgenic and genomic recombination strategies could reduce the median lethal concentration LC_{50} significantly, then infection rates would be improved and equivalent control could be achieved with fewer products, providing scope for reduction of application rates and, hence, costs. Similarly, if survival time could be reduced, *M. anisopliae* might be applied later as a rescue treatment to suppress adult swarms. The effective persistence of the biopesticide could be also increased since even if spores of a genetically modified and wild-type fungi decay at the same rate, there is a greater probability that an insect will come into contact with enough propagules to exceed the inoculum threshold (Thomas and Read 2007b).

Improving efficacy by genetic engineering

If the production and formulation techniques developed for *M. anisopliae* var. *acridum* are applicable to other strains of *M. anisopliae* with different host ranges, then this leaves the need to improve efficacy as the principal barrier remaining to cost effective biocontrol of many pests. Furthermore, many of the new application techniques being developed for entomopathogenic fungi involve some kind of bait station where virulence and effective persistence are particularly important. Lack of efficacy is probably inbuilt in pathogens because an evolutionary balance will have developed with their hosts so that quick kill, even at high doses, is not adaptive for the pathogen. In which case, cost-effective biocontrol will require transferring genes to the fungi (Gressel 2001). The most attractive initial candidate genes for this approach include cuticle-degrading enzymes and toxins that are encoded by single genes as they are highly amenable to manipulation by gene transfer. Many of the cuticle-degrading enzymes that act synergistically to solubilize cuticles are multiple-gene products with distinctive activity profiles (St. Leger et al. 1996a, b, c; Bagga et al. 2004). In the first example of a recombinant fungal pathogen with enhanced virulence, additional copies of the gene encoding the regulated cuticle-degrading protease Pr1 were inserted into the genome of *M. anisopliae* and constitutively overexpressed. The resultant strain showed a 25% mean reduced survival times (LT_{50}) towards *Manduca sexta* as compared to parent wild-type strain (St. Leger et al. 1996a).

Over one million arachnid and scorpion toxins include many insect-specific ones that will likely remain a major resource for genetically modified plants and biopesticidal delivery systems (Edwards and Gatehouse 2007; Whetstone and Hammock 2007). The remarkable extent to which virulence can be increased was shown by expressing a scorpion toxin (AaIT) in *M. anisopliae* strain ARSEF 549. We chose to test the effects of insect-specific AaIT because it is very well studied and very potent and so would provide a benchmark for efficacy (Zlotkin et al. 2000). Indeed, the modified fungus achieved the same mortality rates in tobacco hornworm (*M. sexta*) at 22-fold lower spore doses than the wild type, and survival times at some doses were reduced by 40% (Wang and St. Leger 2007a). Similar results were obtained with mosquitoes (LC_{50} reduced nine-fold) and Broca (coffee berry borer beetle; LC_{50} reduced 16-fold) (Pava-Ripoll et al. 2008). At high spore doses, Broca were killed in less than 3 days, but the effective spore dose was less than five spores. Interestingly, however, coexpression of Pr1 and AaIT in *B. bassiana* failed to achieve any synergistic virulence effect since the expressed AaIT was digested by Pr1 produced in the hemolymph (Lu et al. 2008).

Selection of virulence genes for enhancing efficacy

As stated above, selection of potent virulence genes is a crucial step for effective genetic engineering. Apart from cuticle-degrading proteinases, a strain of *M. anisopliae* has been identified that produces an acute protein toxin active

at 0.7 μg/100 mg, and other toxins from *M. anisopliae* and *B. bassiana* are being isolated (Quesada-Moraga et al. 2006). A toxic protein from *B. bassiana* (bassiacridin) had an LT_{50} of 3 μg per insect when injected into fourth instar locust nymphs *Locusta migratoria* (Quesada-Moraga and Vey 2004). The various toxins from *M. anisopliae* and *B. bassiana* affect different aspects of insect biology and, therefore, could be used synergistically to increase the magnitude of hypervirulence and to reduce the probability of resistance evolving to a single transgene product.

Aside from toxic proteins, the range of exploitable fungal virulence genes is enormous. The employment of various techniques, e.g., microarray, gene knockout, RNA interference, etc. (St. Leger and Wang 2009) has identified adhesins, extracellular enzymes, and systems for evading host immunity. These can be used to create novel combinations of insect specificity and virulence by recombining them in other fungi, bacteria, or viruses to produce improved pathogens. Thus, the Pr1 gene and an esterase gene from *M. anisopliae* have each been used to increase virulence of *B. bassiana* (Gongora 2004; Lu et al. 2008). Subtilases similar to Pr1 have improved the biocontrol potential of fungal pathogens of other fungi, e.g., the nematophagous fungus (Ahman et al. 2002).

In recent years, hypothesis-driven cloning of genes assumed to be involved in pathogenicity has been replaced by expressed sequence tag (EST) and microarray approaches that let the pathogen inform on what it is doing during infection processes (Cho et al. 2006a, b; St. Leger 2007). Although they dramatically accelerate the gain of information, the bottleneck is in translating gene expression profiles into hypothesis-driven approaches which result in clearly proven functions for single genes and new ideas for genetically enhancing virulence. Nevertheless, construction of deletion strains for highly expressed genes has already led to the identification of virulence genes, some of which can be annotated based on sequences in other organisms, e.g., regulators such as the protein kinase A that controls expression of some secreted virulence factors (Fang et al. 2009), an osmosensor MOS1 that signals to penetrant hyphae that they have reached the hemocoel (Wang et al. 2008), and a perilipin MPL1 (the first characterized in a fungus) that regulates lipolysis, turgor pressure, and formation of infection structures (Wang and St. Leger 2007b). Some genes are highly adapted to the specific needs of *M. anisopliae*, e.g., separate adhesins (*Mad1* and *Mad2*) allow it to stick to insect cuticle (Fig. 1) and plant cells, respectively (Wang and St. Leger 2007c). However, the properties that make pathogens unique are often organism- or species-specific. Thus, *Mcl1* (immune evasion) with its collagen domain is so far unique to *M. anisopliae* (Wang and St. Leger 2006). Horizontally transferred genes from bacteria including a chymotrypsin for cuticle digestion (Screen and St. Leger 2000) and a phosphoketolase involved in pentose metabolism in insect hemolymph (Duan et al. 2009) have also been established as virulence factors. These genes all have as yet unrealized potential to engineer changes in host range, either increasing it or diminishing it and illustrate the power of expression profiling for revealing previously unsuspected stratagems of infection. Similarly, knowledge concerning the surface properties, mechanisms of adhesion, and nutrient uptake in *B. bassiana* could be exploited towards enhancing its virulence and/or changing specificity (Holder and Keyhani 2005; Cho et al. 2007; Holder et al. 2007; Lewis et al. 2009; Wanchoo et al. 2009).

Synthetic genes

Scientists today are not limited to the immense diversity of peptide and protein sequences that already occur in nature as they can derive synthetic multifunctional genes that are hybrids of different activities. Intriguingly, the chitinases isolated from *B. bassiana*, thus far, appear to lack chitin-binding domains. Fan et al. (2007a) constructed several *B. bassiana* hybrid chitinases where the chitinase was fused to chitin-binding domains derived from plant, bacterial, or insect sources. A hybrid chitinase containing the chitin-binding domain from the silkworm *Bombyx mori* chitinase fused to the *B. bassiana* chitinase showed the greatest ability to bind to chitin compared to other hybrid chitinases. Constitutive expression of this hybrid chitinase gene by *B. bassiana* reduced time to death of insect hosts by 23% compared to the wild-type fungus showing that genetic components of the host insect incorporated into the fungal pathogen can increase virulence. The same group has produced synthetic chitinases with improved enzyme activities through DNA shuffling (Fan et al. 2007b).

As expression systems, *Metarhizium* and *Beauveria* are as easy to use as the commercially available yeasts but with the added advantage of also providing a delivery system into the insects. This contrasts sharply with insect transgenics that requires specialized training and expertise. Insect pathogenic fungi could provide a tractable model system for screening novel effectors or fusion products produced by gene shuffling. After screening, the most potent effectors could be delivered by the fungus, another microbe, and/or in a transgenic insect or plant.

Tools and utensils for genetic engineering-host specific promoters

One of the most important factors for successful fungal transgenesis is the availability of suitable promoters to drive the expression of exogenous or endogenous genes. The

promoter of a glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene originally from *Aspergillus nidulans* has been widely and successfully used to express transgenes in *M. anisopliae* and *B. bassiana* (e.g., St. Leger et al. 1996a; Lu et al. 2008). However, using the orthologous *gpd* promoter from *B. bassiana* increased expression of transgenes in *B. bassiana* by two- to three-fold compared to the *Aspergillus gpd* promoter (Liao et al. 2008). EST and microarray studies were used to identify *M. anisopliae* promoters that are capable of expressing homologous and heterologous genes in a regulated fashion and that vary in levels of expression. The most highly expressed gene (*Mcl1*) during growth in hemolymph encodes a cell wall protein with a long collagenous domain (Wang et al. 2005). Immunofluorescence demonstrated that the protein coats fungal cells during growth in hemolymph, and gene knockout confirmed that MCL1 is required for immune evasion (Wang and St. Leger 2006). The mutant is rapidly attacked by hemocytes and has reduced virulence to *M. sexta*. Reverse transcription-PCR confirmed that *Mcl1* is expressed during growth in the hemolymph of a diverse array of insect species, consistent with the broad host range of the *M. anisopliae* strain (ARSEF 2575) that it was acquired from. However, it was not expressed in other media, consistent with it being involved in hemolymph-specific growth and pathogenesis (Wang and St. Leger 2006).

The highly expressed *Mcl1* promoter seems optimal for targeted expression of transgenes encoding toxins to the hemolymph. Aside from the possibility of increasing virulence, restriction of toxin expression to growth in the hemolymph has safety considerations, by precluding casual release of the toxin by the fungus during saprophytic growth. In addition, specificity is usually controlled by infection events at the level of the cuticle (St. Leger and Screen 2001), so altering post-penetration events should not reduce environmental safety considerations derived from species selectivity. Precise information on host-related signals that induce the promoter is required for engineering purposes, as well as for regulatory bodies to determine whether the specificity of the promoter can be relied on in field conditions. In this regard, targeted expression to the hemocoel was confirmed by using the *Mcl1* promoter to drive expression of a green fluorescent protein gene. Subsequently, the *Mcl1* promoter was used to drive the expression of transgenes in *M. anisopliae*, including the insect-selective 70 aa AaIT neurotoxin from the scorpion *Androctonus australis* (Wang and St. Leger 2007a).

Concluding remarks and future directions

Success in developing transgenic organisms will benefit from knowledge of the signal transduction pathways that regulate pathogenesis, particularly host range and the availability of a wide range of suitable genes that can be used to increase virulence. Genetic engineering strategies require information on the roles and consequences of these genes, leading to enhanced exploitation of the resources present in insect pathogenic fungi. The complete sequencing of four exemplar pathogenic fungi including *B. bassiana*, *M. anisopliae* var. *acridium*, *M. anisopliae* var. *anisopliae*, and *Cordyceps militaris* will be completed in 2010 by international collaborations. Combining transcriptome analysis with an insertional mutagenesis strategy to tag virulence genes will significantly increase the value of genome data. Linking a genome with the mutant library will require extensive sequencing of the insertion sites, but it will then be a seamless process to go from identifying a novel gene sequence to finding the corresponding insertion sequence.

Coverage of these genomes will help determine the identity, origin, and evolution of traits needed for diverse lifestyles and the theorized host switching of these fungi from plants to insects. We have already identified signal transduction pathways including a protein kinase A (MaPka1) that are master regulators of insect infection processes (Fang et al. 2009). However, EST approaches have not identified the downstream regulators of differentiation perhaps because of very transient expression. This is a crucial information gap, as we do not know how to target fungi to specific hosts, although the machinery for such targeting is present in natural strains. Even strains with broad overlapping host ranges differ in patterns of growth and differentiation depending on the composition of the cuticle of their preferred hosts (St. Leger et al. 1992). Immune evasion and suppression are also critical for successful pathogenicity, and the insect immune system is a potentially excellent target for disruption as a means to control pests. Identifying insect-specific immunosuppressive molecules could reveal new vulnerabilities in the insect, and the pathogen molecules could be useful whether used in biopesticides, in transgenic plants, or as a conditional lethal trait in insect-release programs. To date, we have identified only one gene (*Mcl1*) important for immune evasion (Wang et al. 1996), but it has also been demonstrated that secondary metabolites known as destruxins inhibit part of the humoral immune system (Vey et al. 2002; Pal et al. 2007). It is unknown whether other small secondary metabolites play important effector-type functions in insect defense modulation instead of (or in addition) to these proteins.

Future advances in our knowledge of the genetic basis of host specificity and virulence will allow development of recombinant strains of insect pathogenic fungi that show narrow specificity for target pests and that persist in the environment, providing sustainable cheap control for much

longer periods than existing chemicals. Producing such an organism could not have been envisioned as recently as a decade ago. However, we will soon have established the conditions and assembled the molecular biological knowledge and techniques that will make creation of these microbes highly feasible. Furthermore, the precision and malleability of molecular techniques will allow design of multiple pathogens with different strategies to be used for different ecosystems and avoid the possibility of the host developing resistance. Upon a better understanding of insect fungal pathogenesis, future efforts in genetic engineering could be focused on producing target-specific and marker-free products which will alleviate any environmental safety concerns.

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