GE and non-GE fungi: Risk Assessment.

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A major deterrent to the development of fungi as pesticides has been that it can take 5 to 15 days post-infection to kill the targeted pest. This not only makes them poorly competitive, but also limits industrial investment in application and formulation technologies for advanced efficacy. Consequently any consideration of the suitability of a pathogen for commercial development inevitably leads to the possibility of improving its performance. Ultimately, various traits of flingal pathogens, including host range, production capacity, stability and virulence, might be enhanced through genetic manipulations. Unfortunately, host specific strains best adapted to an IPM program in particular kill slowly and produce fewer toxins than generalist strains that could potentially kill natural predators as well as target pests. Presumably strains that are not specifically adapted to subvert/avoid/overcome the immune response of a particular insect are best served by achieving a rapid kill with toxins. An adapted strain may optimize utilization of host nutrients and production of infectious propagules by growing within the living host. Adding new genes to the fungus that will allow it to kill the insect host more quickly is a solution. Attractive initial candidates 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.

Recombinant Metarhizium strains that constitutively overexpress the subtiisin protease Pria have improved pathogenic qualities at all stages of infection. In contrast to the wild-type, transgenic strains continued to produce Pri in the haemocoel of Manduca sexta caterpillars following penetration of the cuticle. This caused extensive melanization in the body cavity, and early cessation of feeding. Insects killed by transgenic strains and extensively melanized were very poor substrates for fungal growth and sporulation. This reduces transmission of the recombinant fungi, which assisted in obtaining permission for a field trial. The trial occurred on a patch of cabbage with an engineered hypervirulent strain carrying extra protease genes plus the gene for EGFP1 (a variant of the green fluorescent protein). The gfp gene is driven by a constitutive promoter and the cytoplasmically located protein strongly labels the whole fungus, with no detectable effects on fungal growth and pathogenicity. Use of GFP to monitor survival and distribution was essential because: a) there were no precedents for the release of such fungal products, and b) there is an inherent paucity of knowledge concerning the fate of fungal genotypes at the population and ecosystem level. This ignorance has helped stir controversy concerning the risks and benefits of releasing transgenic (or foreign) fungi for disease control, insect, and plant pest management or bioremediation, and provides a powerful motivation for studies on their ecology. The field test confirmed that GFP is a very convenient way to monitor pathogen strains in field populations and demonstrated short term effects of insect transmission (non-target insects).

The most interesting result of the field trial was that it documented rhizosphere competence of an entomopathogenic fungus. This emphasizes that for many economically important pathogens the most understudied aspect of their biology involves the extended periods they survive in soil in the absence of a suitable host. Such knowledge is clearly of crucial importance for being able to predict and control outbreaks of plant or animal disease. Rhizosphere competence could be considered as a feature for selecting fungal strains for biocontrol. This would dovetail with attempts in 1PM to manipulate the environment of the plant and insect to enhance insect biocontrol. However, there are many environmental and economic reasons why researchers and industry would not seek to permanently establish an engineered microbial agent in the environment. Rhizosphere

competence might increase the difficulty of eliminating the pathogen following unanticipated and deleterious environmental effects.

Unfortunately, the current predictive data base for risk assessment issues regarding future releases of genetically engineered fungi remains small and very little is known concerning the survival of individual genotypes in the field. We still need to identify the lifestyle (saprotrophy or pathogenicity) responsible for maintaining the large populations of insect pathogens in soil. We also need to provide the knowledge required to predict and improve fungal responses to various environmental stimuli. In particular, to determine side-effects of genetic alterations on the survival of transgenics in soil, their interactions with other soil organisms, transmission to insects and genetic stability. Such knowledge might facilitate genetically based containment by reducing the ability of the organism to spread through a lack of saprophytic competence. I will discuss a recently initiated BRAG funded field trial that will address these issues.

Recently we have supplemented toxic proteins from the generalist *M anisopliae* strain 2575 with the insect-selective 70 an AaIT neurotoxin from the scorpion *Androctonus australis.* This toxin has already provided promising recombinant baculovruses, These studies are providing an opportunity to diversify the deployment of this useful, very well studied toxin, which like M. *anisopliae* has already passed many regulatory hurdles. Our results indicate that fungal and arthropod toxins have good killing power singly, but synergistic effects derived from combining them in a single strain produce a large magnitude of hypervirulence. One of our principal candidates for genetic enhancement is *M anisopliae sf acridum.* Its development as a locust mycoinsecticide is being hindered by its slow speed of kill. Strain 324 does not express several lytic enzymes/toxins produced by strain 2575, including phospholipases. Thus, we are investigating the extent of increases in virulence that result from appropriate combinations of several genes from *M anisopliae* strain 2575 encoding enzymes and toxins that act additively or synergistically to quickly kill insects or to prevent them from feeding. To analyze gene interactions, and the comparative efficacy of the AaIT with flingal toxins, we are comparing disease development (particularly speed of kill) by *acridum* transformed with two or more transgenes with equivalent *acridum* strains transformed with the Pri subtilisin gene or AaIT separately. Changes to LT_{50} values indicate faster kill consistent with toxicosis, while reductions in the median lethal dose (LC_{50}) values indicate that inoculum loads and efficiency of infection (attachment and penetration) are improved. We are also determining if any of the transformations broaden the conditions under which 324 or other strains can produce infection structures. Although we do not expect host range to change, we are evaluating the specificity of transgenic 324 against non-hosts compared with the wild-type (including *Apis mellifera, M sexta, Acheta domestica, D. melanogaster, Galleria mellonella* and *Tenebrio molitor).* The minimum dosage applied to an insect is 100-fold above the LC_{50} for the susceptible grasshopper host. By varying host density, relative humidity, and temperature, we are attempting to optimize the infection level within an insect population. Low infection rates using these procedures would probably translate into virtually undetectable infection rates under natural conditions.