8 Prospects for Strain Improvement of Fungal Pathogens of Insects and Weeds

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Introduction

Fungal pathogens have been recorded for virtually all groups of multicellular organisms. Plant pathologists and insect pathologists have long been interested in the fungal pathogens of their respective host groups. All classes of *Eumycotina* contain at least a few plant pathogens. Likewise, at least 90 genera and more than 700 species of fungi have been identified as closely associated with invertebrates, principally insects. Virtually every major fungal taxonomic group, except the higher basidiomycetes and dematiaceous hyphomycetes, has members pathogenic to insects (Roberts and Humber, 1981). Initially, plant pathologists were interested in protecting crop plants from fungal diseases, but more recently they have selected and/or engineered fungi for weed and plant pathogen (primarily fungi) control. A similar evolution was followed by insect pathologists. Originally, they were interested in protecting beneficial insects (primarily honey-bee and silkworm), but currently there is considerable interest in using fungal pathogens to control insect pests. Recent widely publicized problems with synthetic chemical insecticides and herbicides have stimulated this increased interest in the development of fungi as biological control agents as supplements or alternatives to these chemicals.

Biological control experiments with fungi have often produced inconsistent results, and the slow speed of kill compared with chemical insecticides has deterred commercial development (see Chapter 1). Consequently, any consideration of the suitability of a fungus for commercial purposes inevitably leads to the possibility of improving its performance, i.e. by incorporating more toxic modes of action and increasing kill rates. However, registration requests to date have been for naturally occurring fungi obtained by standard selection procedures and improved as pathogenic agents against insects and weeds by developing the techniques required for optimizing the production and stability of the inoculum (TeBeest, 1991; Roberts and Hajek, 1992; Gressel, 2000). Improvements of pathogens have been attempted through parasexual crossing and protoplast fusion (Heale *et al.*, 1989) or by conventional mutagenesis (Miller *et*

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al., 1989), but genetic engineering by directed addition of one or more genes coding for pathogenicity determinants provides the most targeted and flexible approach to altering the physiology of pathogenic fungi and producing combinations of traits that are not readily identifiable in nature without the co-transfer of possibly undesirable linked characteristics.

Until recently, recombinant DNA techniques had no application in a strain improvement programme involving pathogenic fungi because there was no information available as to the nature of the genes that control either pathogenesis or specificity. This has changed dramatically and molecular biology methods have elucidated pathogenic processes in several important biocontrol agents, with the cloning of genes that are expressed when these fungi are induced by physical and chemical stimuli to alter their saprobic growth habit, develop a specialized infection structure (the appressorium) and attack the host. Some of these genes encode enzymes and toxins with demonstrated targets in the hosts. Other genes have been identified as virulence determinants because of their role in signal transduction during the production of infection structures. As a result, we have entered an era when techniques for the isolation, identification and subsequent manipulation of expression of individual genes implicated in the disease process will allow the production of transgenic fungi with improved pathogenic qualities and hopefully generate a wider interest in fungi as sources of pesticidal genes. The availability of these diverse pathogenicity genes may supplement currently used genes in producing recombinant viral, bacterial, fungal and, eventually, plant products to add to the collection of 'softer' environmentally friendly tools for integrated pest management. Up till now, pathogenic fungi have played little part in providing 'useful' pesticidal genes for transfer, which is surprising given the vast array of biologically active metabolites they produce.

Characteristics of Fungal Candidates for Biotechnological Manipulation

Several different approaches have been used for introducing pathogenic fungi into insect and weed populations. Classical biological control entails the establishment of a fungal species in an area with host populations (usually where the pathogen does not occur) and relies on the pathogen permanently cycling in pest populations. This strategy is frequently applied to the many insects and the great majority of weeds of economic importance that are not native but have been introduced from other continents or geographical areas where they are subject to fungus-induced disease. Obligate pathogens, including members of the Uredinales, Peronosporales and Ustilaginales (plant pathogens) and Zygomycetes (insect pathogens), in general make excellent classical biological control agents, as they are often restricted in their host range and are capable of aggressive pathogenicity (Roberts and Hajek, 1992; Hajek and St Leger, 1994; Quimby and Birdsall, 1995). Biological control of a weed with a fungus began in the early 1970s with the introduction of a rust fungus to control rush skeleton weed (Adams and Line, 1984). The greatest success has been with the use of rusts (*Puccinia jaceae*) for the control of diffuse knapweed (*Centuria diffusa*) and skeleton-weed (*Chondrilla juncea*) control with the use of *Puccinia chondrillina* (Quimby *et al.*, 1991; Kennedy, 1996). Biological control of insects with fungi dates back at least 100 years. Notable recent successes include *Entomophaga maimaiga* to control gypsy-moth (*Lymantria dispar*) populations (Hajek *et al.*, 1990) and *Erynia radicans* to control the

spotted alfalfa aphid invasion of Australia in the mid-1970s (Milner *et al.*, 1982; Carruthers and Soper, 1987). These mostly obligate pathogens are not suitable for use as mycoherbicides or mycoinsecticides in an inundative strategy because they are not readily culturable and/or mass production must be done on the living host, which is not economically feasible.

Pathogens suitable for use in inundative inoculations are generally readily culturable in natural or artificial substrates and are able to produce infective units readily in culture. These properties, combined with the ease with which many facultative pathogens can be genetically altered, make them more amenable to analysis and manipulation at the molecular level than more fastidious pathogens. A further related consideration is that commercial products, including Collego (*Colletotrichum gloeosporiodes* f. sp. *aeschynomene* vs. northern joint-vetch) (developed by Upjohn Corp. with rights currently held by Ecogen), *C. gloeosporioides* f. sp. *malvae* vs. round-leaved mallow (developed by PhilomBios) and DeVine (*Phytophthora palmivora* vs. strangle-vine) (Abbott laboratories), are hemibiotrophic pathogens. They initially colonize plants in a biotrophic manner (a characteristic of many obligate pathogens) and hence do not kill cells or invoke the immune response. However, having invaded large amounts of tissue, they then kill plant tissues and enter a destructive necrotrophic phase, killing the plant. The combination of biotrophy and necrotrophy makes these pathogens highly destructive (Greaves *et al.*, 1989).

Insect pathogens currently employed as inundative control agents are all *Deuteromycetes* (class Hyphomycetes). Insect-pathogenic fungi are of special relevance for biological control as: (i) they are the major natural means of control for many insect pest populations; and (ii) they provide the only practical means of microbial control of insects that feed by sucking plant or animal juices and for the many coleopteran pests that have no known viral or bacterial diseases. The largest programme entailing fungi for insect control is that of the People's Republic of China, where at least 1,000,000 ha of pine forest are treated every 3 years with conidia of *Beauveria bassiana* to control pine moth (Xu, 1988). Other large programmes occur in the former USSR and in the Philippines. In Brazil, *Metarhizium anisopliae* is produced by small companies or grower cooperatives and used to treat approximately 100,000 ha of sugar cane annually for spittlebug control (see Roberts and Hajek, 1992, for review). Entomopathogenic fungi may also assume importance in US agriculture and household entomology. *M. anisopliae* was registered by the US Environmental Protection Agency (EPA) in 1993 for cockroach control and in 1995 for termite control, and registration packages for the use of *B. bassiana*, another imperfect fungus, were recently approved for grasshoppers and whiteflies. *Verticillium lecanii* is registered in Europe, and there is interest in it in the USA, particularly for aphid and whitefly control.

More than 100 pathogens have been identified as having the potential for biological control of weeds and many institutions and universities are now involved in research (see Quimby and Birdsall, 1995, for review).

Biotechnology – the Potential to Provide a Vast Array of New Products

Almost all pathogens currently being used or tested as commercial products have been selected from among wild-type field strains following screening against the pest insect or weed. Acceptance of these products has been limited and they represent less than

2% of the total insecticide and herbicide market. There is a perception among farmers that, compared with conventional chemical products, biologicals are not as fast-acting, lose their effectiveness more rapidly, have a narrower host spectrum and require more knowledge to use effectively. The advanced engineered approach begins by attempting to remedy these deficiencies and could lead to designing the ideal biocontrol organism using genetic engineering. It relies on molecular biology's power of specificity to identify genes conferring pathogenicity to diverse hosts and the development of a gene bank of cloned pathogen genes, each of which controls a different virulence trait. Genetic engineering would employ these and other genes to produce a genetic fusion of many desirable characteristics into the microbe stocks. Cloning of genes from many fungi, as well as their addition and expression in fungi, have become rather simple and straightforward (reviewed by St Leger and Joshi, 1997). Genes encoding biochemical entities already implicated in pathogenicity have been cloned by a range of approaches, which include the use of heterologous DNA probes (Desjardin *et al.*, 1992; Joshi *et al.*, 1995), oligonucleotide probes or primers based on conserved regions of genes (Kusserow and Schafer, 1994) or heterologous expression (Froeliger and Leong, 1991) or the screening of expression libraries with antibodies (Osbourn *et al.*, 1994). These techniques require that pathogenicity determinants be predicted from a prior knowledge of gene function. An alternative cloning strategy is to isolate pathogenicity genes that are specifically expressed during invasion processes, as amongst these may be genes that have a key role in allowing the pathogen to establish itself in the plant or insect host. Differential hybridization (St Leger *et al.*, 1992a, c; Talbot *et al.*, 1993; Pieterse *et al.*, 1994) and differential display (Joshi *et al.*, 1998) techniques allow isolation of infection-regulated genes without making any assumptions about their products. A similar approach – technically less demanding but considerably more expensive – is Expressed Sequence Tag (EST) analysis. Our ongoing EST project to identify the full range of genes expressed during the infection process by the 'generalist' (wide-host-range entomopathogen) *M. anisopliae* strain ME1 and the 'specialist' (narrow-host-range entomopathogen) *Metarhizium flavoviride* strain 324 has allowed us to identify thousands of genes expressed during pathogenicity (www.http:tegr.umd.edu). This collection provides a resource of genes both for the genetic improvement of entomopathogenic fungi and for other biotechnological applications (e.g. insect-resistant plants). Complementary approaches for the identification of pathogenicity genes involve the generation of pathogenicity mutants by random mutagenesis (e.g. REMI), with subsequent characterization of the induced mutations (Bolker *et al.*, 1995). The great promise of these 'Black box' techniques is that they will identify currently unsupected stratagems of pathogen attack.

Strain improvement can be achieved in a variety of ways, from random selection of (ultraviolet (UV)/chemical-induced) mutants to site-directed homologous gene replacement techniques. The technique chosen depends upon the availability of suitable selectable markers (e.g. antibiotic resistance), transformation systems and the desired phenotypic change. In recent years a number of robust methodologies for fungal transformation have been developed, including Ca^{2+}/polyethylene glycol (PEG)-mediated protoplast transformation, electroporation and particle bombardment (reviewed by St Leger and Joshi, 1997). Combinations of desired genes can then be created by the mating of suitable strains (not available for deuteromycete fungi). In the absence of a sexual stage, the parasexual cycle (anastomosis) may be used (Messias and Azevedo, 1980; Bello and Paccola-Meirelles,1998), or a 'forced' union can be

achieved by means of protoplast fusion techniques (Viaud *et al.*, 1998). Unfortunately, there is still no effective way to stably transform biotrophic fungi.

Characteristics that might Benefit from Genetic Manipulation

There are many common threads running through previous studies on plant and insect pathogens and many of the insights, research methods and aims developed for one system also apply to the other. Both entomopathogens, e.g. *Metarhizium* spp., and plant pathogens, e.g. *Colletotrichum* spp., infect their hosts via conidia, which attach, swell and form a germ tube upon contact with a suitable host (St Leger *et al.*, 1989; Dickman *et al.*, 1995). The germling then develops an appressorium, a terminal swelling in the germ tube, from which a narrow infection peg eventually penetrates the external cuticular surface. Many of the apparent differences between these two pathogens arise from the fact that *M. anisopliae* penetrates a (mostly) proteinaceous insect cuticle, while *Colletotrichum trifolii* penetrates a (mostly) carbohydrate plant cuticle. For both plant and insect pathogens, however, fungal perception of and response to its host are likely to be critical in dictating the sequence of events that culminate in a successful infection and will therefore be important targets for molecular manipulation. It is likely that several broad classes of pathogenicity genes are involved in these processes. Some genes encode receptors that detect either directly or indirectly the presence of the host (e.g. a guanosine triphosphate (GTP)-regulated adenylate cyclase, tyrosine protein kinases, serine and threonine protein kinases, and phosphoprotein phosphatases (reviewed in St Leger, 1993; Dickman *et al.*, 1995)). These act to change second-messenger levels or are themselves activated by second messengers to trigger differentiation. Activation of such receptors and signal transduction pathways may result in the induction of generic pathogenicity genes. These could include another class of pathogenicity genes that inactivate host defences, such as the detoxifying enzymes produced by *Gaemannomyces graminis* and *Gloeocercospora sorghi* (which both detoxify preformed inhibitors of fungal growth) (Osbourn *et al.*, 1994; Van Etten *et al.*, 1994) and *Nectria haematococca* (which detoxifies the pea phytoalexin pisatin) (Van Etten *et al.*, 1994). Other pathogenicity genes may encode toxins that are required for disease symptoms, e.g. non-specific toxins, such as trichothecenes, produced by a number of *Fusarium* spp. (Oliver and Osbourn, 1995), and cytochalasins and destruxins, produced by *M. anisopliae* or host-selective toxins, produced by members of the genus *Cochliobolus* (reviewed by Oliver and Osbourn, 1995). A fourth category of pathogenicity genes encodes enzymes that allow the fungus to overcome host barriers. To determine whether such pathogenicity genes exist and what the characteristics of each class are, it is necessary to characterize multiple genes conferring pathogenicity to diverse hosts, preferably from several pathogen species. This will allow the development of a gene bank of cloned pathogen genes, each of which controls a different virulence trait. The availability of these genes raises the possibility of creating novel combinations of insect specificity by expressing them in other fungi, as well perhaps as bacteria or viruses, if that would produce an improved pathogen.

The broad classes of pathogenicity genes detailed above suggest that directed changes to alter virulence could result from the manipulation of nearly every aspect of fungal developmental biology (summarized in Table 8.1).

We have now isolated genes (as mentioned above) from the entomopathogenic fungus *Metarhizium* spp. involved in all of these developmental processes (S. Screen

Table 8.1. Gene products that could be manipulated to enhance virulence

Receptors that detect the presence of the host
Enzymes that facilitate penetration of the host
Gene products that inactivate host defences
Toxins that are required for disease symptoms

and R. St Leger, unpublished), providing a resource of useful genes for strain improvement. In addition, we have analysed the biochemical and molecular mechanisms underlying the complex regulatory mechanisms controlling appressorium formation, enzyme production and penetration in *M. anisopliae* (Screen *et al.*, 1997, 1998; St Leger *et al.*, 1998). Understanding these molecular mechanisms is a prerequisite for making full use of this resource.

An immediate issue of prime importance is how to select those genes which offer the greatest immediate potential in improving the efficacy and reliability of fungi for pest control. The following is a wish list of characteristics that might benefit from genetic improvement.

Improving virulence – speed of kill

A major deterrent to the development of fungi as pesticides has been that it can take 5–15 days post-infection to kill the targeted pest. Based on this slow speed of action, fungi, as well as many other microbial control agents, were considered to have poor commercial efficacy. An obvious solution to this problem is genetic engineering, the idea being to add a new gene to the fungus that would allow the fungus to kill the plant or insect host more quickly and/or to prevent insects from feeding after infection. Most attention has been focused on the speed with which pathogens are able to infect the host, as this is believed to contribute significantly to escape from environmental hazards and to aggressive pathogenicity. For phytopathogens, the plant cell wall is the major barrier to infection and is composed of an array of polysaccharides and protein (Walton, 1994). Penetration of this barrier is believed to involve a combination of mechanical pressure and enzymatic degradation of cell-wall components. Cell-wall-degrading enzymes are produced by single genes and hence genetic manipulation offers attractive possibilities for enhancing pathogenesis by improving the ability of fungi to penetrate and colonize host tissues. However, disruptions of genes encoding cutinases (Stahl and Schafer, 1992; Crowhurst *et al.*, 1997; van Kan *et al.*, 1997), xylanases (Abel-Birkhold and Walton, 1996), pectinase (Scott-Craig *et al.*, 1990; Centis *et al.*, 1997) and cellulase (Sposato *et al.*, 1995) have not dramatically reduced pathogenicity, perhaps due to the redundancy of the encoding genes, i.e. residual activities remain after gene disruption (Mendgen *et al.*, 1996). Presumably, production of mixtures of compounds that affect a number of systems increases the adaptability of the pathogen and minimizes the possibility of resistance developing to the principal toxin(s). In any event, gene disruption is unlikely to provide an efficient way of assessing function of these secreted proteins if their effects can substitute for each other. The fact that the role of these genes in pathogenicity remains uncertain does not preclude them from being used for strain improvement of weed pathogens. In fact, while the multiplicity of these molecules provides a major challenge with respect to establishing

the function of each molecule in pathogenicity, the variability of molecules with activity against host substrates increases the range of tools naturally available for developing biotechnological procedures for pest control.

We have developed a strategy of developing transformation and vector systems to introduce depolymerases and toxins into insect pathogens that normally lack them or to alter their mode of action in a way that would increase speed of kill. For example, as activation of fungal infection processes involves the expression of many inducible proteins, constitutive expression provides a direct strategy for engineering enhanced virulence. This may override effects produced by physical and chemical signals that induce a transient expression of actions of the gene. The most attractive initial candidates for this approach include genes encoding cuticle-degrading enzymes and toxins, as these have often been shown to be active synergistically *in vitro* against insects (reviewed in St Leger, 1993), and, since the active agents are encoded by single genes, they should be highly amenable to manipulation by gene transfer.

The insect pathogen *M. anisopliae* produces multiple cuticle-degrading proteases that are encoded by several gene families. We used *M. anisopliae* to develop the first genetically improved entomopathogenic fungus (St Leger *et al.*, 1996c). Additional copies of the gene encoding the regulated cuticle-degrading Pr1 protease were inserted into the genome of *M. anisopliae* under the control of an *Aspergillus pgd* promoter such that the gene was constitutively overexpressed.

In contrast to the wild type, transgenic strains continued to produce Pr1 in the haemocoel of *Manduca sexta* caterpillars following penetration of the cuticle. This caused extensive melanization in the body cavity and cessation of feeding 40 h earlier than controls infected with the wild type. Pr1 was found to act indirectly by activating a trypsin-like enzyme that is involved in a cascade terminating in prophenoloxidase activation. This was facilitated by Pr1 possessing pathogenic specializations that distinguish it from similar molecules produced by saprophytes. Thus Pr1 is resistant to proteinase inhibitors (serpins) present in insect blood and even to being in a melanizing milieu, mimicking the insect defence response (St Leger *et al.*, 1988). Insects killed by transgenic strains and extensively melanized were very poor substrates for fungal growth and sporulation. This reduces transmission of the recombinant fungi providing a degree of biological containment (St Leger *et al.*, 1996b). It is also consistent with the new emphasis of using entomopathogenic fungi as 'contact insecticides' that achieve a quick kill (Prior, 1992).

Other *M. anisopliae* molecules also show pathogenic specializations, symptomatic of the fact that fungi may have spent millions of years of evolution refining chemicals that subdue their hosts; this makes their proteins choice candidates for producing improved transgenic organisms (St Leger and Bidochka, 1996). This suggests that we can use the multifarious secreted compounds produced by the entomopathogens themselves as a resource for their genetic improvement. This is important, as the use of homologous genes, albeit under altered regulation, provides an experimental design that seems inherently unlikely to raise public concern.

Restricting or widening their specificities

One of the positive environmental attributes of many naturally occurring fungi is that their reported host ranges are limited to a small number of plants or insects that do not include beneficial species. In this way, they are seen as environmentally safer than

chemicals, which may have more widespread effects. Certainly, many obligate pathogens show a specificity that is unequivocal. For example, *Erynia variabilis* is restricted to certain small dipteran flies, in part by a requirement for oleic acid to induce germination (Kerwin, 1984). However, many of the facultative pathogens currently being considered for pest control are less fastidious and have a broad host range. Even *C. gloeosporioides* f. sp. *aeschynomene* (Collego), which was initially thought to be highly specific to its leguminous target, northern joint-vetch (*Aeschynomene virginica*), can also cause low-level infections in several crop legumes (TeBeest, 1988). Fortunately, environmental risks can be reduced by strain selection or by innovative techniques such as the development of auxotrophs (Quimby and Birdsall, 1995). For example, *Rhizoctonia solani* is a broad-spectrum pathogen with the potential to control difficult weeds. Selected strains vary in their mode of attack and in their pathogenicity and may be able to be used against target weed species with low risk to non-target species (Caeser *et al.*, 1993). However, the limited range of some insect pathogens has also been a deterrent to commercial development, because this generally limits the potential market size. Furthermore, a narrow host range limits the usefulness of a microbial pesticide if a crop is attacked by a diverse group of pests, as is often the case. The interests of commercial profitability and protection of non-target species may collide over the issue of target range. A grower wants to apply just one agent to control all insect pests and one agent to control weeds on his/her crop. At present, when developing specific pathogens, industry has to cover the costs of registration, production and marketing for a pathogen for each pest. Very few pests are important enough to justify such an effort. Clearly, a major goal in developing fungal pathogens of plants and insects is to restrict or widen their specificity. This has been achieved with bacteria. Avirulence genes have been isolated from several plant-pathogenic bacteria, and the transfer of avirulence genes from one strain of bacterium to another has been shown to restrict the host range of the recipient organism (Staskawicz *et al.*, 1984). Likewise, Sandoz Agro (Palo Alto, California) used recombinant DNA techniques to transfer delta-endotoxin genes between *Bacillus thuringiensis* (*Bt*) strains to produce varieties that kill multiple types of insects. Compared with bacteria, less is known concerning the basis of host specificity for fungal pathogens, and what is known is limited to a very few model organisms. In some strains, adhesion of spores is host-specific. For example, conidia of an *M. anisopliae* isolated from the scarab beetle *Cetonia aurata* readily attached to *C. aurata* cuticle, but failed to adhere to a related non-host scarab (Fargues, 1984), indicating that specific attachment may be the earliest event in this host–pathogen interaction (Boucias and Pendland, 1991), as in fungal–plant interactions (Nicholson, 1996). Al-Aidroos and Bergeran (1981) reported that a gene determining specific adhesion by *M. anisopliae* spores was linked to that for brown spore colour, but the molecular basis for such specific adhesion remains to be established.

The absence of genes responsible for cuticle-degrading enzymes would presumably prevent penetration of host barriers. For example, while several studies employing gene disruption have not confirmed a role for cutinase in pathogenicity to plants, insertion of a cutinase gene from *N. haematococca* into an opportunistic wound pathogen enabled it to infect an intact host (Dickman *et al.*, 1989). Many studies on host range and specificity in plant pathogens have focused on detoxification of plant substances by fungi. For example, oat-attacking isolates of *G. graminis* are insensitive to the toxic effects of the oat saponin, avenacin, which is degraded by the pathogen enzyme avenacinase. Gene disruption of avenacinase produced mutants incapable of

infecting oats but fully pathogenic to a non-saponin-containing host (wheat), providing genetic evidence that saponin detoxification determines host range (Osbourn *et al.*, 1994). Interestingly, while *N. haematococca* mutants disrupted in the pisatin demethylase (PDA) gene do not show a reduction in pathogenicity (van Etten *et al.*, 1994), introduction of the gene into PDA-deficient isolates with a low level of pathogenicity to pea conferred PDA activity and increased pathogenicity (Ciufetti and van Etten, 1996). This supports the contention that negative results following gene disruption do not exclude a role for a gene in strain improvement and that genes encoding detoxifying proteins could be used to broaden the host range of a microorganism that is otherwise not pathogenic. Toxins produced by fungi can also influence specificity. Within members of the genus *Cochliobolus* (reviewed by Oliver and Osbourn, 1995), toxin production has been implicated in determining: (i) the virulence of race T of *Cochliobolus heterostrophus* to maize lines with Texas-type cytoplasmic male sterility (*cms-T*) (T-toxin); (ii) the pathogenicity of *Cochliobolus carbonum* race 1 to certain maize varieties bearing the dominant allele of the *Hm* gene (HC-toxin) – these varieties are resistant to non-toxin-producing races; and (iii) the ability of *Cochliobolus victoriae* to cause blight on Victoria oats (victorin). Toxin production is controlled by a single gene in each of the three species and hence readily amenable to genetic manipulation.

To date, little is known concerning the biochemical or molecular basis of host specificity for weed and insect pathogens. However, it seems likely from the above that responsible genes could control: adhesion (surface features favourable to surface attachment); the ability to exploit conditions (nutrients, humidity, specific recognition factors) on the cuticle surface; resistance to inhibitory compounds; the ability to overcome structural and chemical barriers to penetration; and the ability to produce toxins that damage hosts and weaken host defenses.

Reducing inoculum

The advantages of lower inoculum levels would make the processes of adhesion and formation of infection structures attractive possibilities for development. Altering events that occur subsequent to this, e.g. the production of cuticle-degrading enzymes, may affect speed of kill but without influencing the amount of inoculum required to kill an insect host (St Leger *et al.*, 1996b). Biochemical studies have shown that appressorium formation by *M. anisopliae* involves disruption of calcium gradients, redirecting cell-wall synthesis from the growing hyphal tip to the entire surface of the cell, reducing extension growth and producing a swollen appressorium (St Leger, 1993). A similar model may also apply to cellular differentiation of the plant pathogen *C. trifolii* (Dickman *et al.*, 1995). In both fungi, various protein kinases, including calcium- and calmodulin-dependent kinases and cyclic AMP (cAMP)-dependent kinases, were shown to function during fungal development and differentiation. It is evident that unifying themes exist in the manner in which disparate plant and animal pathogens respond to environmental signals. To utilize the specific molecular machinery involved in signal transduction for biotechnology it will be necessary: (i) to understand the relationship between the formation of infection structures and the expression of virulence; (ii) to determine which signal transduction mechanisms operate in the early stages of infection as compared with the later stages of development; and (iii) to determine how utilization of a complex array of host signals (nutrients, thigmotropic stimuli, chemical recognition factors) may facilitate the deployment of pathogen responses. Exploiting

this, it may be possible to produce transgenic pathogens expressing the relevant genes necessary to reduce the time expended by the fungus in penetrating host surfaces. This could reduce the susceptibility of the fungus to hostile environmental conditions and inoculum loads, hasten host death and provide a more effective strategy for pest control by fungal pathogens. To date, this has not been done, but several candidate genes have been isolated. Exogenously added cAMP stimulated appressorium formation by the rice blast fungus, *Magnaporthe grisea*, while gene disruption reduced formation of infection structures (Mitchell and Dean, 1995) and demonstrated an additional role for cAMP signalling in plant penetration (Xu and Mengden, 1997). Another signal transduction pathway required for appressorium formation involves a MAP kinase called Pmk1 (Xu and Hamer, 1996). A similar MAP-kinase signalling pathway may operate in the unrelated plant pathogen *Ustilago maydis* (for review see Kahmann *et al.*, 1995), the human pathogen *Candida albicans* (Kohler and Fink, 1996) and the insect pathogen *M. anisopliae* (S. Screen and R. St Leger, unpublished data), suggesting that a common signal transduction pathway may have evolved to regulate pathogenic growth in a variety of fungi (Hamer and Holden, 1997). However, elements of this pathway are also present in non-pathogens, so it will be important to learn what components are specific to pathogens and what signals activate these pathways. This would facilitate exploitation of this pathway for biotechnology.

Altering persistence

The development of pathogens for classical biological control depends on their being able to recycle through host populations. However, most pathogens currently being considered for genetic enhancement would be applied in an inundative manner and there are both environmental and commercial reasons why it would not be beneficial for an introduced or transgenic pathogen to persist into the next season. Thus, commercial interests in developing pest control agents rely on the probability of achieving profitability through repeat sales. The ecological relationships between fungi and their hosts are not well understood, so it is difficult to access the possibility that foreign or engineered fungi will displace native populations or possess some other unanticipated properties that would warrant mitigation. Elimination of a fungus from nature would be highly problematic and fungal spores can survive for years in soil. Therefore, it would seem prudent to engineer fungi in such a way that they would be at a selective disadvantage in nature. In this context, spore-killing factors, double-stranded RNA and viruses and specific metabolite control of the introduced genes suggest possible approaches (Koltin *et al.*, 1987). Auxotrophs of *Sclerotinia sclerotiorum* have been developed which require pyrimidine to grow. These auxotrophs can be spot-applied to a broad spectrum of weeds, with pyrimidine added to allow activity. When the pyrimidine is depleted, the fungus dies out (Miller *et al.*, 1989).

Obtaining tolerance to environmental constraints

Genetically based resistance to desiccation and temperature extremes would be a distinct advantage, both during infection and during product preparation and storage. However, these properties appear to be governed by polygenic mechanisms too complex to be readily amenable to genetic manipulation. Immediate advances are likely to

come from strain selection, and the discovery of an isolate of *V. lecanii* from aphids that can grow at unusually low humidities (Drummond *et al.*, 1987) suggests that this trait would be worth seeking in other fungi (Prior, 1992).

Resistance to fungicides

If a fungus is to be used as part of an integrated pest management programme, it will be advantageous for the fungus to be resistant to certain fungicides (Greaves *et al.*, 1989). This can be accomplished by strain selection, mutation or gene transfer.

Tracking Transgenic Fungi in the Field

Unlike most classical biocontrol procedures, it is unlikely that researchers would seek to permanently establish an engineered agent in the environment. The 'first'-generation product would probably be localized and temporary because of reduced potential for secondary infection. There is an inherent uncertainty because of the paucity of our knowledge concerning the fate of fungal genotypes at the population and ecosystem level. In fact, there is no information available on survival of individual genotypes (clones) of entomopathogenic fungi in nature, nor is there experimentally derived information on gene transfer from populations of genetically engineered pathogenic fungi to wild-type or other fungal species.

The desire to release transgenic organisms into the environment is providing a powerful motivation for studies on microbial ecology. Regulatory bodies, businesses developing products, as well as scientists themselves, are seeking systems that balance relative benefits with relative risks. These risks include potential effects on human health and environment, concerns that engineered organisms might cause ecological perturbations by replacing related organisms and the potential for the foreign material to be transferred to other organisms (Wood, 1994).

To obtain information on the survival of specific genotypes of entomopathogenic fungi in nature and to quantify with precision the epidemiological effects of genetic modifications, it will be necessary to develop methods to monitor the pathogen's survival and migration within the background of the complex microbial communities into which they will be introduced. Allozymes provided the first unambiguous markers available in sufficient numbers to enable reliable genetic studies of entomopathogenic fungi (St Leger *et al.*, 1992b). Random amplification of polymorphic DNA (RAPD) provided additional markers (Bidochka *et al.*, 1994). Strain-specific DNA probes were produced to track a wild-type Australian strain of *Entomophaga grylli* introduced into the USA for grasshopper control (Bidochka *et al.*, 1996).

Nucleic acid probing is highly specific and allows inocula to be tracked if the detected DNA sequence is stable, but gives no indication of the viability or activity of a specific population and is a poor tool for predicting the environmental impact of an inoculum. The application of molecular markers (i.e. introduced genes conferring distinctive phenotype properties) has greater potential for increasing our understanding of environmental microbiology. GUS expression has been found to be a practical means of identifying and localizing the active biomass of marked strains under different environmental conditions (St Leger *et al.*, 1995). In combination with techniques such as DNA probes and clamped homogeneous electrical field (CHEF) pulsed-field gel

electrophoresis analysis, marker genes will allow the analysis of potential gene transfer to indigenous fungal strains, i.e. by determining whether fungi retain the marker elements in their original form. To this end, it would probably be a wise precaution to construct transformants containing two or three different marker genes in the genome, as it is unlikely that multiple markers would all be lost at once if gene transfer occurred.

Problems in the Development and Commercialization of Genetically Engineered Fungi

The unacceptable broad chemical toxicity of many pesticides, regulatory considerations, economics and pest resistance have led to a marked decline in the number of chemical options available to growers for insect and weed control (see Krimsky and Wrubel, 1996, for review). Microbial pesticide producers regard this as an opportunity to promote the advantageous qualities of their products to farmers. Furthermore, while it costs $40 million and takes 4 years to develop and register a new chemical insecticide in the USA, it cost Mycogen only $1 million to bring each of its recombinant *Bt* products to market. However, even with the incentives to reduce reliance on chemical insecticides, microbial pesticides have had very little market success. At present, two commercial mycoherbicides are used on a relatively large scale in the USA, while worldwide only six species of insect pathogen are employed for pest control (TeBeest, 1991; Roberts and Hajek, 1992; Charnley, 1997; Gressel, 2000). To date, the promising techniques of genetic engineering have not produced any new commercial fungal products. It is necessary to consider why their application is so limited. A review of the literature, in particular articles by Wood (1994), Krimsky and Wrubel (1996), NABC6 and the US Congress, Office of Technology assessment OTA-ENV-636, reveals the following key points:

1. Fuelled by lavish venture capital and enthusiasm for biotechnology, a flush of biocontrol companies went public in the 1980s to exploit the potential biologicals offered as environmentally benign alternatives to chemicals.
2. The widely predicted demand for biologicals never materialized and many companies downsized. Biocontrol now makes up less than 2% of the global pesticide market.
3. Even this fraction is threatened as new pesticide chemistries come on-line.
4. On the plus side, current federal biotechnology policy is designed to stimulate innovation and to enable the US biotechnology industry to achieve hegomony in global markets. The thrust is a minimalist, cost-effective, priority-driven approach, requiring a burden of proof that regulation is warranted. The burden of proof is then on those who advance a risk scenario and, as agency resources are scarce, responsible officials carefully choose the risks of highest concern.
5. Evidence suggests that early regulatory inaction or confusion kept firms from investing in transgenic microorganisms. Companies do not make a similar case today, as biotechnology in the USA is not burdened with over-regulation. There is, however, significant divergence between American and European regulations.
6. Technical problems, especially lack of efficacy, are probably more important than any government constraints in explaining the relatively slow progress of the industry. Environmental, health and safety considerations do not sell pest control products to most farmers. The bottom line for most farmers is how well the new products work, how easy they are to use and how much they cost.

7. Companies developing biological pesticides see themselves as part of the larger pest control industry and not as an alternative industry seeking to replace conventional insecticides.
8. Many innovations in agricultural biotechnology are science-driven rather than need-driven. Industry has developed powerful tools to manipulate organisms and is seeking ways to develop products using these techniques that will generate economic value. Hence the thrust of the biotechnology industry is not to solve agricultural problems as much as it is to create profitability.
9. Major companies have targeted larger mainstream farmers rather than small organic operations (traditional mainstay of biocontrol products) – even though large farms and their customers (grocery chains, food processors) require the high cosmetic standards achievable with chemical products.
10. The chemical model emphasizes major crops and cheap, stable products that are easy to scale up and use. Most biocontrol agents fit this model poorly. Their success will depend on the ability to improve the efficacy and consistence of products and to provide consistent support to farmers and extension personnel on the techniques needed to maximize effectiveness and avoid pest resistance problems.
11. If genetic engineering succeeds in creating microbial pesticides that are more equivalent to conventional pesticides (more toxic modes of action, increased kill rates and extended environmental persistence), scientists will have engineered out the very characteristics of target specificity and short field persistence that make current microbial pesticides relatively benign.

A further consideration is that microbial pesticides will have to compete with genetically engineered plants and the number of field tests of transgenic plants dwarfs that of genetically engineered microbes. Two factors may help explain this. First, our understanding of the biology and ecology of microorganisms is limited, especially when compared with higher plants and animals. Therefore more uncertainty is associated with predicting the probability of untoward effects associated with the environmental release of microbes. Secondly, unlike field tests of transgenic plants, even small-scale field releases of genetically engineered microbes are difficult to contain (Wood, 1994; Krimsky and Wrubel, 1996).

The US agricultural market for herbicides in 1994 was more than $3.9 billion (thousand million) annually, compared with $1.1 billion for insecticides and $0.9 billion for fungicides (Krimsky and Wrubel, 1996). The greatest opportunity in herbicide usage presented by the advent of gene transfer technology has been to create new crop strains that are resistant to herbicides so that broad-spectrum weed killers, such as Round-Up, will selectively control weeds in crops (see Cole, 1994, for review). Transformation of crops to herbicide resistance is among the most controversial applications of biotechnology to agriculture. A prominent theme of the agricultural biotechnology industry is that genetically engineered crops will reduce the use of pesticides and are thus environmentally beneficial and should aid in the development of sustainable agriculture. Crops engineered with the *Bt* protein to kill insects have been cited by industry as an example. Likewise, entomopathogenic fungi represent an unconsidered, and therefore untapped, reservoir of pesticidal genes for the production of insect-resistant plants – an important consideration, given that the lack of 'useful' pesticidal genes for transfer has been a major constraint in the implementation of biotechnology in crop protection (Gatehouse *et al.*, 1992). In contrast, the ability of herbicide-resistant crops to reduce herbicide use is very doubtful. In fact,

agrochemical companies see herbicide-resistant crops as a way of increasing the value of certain herbicides by expanding their range of uses to fields containing growing crops (Krimsky and Wrubel, 1996). If herbicide-resistant crops provide an extension of high-input chemically intensive agriculture in the USA then the genetic enhancement of mycoherbicides provides an opportunity for biotechnology to reduce herbicide use.

Pathogenic Fungi as a Possible Resource of Genes in Producing Recombinant Viral and Plant Products

Producing an engineered biocontrol insect-pathogenic fungus may not always be the most effective means of delivering a fungal anti-insect gene (depending on the insect pest and host plant in question). A very profitable direction for research could be to use genes encoding the battery of hydrolytic enzymes, toxins and other anti-insect components produced by various entomopathogenic fungi to improve the performance of pathogens that already possess a set of suicide mechanisms (thus alleviating public concerns and reducing the possibility of environmental damage), or to directly introduce anti-insect genes into the genome of plants. In this case, pioneer products have already paved the way for registration. One such strategy for containing recombinant strains is the co-occlusion process for baculovirus populations (reviewed by Wood, 1994). The potential of using baculoviruses for insect pest control has long been recognized as they have minimal environmental impact and high target specificity. At present, baculoviruses compete inadequately with classical insecticides, partly because of their slow speed of action. An important goal of genetically engineering these viruses has been to improve their ability to kill target insects rapidly. An example of this type of approach has been the construction of improved baculovirus insecticides containing toxin genes from spiders or mites (Stewart *et al.*, 1991). Several groups are also investigating the potential of insect hormones or hormone regulators, but with little success so far at improving pathogen performance (Possee, 1993).

Based on the potential utility of foreign gene inserts constructed to date, particularly toxins, the search for additional pesticidal genes is clearly a commercial priority. The insertion and expression of additional genes is performed very simply. The primary limitation in this area has been the availability of pesticidal genes (Stowell, 1994; Wood, 1994). Arthropods have provided a major resource for toxins, but analysis of these is usually hampered by the extremely small quantities of material available. Fungi are much more amenable to molecular analysis, and the screening of fungal genes encoding fast-acting proteins could greatly increase the scope of such studies. To confirm that the baculovirus expression system can produce a biologically active toxin from *M. anisopliae* complementary DNA (cDNA), we have introduced the Pr1 gene into the *Autographa californica* nuclear polyhedrosis virus (ACMNPV) to produce Pr1-BEV. Time to death following infection by Pr1-BEV was reduced by about 50% compared with controls, indicating that the recombinant Pr1 retained its toxic capacity (Huang *et al.*, 1999).

Protection of crops from their insect pests is already a major goal of plant genetic engineering. Fungal genes with anti-insect activity could be used in plant transformation procedures so that the insect will be forced to encounter the gene product when feeding or interacting with the specific host plant. Future success in developing transgenic plants will benefit from the availability of as wide as possible a range of suitable genes. In spite of the vast range of compounds available from fungi, up to now fun-

gal genes have played little part in the 'invisible revolution' resulting from the implementation of biotechnology in crop protection. To date, the vast majority of the work on incorporation of microbial genes, as well as on all other aspects of microbially mediated biocontrol, centres around *Bt* toxins. As a result of relying on a single agent for biocontrol, there is mounting concern that the evolution of resistance to *Bt* toxin will greatly reduce its utility (Krimsky and Wrubel, 1996). Thus far, there are few alternative insecticidal genes and the lack of useful genes for transfer has become a major constraint on this technology. This again is a strong reason for widening the scope of searches for genes, and entomopathogenic and other fungi could provide a major untapped reservoir of insect resistance genes to supplement *Bt* toxin. Fortunately, pioneering products engineered with *Bt* crystal protein genes have already paved the way for registration and for strain acceptance by breeders, farmers and consumers (Krimsky and Wrubel, 1996). Recombinant microbial products and transgenic plants could potentially have complementary roles to play in plant protection. Combinational integration of the large repertoire of anti-insect genes from fungi into ongoing plant breeding programmes or into alternative vectors should make an important contribution to effective, durable crop protection.

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