

Potential failsafe mechanisms against the spread and introgression of transgenic hypervirulent biocontrol fungi

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Microbial biocontrol agents are typically inefficient owing to the evolutionary necessity to be in balance with their hosts to survive. If transgenetically rendered hypervirulent, however, they could be competitive alternatives to pesticides. Potential means are delineated to prevent, contain or mitigate uncontrollable spread of hypervirulent biocontrol organisms, mutations that increase their host range, and the sexual or asexual introgression of hypervirulence genes into pathogens of other organisms. The use of asporogenic deletion mutants as a platform for generating transgenic hypervirulent biopesticides would prevent such spread. Hypervirulence genes flanked with available 'transgenetic mitigator' (TM) genes (genes that are neutral or positive to the biocontrol agent but deleterious to recombinants) would decrease virulence to non-target species.

Microbial biocontrol agents have the potential of controlling specific pests without affecting non-target organisms¹ but it is doubtful whether many such agents can be developed without transgenetically enhancing their virulence². There have been few successful commercial releases of biocontrol agents, except for 'classical biocontrol' agents introduced from the center of origin of the particular pest, which had been lacking since the pest was introduced. Many attempts at the commercial introduction of indigenous biocontrol agents have failed because of the requirements for high inoculum levels that still did not provide adequate control. Pathogens typically exist in balance with their hosts because killing a host would be suicidal for a host-specific pathogen from an evolutionary perspective. Hypervirulence has been obtained in many organisms by engineering genes into a biocontrol agent that will each incrementally enhance the extent or rapidity of its establishment, lower the level of inoculum needed, or increase its speed of debilitating the host (as extensively reviewed in Ref. 2). Enhanced virulence can be gained through the overexpression of known functional genes from the biocontrol agent itself, or from other organisms. Hypervirulence does not require the biochemical function of the gene to be known. The genes can also be unknown virulence genes from the biocontrol pathogen or from other pathogens, which are discovered using genomic techniques²⁻⁶. None of these studies, or those reviewed therein, discusses the possibilities that hypervirulence genes might

introgress into the genomes of crop or other pathogens. The products of such genes can enhance virulence by inactivating constitutive inhibitors, pathogen-induced phytoalexins, or toxins produced by the host as defenses. The hypervirulence gene products could also solubilize host cell components, or could activate indigenous multiple virulence factors after detecting the host.

A hyperpathogenic biocontrol agent would have the advantage of retaining the narrow host specificity to the target pest (which could be a crop-pathogenic fungus, an insect or a weed species) without affecting non-target organisms, while being more potent than 'wild type' biocontrol agents. If a biocontrol agent is highly lethal, it will have to be reapplied whenever the pest reappears. Various groups have generated hypervirulent bacteria, viruses⁷ and fungi⁶ or have changed the host range of a pathogen⁸, potentially allowing the biocontrol of insects and plant disease pathogens. The genes used are unlikely to enhance the virulence of biocidal crop pathogens but they could increase virulence of pathogens to beneficial insects or microorganisms. The first experimental field release of a hypervirulent insect-controlling fungus will be in 2001 (St. Leger, R. pers. comm).

Three potential biological constraints have limited the interest in developing transgenic biocontrol agents because the hazards might pose unacceptable risks: (1) there is the possibility that the agent could persist in the environment and spread, affecting non-target hosts that were not assayed when the host range was checked; (2) they could mutate resulting in a change in host range. Such evolutionary change in host range is potentially more hazardous in a hyperpathogenic organism than in the native pathogen; and (3) the biocontrol agent might sexually or asexually recombine with a related pathogenic species that attacks desirable species, and by gene introgression, confer it with hypervirulence.

Examples of movement of genes among fungi

Many fungi that have the potential to act as biocontrol agents, exist as narrow host-range-specific pathogens that are related to pathogens of beneficial

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species. These *formae specialis* or pathovars have often been shown by DNA comparisons to have evolutionarily diverged a very long time ago from related pathogens. A case in point is the imperfect (having no known sexual stage) *Fusarium oxysporum* with its thousands of *formae specialis* and even more vegetative compatibility groups⁹. There is evidence that parasexual recombination between closely related species, and supposedly incompatible strains of the same species, is more prevalent when environmental stresses are exerted^{10,11}. Indeed, incompatibility between strains and even between species can be overcome by the mutation of a single gene¹². This has occurred in both imperfect strains of *Fusarium* species and *Rhizoctonia* (Basidiomycetes) that include major crop pathogens as well as host specific pathogens of weeds, some of which could be used as biocontrol agents if their virulence was enhanced. Some imperfect *Fusarium* spp. can be physiologically stimulated to sexuality (with the sexual forms called *Gibberella* spp.) and a gene for toxin production could then be moved sexually among strains that do not parasexually recombine by heterokaryon formation¹³. Interspecific protoplast fusion with the appearance of stable hybrids is known (e.g. between an insect-killing *Beauveria* sp. and a toxigenic species). Some of the somatic hybrids formed are hypervirulent¹⁴.

The possibility of gene movement by horizontal gene transfer (asexual exchange among different species or incompatible strains) has been suggested to be the cause for many anecdotal appearances of improbable genes or gene sequences in various fungi. Recently, the 'implied' cases and the supporting evidence have been reviewed by Rosewich and Kistler¹⁵. They endeavored to exclude cases in which parasexual heterokaryon formation and protoplast fusion might be the cause but, as seen above, mutations and stress can alter sexuality and compatibility. Therefore, it was recently proposed to refer to this 'gray' area between vertical (sexual or parasexual) and horizontal exchange between closely related strains and organisms as 'diagonal' gene transfer¹⁶. An example of what might be true horizontal gene transfer is the circumstantial (yet convincing) expressed sequence tag (EST) evidence for the appearance of a bacterial chymotrypsin in a fungus¹⁷. Chymotrypsins were previously unknown in fungi, and sequence analysis showed that the intron-free gene of the fungus and that of a soil bacterium are related¹⁷. Rosewich and Kistler, after an exhaustive discussion of all the extant reports, conclude that horizontal gene transfer in fungi has "not been proven beyond reasonable doubt"¹⁵, but diagonal gene transfer is a clear possibility.

Needs to obviate movement

In nature, genes can move among fungal species by whatever means but there are many good reasons to desire that organisms with transfers of hypervirulent

transgenes from a biocontrol agent to a pathogen of a crop (or of a beneficial insect or fungus) will not become established.

Recently, it has been suggested that there is an advantage to the often-found clustering of complete sets of genes that are needed for secondary metabolism pathways in fungi¹⁸. The fact that such genes are clustered in many species is easier to explain by interspecific introgression as clusters, than by concurrent evolution of clusters of the same genes in the same order. This gene clustering is further considered to be an indication of horizontal gene transfer because if only part of the cluster is transferred, then the offspring cannot produce the particular secondary metabolite and there would be no advantage to the other genes in the cluster¹⁸. Gene clusters of secondary metabolic pathways that confer some selective advantage would also remain intact after sexual or parasexual gene transfer where there is crossing-over of similar, but not identical (but partly homologous) 'homologous' chromosomes of related organisms. Indeed, whole plasmids have been dispersed among fungal species by heterokaryon formation and integration¹⁹. It is suggested later that clustering of transgenes can provide a selective disadvantage to many recipients and can therefore be used as part of a failsafe mechanism.

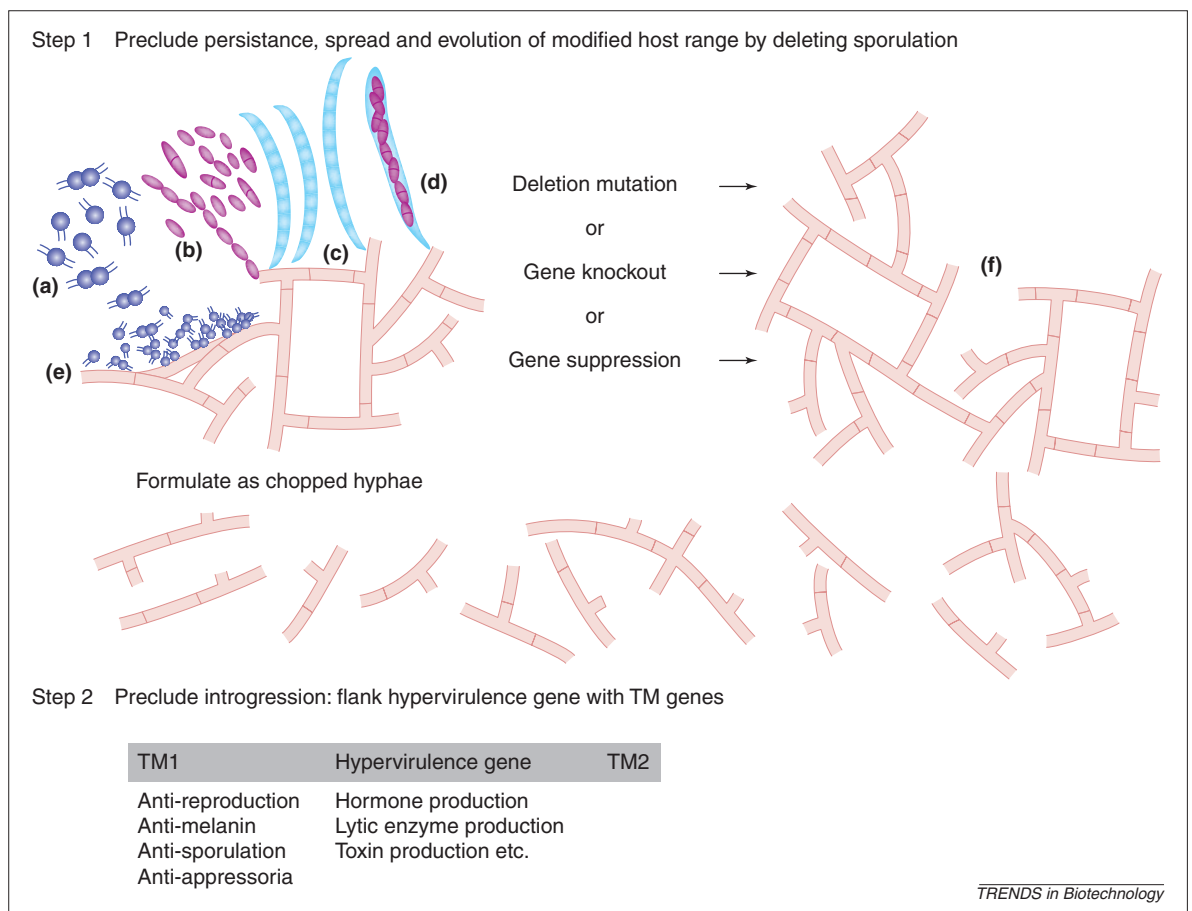
Because of the risk of gene movement among fungi, it might be wise to consider failsafe methods to prevent or mitigate such movement. This requires containment (prevention of spread from the site of application), decreasing environmental persistence and rendering gene flow disadvantageous to the recipient. Decreasing environmental persistence might be construed as a ploy to increase the use of and expenditure on biocontrol agents, but it is actually an ecological prerequisite to preclude widespread dispersal.

A two-stage procedure to obviate the spread of native, host-range mutated, or hypervirulent transgenic fungal biocontrol agents and then to mitigate introgression is illustrated in Fig. 1. The general concepts described might be broadly applicable to many agents including parasitic insects used to control insect pests. However, the specific examples presented are limited to fungal pathogens of weeds (mycoherbicides).

Prevention of persistence and spread of transgenic biocontrol agents

Except in humid, constant tropical environments, most fungi and bacteria must spend a part of their life cycle in dormant resting structures that are resistant to heat, cold, desiccation or other environmental tribulations. These same resting propagules (such as spores, conidia, sclerotia, pycnidia and ascospores) are a major form of dispersal, whether by wind, water or animal movement. The suppression of spore formation in most hypervirulent biocontrol agents can prevent both persistence and spread (Fig. 1, Step 1). Non-sporulating mutants are relatively easy

Fig. 1. The figure shows failsafe mechanisms for hypervirulent biocontrol agents. Dual failsafe mechanisms to prevent (Step 1) spread of biocontrol agents, and (Step 2) their introgression into other organisms. Key: a, chlamydospores; b, microconidia; c, macroconidia; d, ascus with ascospores; e, sclerotia; f, asporogenic mycelia



to isolate; in such a case it is probably best to use a physical mutagen (gamma or neutron radiation) that causes a loss of gene fragments. Point mutations caused by ultraviolet light or by most chemical mutagens can revert, whereas deletions cannot. A similar concept was suggested to prevent the persistence of a wide host range (non-transgenic) pathogen, *Sclerotinia* that was proposed for general weed control²⁰. In addition, this proposal suggested using auxotrophic mutants of the biocontrol agent that could exist only on the culture medium and on the pest host, but could not exist independently outside them²⁰. This concept was never really brought to fruition because it was assumed that efficacious commercial inoculation with any biocontrol agent could not be with fresh mycelia, it had to be with dormant spores.

For two fungal species, it has recently been shown that fragmented mycelia can be dried, stored for more than a year and then be rehydrated and found to be viable²¹. The rehydrated mycelia were more virulent than spores of the same species because the mycelia establish more quickly in the pest²¹. They can, however, be used in the same ways as spores. This procedure has an added asset in that it is usually far more efficient to produce mycelia in liquid culture than spores in liquid or on solid media.

Rendering potential microbial biocontrol agents transgenetically asporogenic (Fig. 1, Step 1) can also

prevent their spread. This can be performed by antisense-type strategies or by gene targeting²² and targeted gene deletion techniques. Many pathogenic species require melanized spores for pathogenicity²³; the germinating spore develops a melanized appressorium that attaches tightly to the host, forming an infection peg that penetrates the host. Unmelanized spores are less viable than melanized spores and therefore, in cases where there is mycelial penetration through stomata in the leaves²⁴, a transgenic suppression of melanization can be akin to deleting sporulation.

Mycelia themselves are not always pathogenic. In cases where only the spores are pathogenic, the spread of a transgenic hypervirulent biocontrol agent can be prevented using a more complex strategy analogous to the 'terminator' strategy^{25,26}, in which chemically induced reproductive suicide genes are activated just prior to field application. The biocontrol agent can grow vegetatively but cannot reproduce. Transgenes that could potentially suppress sporulation could be engineered into the biocontrol organism under the control of a chemically inducible promoter. To suppress sporulation, sporulation genes could be overexpressed to cause cosuppression or arranged in an antisense configuration. Spores or mycelia to be used as inoculum could then be treated with the chemical inducer before application to the target pest. The

Table 1. Potential transgenic mitigator (TM) genes for introgressing into non-target pathogens

Target Potential TM gene	(Presumed) mode of gene action	Refs.
Reproduction		
<i>hetC</i>	Heterokaryon incompatibility	38
<i>sfaD^a</i>	Repressing G protein β subunit	39
<i>stuA</i>	Transcription factor	32
Appressorium formation^b		
<i>mpg1</i>	Hydrophobic surface recognition protein	40
<i>mac1^c</i>	adenylate cyclase	41
<i>pmk1</i>	MAP kinase	42
Spore stalk formation		
<i>fluG</i>	Unknown diffusible factor	43
<i>brlA</i>	Contains Zn finger motifs	44
<i>chsA/chsE</i>	Chitin formation	45
<i>chk1^d</i>	Mitogen activated protein kinase	46
Viable spore formation^b		
<i>abaA</i>	Regulates phialide to spore transition	47
<i>magC</i>	G protein μ subunit-decreases conidiation	27
<i>cmp1</i>	Spore surface protein	48
<i>acr1^d</i>	Prevents regulation of <i>mpg1</i> for spore maturation	49
<i>chsA/chsD</i>	Chitin synthesis	50
Spore germination		
<i>cmk1^d</i>	MAP kinase (regulator gene)	51
<i>ctg1</i>	G protein μ subunit required for germination	28
Melanin formation		
<i>alb1</i>	Polyketide synthase	33, 51-54
<i>arp1</i>	Scytalone dehydratase	52, 55, 56
<i>arp2</i>	Hydroxynaphthalene reductase	51

^aThere are other spore-specific genes that are expressed, with yet unclassified phenotypes that may prove to be vital for spore function. These include SpoC1-c1c57, which must be used in the constitutive sense form to suppress reproduction. ^bNo spores are produced when there are no spore stalks. ^cThese genes also reduce sporulation. ^dThese genes also prevent appressorium formation.

chemical inducer could be in the micropellet containing inoculum used for application²¹, or could be a specific endogenous compound in the pest host. Thus, the biocontrol agent could be contained to the single, purposely infested pest population. The use of transgenic sporulation suppression is less appealing than the use of deletion mutations because of the possibility of transgene silencing, allowing the organism to revert back to wild type.

Obviating recombination

The above strategies can be used to prevent persistence and spread but do not preclude introgression with organisms from the same or related species (after sexual conjugation or heterokaryon formation). Such conjugation might provide genes that support spore formation, resulting in hypervirulent, persistent and spreading pathogens. Thus, it is necessary to prevent the possibility that recombined, introgressed, hypervirulent organisms become 'superbugs' and attack non-targeted species.

It is proposed that the hypervirulence gene should be flanked with transgenic mitigator (TM) genes that are positive or neutral to the biocontrol agent in the form to be used but would be detrimental to any recombinant (Fig. 1, Step 2). Considering the large

amount of intraspecific and interspecific competition in nature, there should be a rapid elimination of individuals that introgress the TM construct owing to the lowered fitness conferred by the mitigator genes. In its simplest form, the hypervirulence gene could be flanked by one or two of the genes listed in Table 1, using genes that do not adversely affect virulence in the particular case. These genes, in an antisense or co-suppressive form, would affect one of the processes that leads to the ability to recombine, to form viable spores or to make efficient infection structures. However, some of these genes might have other deleterious effects that might render them inappropriate for this purpose.

An antisense gene suppressing sporulation should prevent sporulation in a heterokaryon or other recombinant organism. The genes that control melanin biosynthesis and/or conidiation might only be applicable for biocontrol agents that do not require spores or melanin for pathogenicity. Spores without melanin do not have the viability in, or resistance to, harsh environments. Thus, if some spores do form they would be without vigor.

Of the three genes that encode the G α protein subunits that control sporulation, the deletion of *magC* has no effect on mycelial growth or appressorium formation, unlike *magA* or *magB* (Ref. 27). Another G α protein subunit, *ctg 1*, controls spore germination²⁸. The concept of using analogous TM constructs was recently proposed as a failsafe method to mitigate introgression of transgenes from crops to weeds²⁹.

Recently, it has been observed that linked genes mutate at approximately the same rate³⁰. Thus, TM genes would be expected to be as susceptible to mutational modification or inactivation as the hypervirulence gene that they flank.

Risk analysis of these failsafe methods

Risk analysis must be performed separately for each transgenic, hypervirulent biocontrol agent with TM genes, paying particular attention to two issues: (1) the limitations on the failsafe mechanisms that can be used; and (2) the biology of the pathogen and its relatedness to other pathogens.

Limitations of various failsafe measures

A safety aspect that must be clearly ascertained early in the development of asporogenic mutants, as well as in the case of antisense spore formation, is that all forms of sporulation must be suppressed. Many fungi produce more than one type of spore; many *Fusarium* species can produce micro- and macro-conidia, as well as chlamydospores, and each is produced under different environmental conditions. Additionally, one spore type can be under more than one form of control and all controls must be blocked for the failsafe mechanism to be effective. For example, light-induced conidiation is precluded in some asporogenic fungal mutants but starvation-induced sporulation is not³¹. It would be interesting

to ascertain whether each of the genes that control spore stalk development (Table 1) can (when under antisense control or deleted) suppress all types of spore forms. The *stuA*-encoded transcription factor controls both sexual and asexual reproduction in *Aspergillus nidulans* (Ref. 32). It will be easier to load more or simpler failsafe mechanisms into organisms that do not require appressoria for penetration, such as those that penetrate through plant stomates or insect alveoli or through cut surfaces. More complex methods, such as modified 'terminator' technology (described above), will have to be considered for organisms that use melanized appressoria, or in cases where hyphae are not typically pathogenic. Not all organisms (e.g. *Alternaria alternata*) utilize melanization of appressoria as part of infection³³. The use of a spore-specific promoter for anti-melanin genes where hyphae form appressoria requiring melanin can be considered. Thus, failsafe mechanisms will have to be more complex with the melanized appressoria-utilizing *Colletotrichum* species²³. However, some *Colletotrichum* spp. can attack plants by stomatal penetration²⁴. Many regulatory genes are activated during sporulation^{34,35}; such genes could be used to activate melanin-suppression or other anti-sporogenesis genes could be used.

Pathogen biology

A knowledge of the relative likelihood of a pathogen mutating its host specificity and its ability or inability to transfer genes 'diagonally' to related organisms will govern the required number and level of failsafe methods. These should then be assessed in the laboratory and in (small) field trials before large-scale field releases are carried out.

The possibility that the pathogen will mutate in a manner that broadens its host spectrum to include non-target species, must be considered (e.g. that a specific pathovar of *F. oxysporum* will attack other organisms). Although there are no documented cases of a member of this species mutating its host range other than in an evolutionary time scale, the species is sub-divided into hundreds of known *formae specialis*, each with its own host specificity. In this case it is probable that evolution over millennia has played a part in its ability to infect different hosts, suggesting that more caution is needed here than, for example, for the *Fusarium arthrosporioides* that is known to be pathogenic for *Orobanche* species²¹. This *Fusarium* does not have known *formae specialis* (pathovars) on known crop species where it was isolated. However, the

possible existence of alternate crop hosts must still be considered.

Some species easily conjugate with their close relatives to form heterokaryotic mycelia with mixed nuclei (e.g. *Trichoderma*) and the resulting mycelia have mixed properties. Problems might arise in cases where spores are multinucleate or where there is recombination among nuclear chromosomes. Further generations will carry the heterokaryotic, complemented properties. In a multigeneration experiment with hundreds of millions of (uninucleate) spores, no recombination was observed among complementing nuclei that enabled a heterokaryon of two different *Trichoderma* auxotrophs to live on minimal media. No spores formed on these heterokaryons could exist on minimal medium (Galun, E., unpublished). This demonstrates that even within the same species there can be seemingly impenetrable barriers to prevent recombination with 'alien' genomes, even when the traits could be beneficial or vital for existence.

Imperfect fungi have less capacity to transfer traits than perfect (sexual) fungi. However, it is impossible to 'prove' that an imperfect fungus does not have a rare sexual form that appears only in special conditions, as discussed above.

Summary

Those interested in risk assessment would be advised to set up a mechanism to formally answer a series of questions that will assist in categorizing risk. Such 'decision trees' have been constructed for evaluating the analogous situation for the introgression of transgenic traits from crops to related weeds³⁶. It is imperative that containment³⁷ is considered when working with transgenic hypervirulent organisms that can spread, until they are transgenetically mitigated or otherwise deemed safe for release into the environment.

Transgenic biocontrol agents have much to offer agriculture, human health and welfare. There are many cases where a minimum of anti-introgressional failsafe methods introduced into asporogenic deletion mutants will lower the risk to an infinitesimal level. These failsafe mechanisms should provide a modicum of safety and containment that is much greater than the risks of the present use of spore forming but inefficient non-transgenic biocontrol agents. The use of failsafe methods that contain the elements previously described might allow a more acceptable level of risk from transgenic biocontrol agents, and enable their increased use to augment and partially replace chemical pesticides.

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