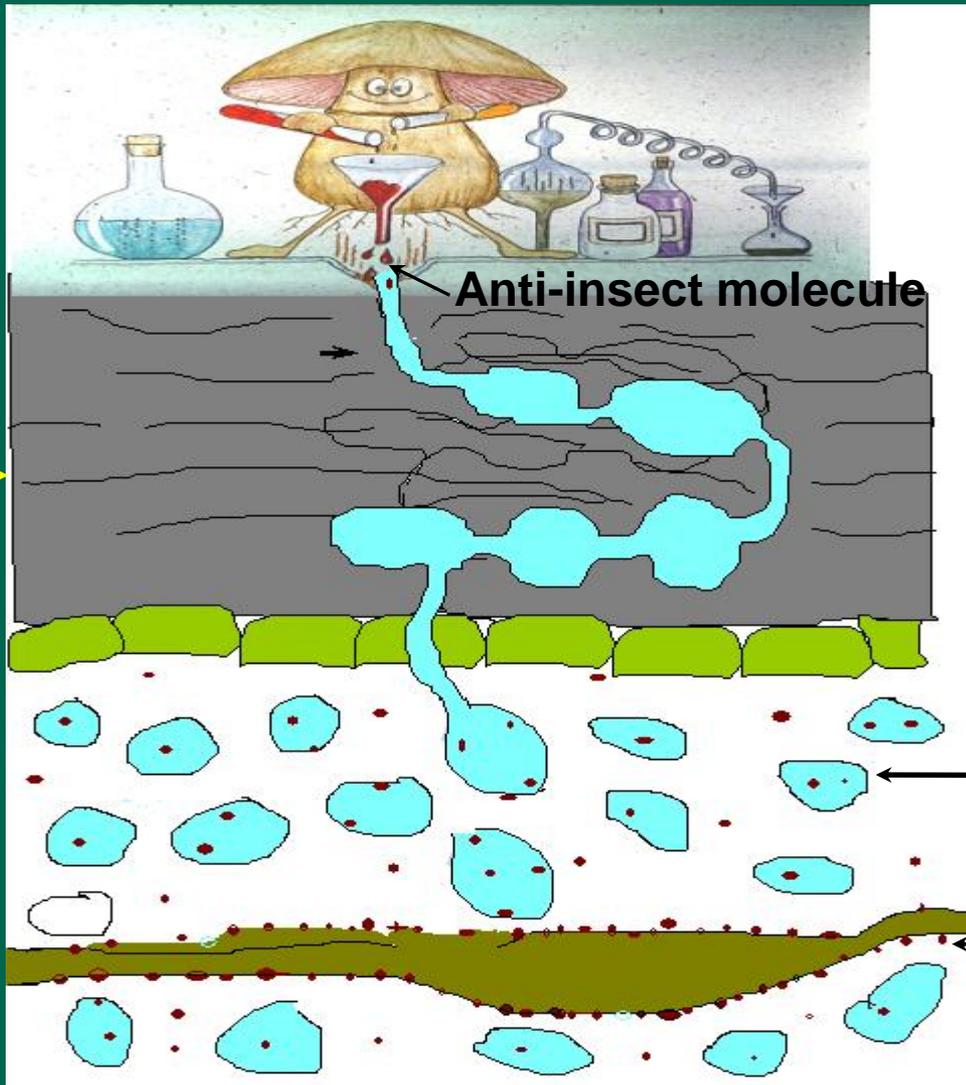




Metarhizium robertsii infecting giant cockroach



Insect cuticle →

Haemocoel →

Insect organ →

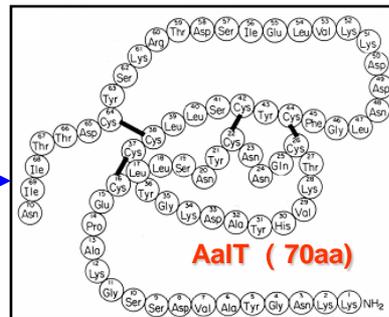
Anti-insect molecule

Blastospores

Anti-insect molecule

Use entomopathogenic fungi to deliver insecticidal molecules

Metarhizium genetic engineering

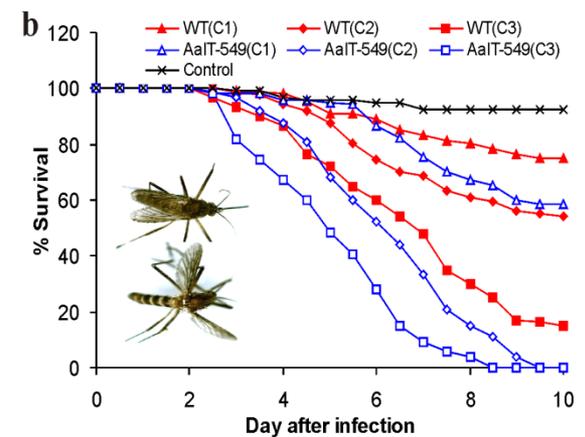
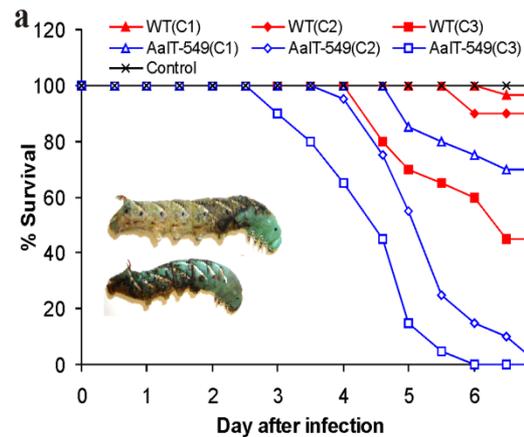
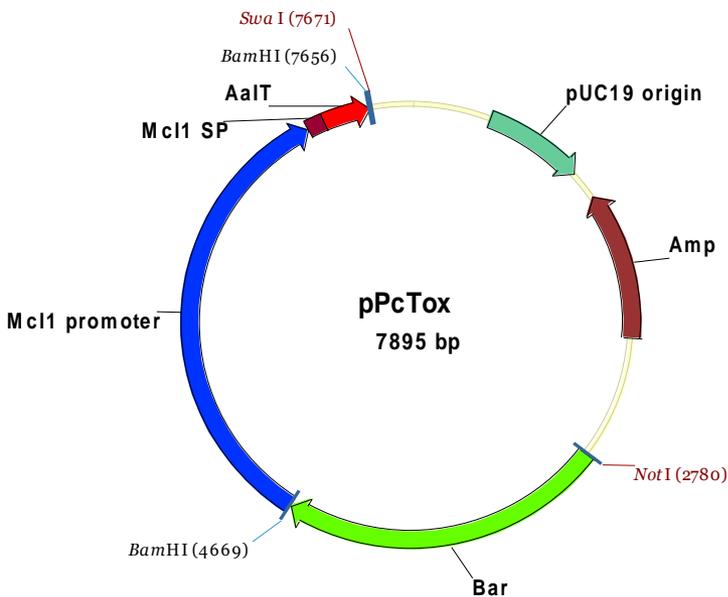


A

```

1  CCGGATCCGTTTCATGGAACATCACACTCGCTGACTCTGGACACCAACTGTATTCACTCG
   (BamHI)
61  CTAARTCCGTTCCCTGGCTCAAATCTTTTCGTTCCCTAGACCATCATGCGTGAACCTTCT
   M R E L S
121 TCGGTTCTCGCCCTTTCGGGCTTTCGTCGGCCCTGGCGTAAAGAAAGAACGGCTACGCC
   S V L A L S G L L A L A S A K K N G Y A
181 GTCGATAGCAGCGGC AAGGCCCCCGAGTGCCTGCTGAGCACTACTGCAACCAACCAAGTGC
26  V D S S G K A P E C L L S N Y C N N Q C
241 ACCAAGTCCACTACGCCGATAAGGGCTACTGCTGCCTGCTGAGCTGCTACTGCTTCGGCC
46  T K V H Y A D K G Y C C L L S C Y C F G
301 CTGAACGATGATAAG AAGGTCTTGGAGATCAGCGATACCCGTAAGAGCTACTGCGATACC
66  L N D D K K V L E I S D T R K S Y C D T
361 ACCATCATCAACTAAGGATCCCG
86  T I I N * (BamHI)
    
```

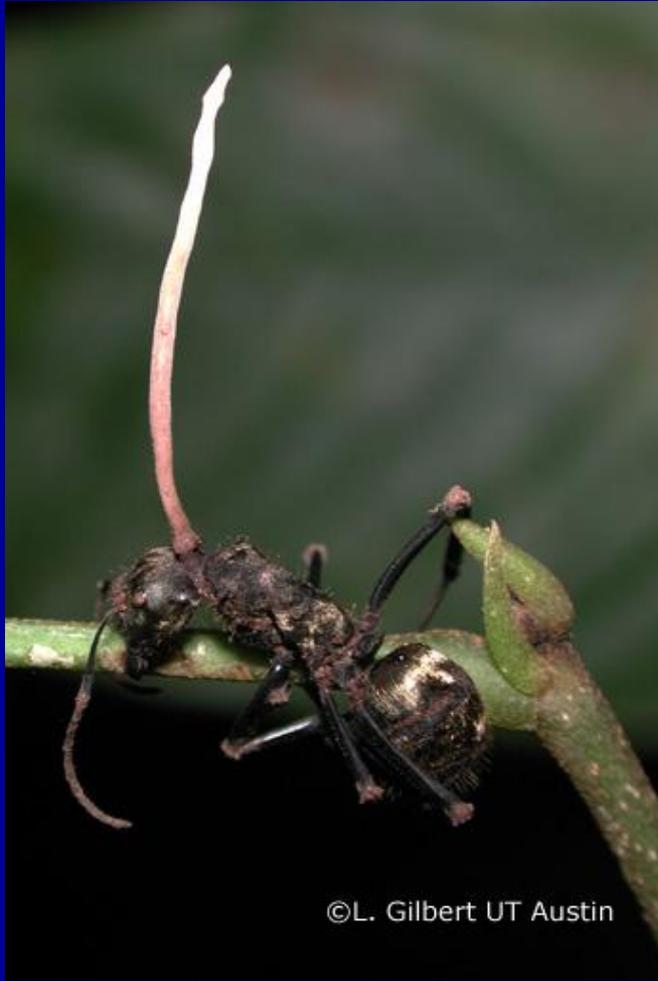
Gene synthesis



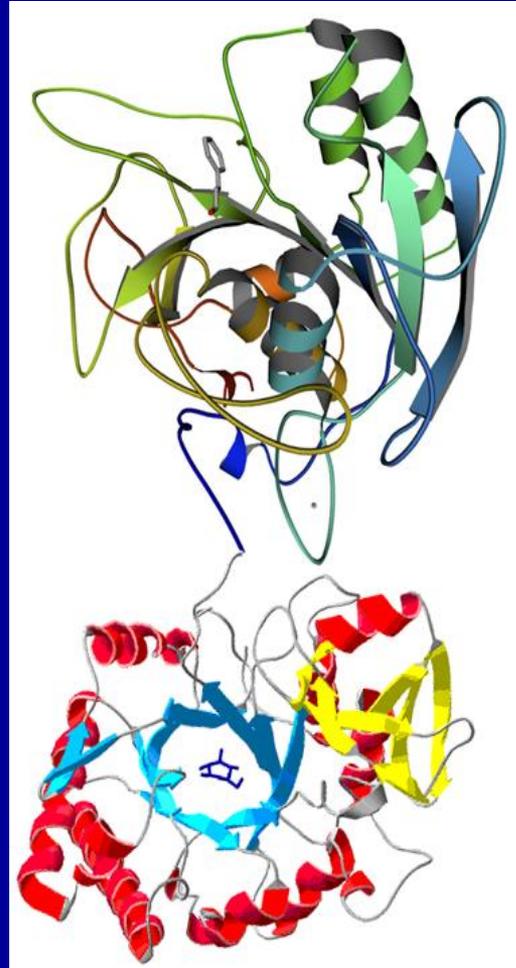
Bioassays

Wang and St. Leger Nature Biotechnology, 2007. 25:1455.

Stacking genes into *Metarhizium*



©L. Gilbert UT Austin



Lqh-dprIT3 (ED50 on blowflies is 3-5ng/100mg body weight).

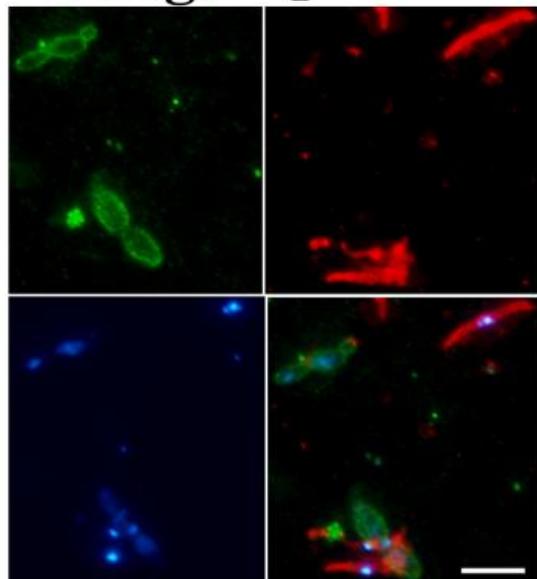


Hirsutellin A-similar to well-characterized ribosomal inhibiting proteins (inhibits protein synthesis)

Effectors	Source	Targets
Scorpine	<i>Pandinus imperator</i>	Toxic to Plasmodium
SM1	Synthesized	Blocks sporozoite invasion of salivary glands
PfNPNA	Human	Agglutinates Plasmodium
SM1:Scorpine	Synthesized	Synergistic or additive effects between SM1 and Scorpine

A

Fungi Sporozoite



DAPI

Merge

B

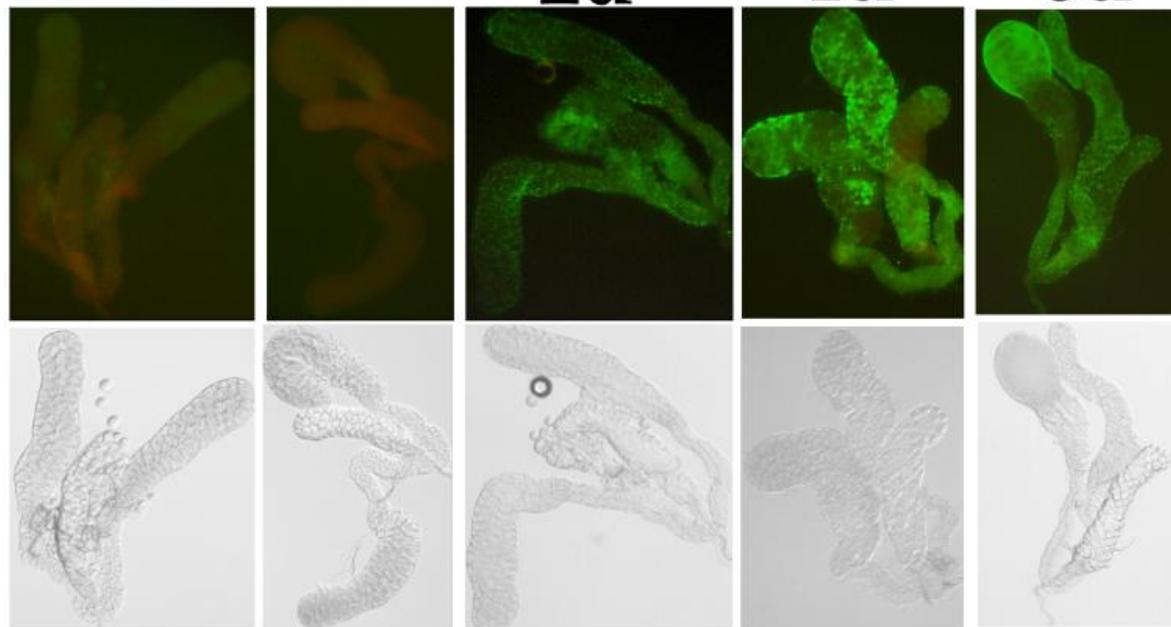
Control WT

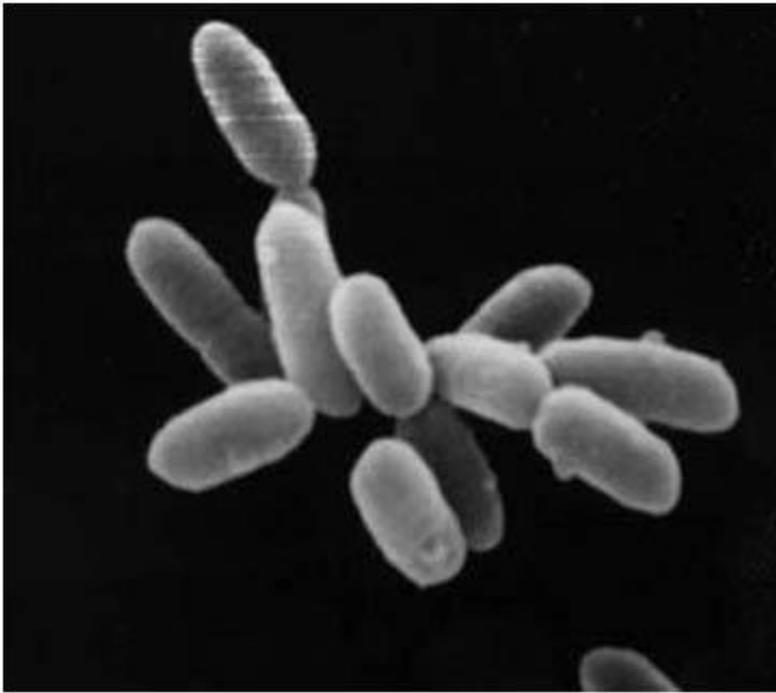
Ma-[SM1]₈

2d

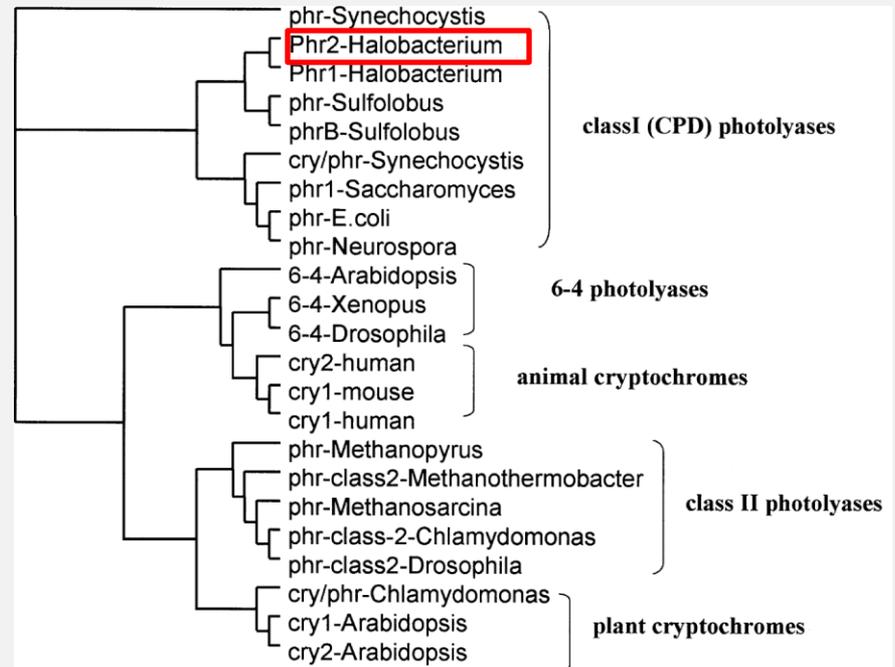
4d

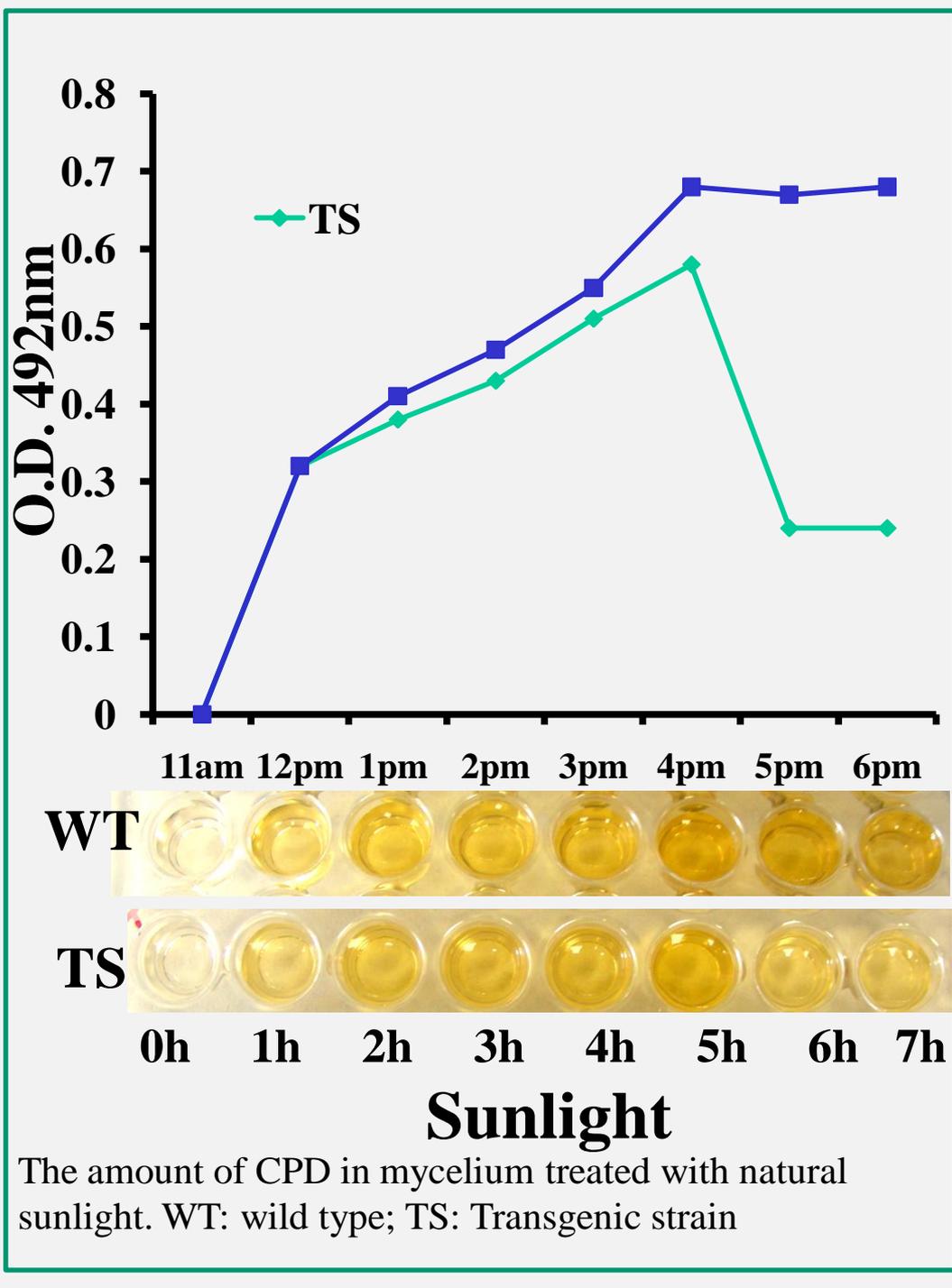
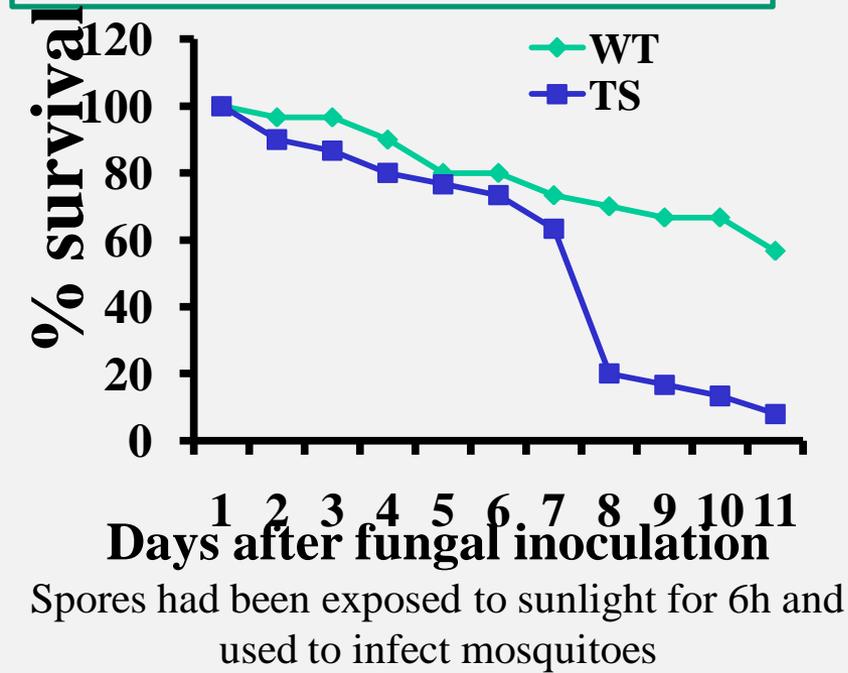
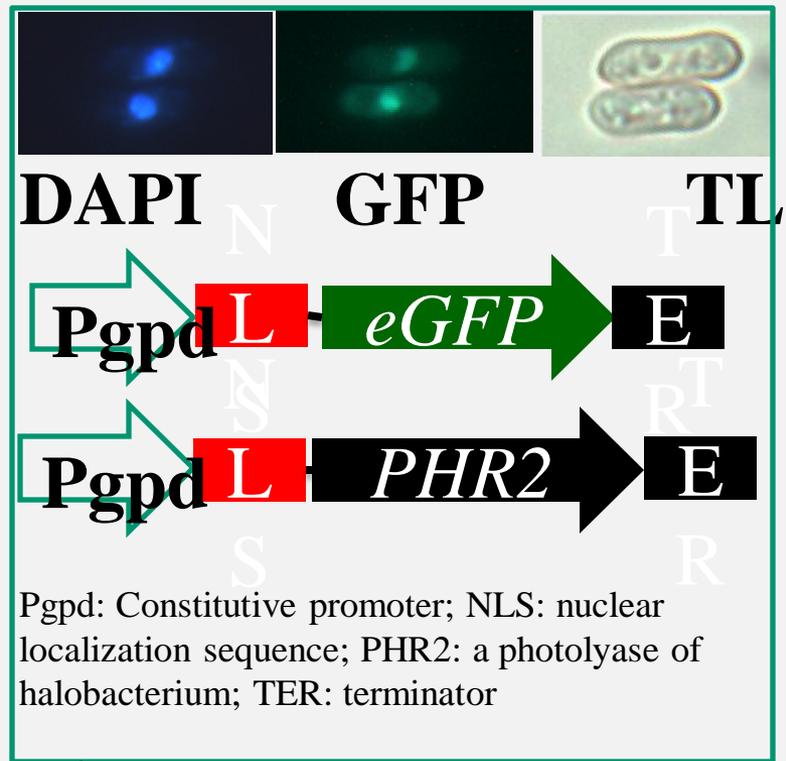
6d





Halobacterium tolerates high levels of sunlight in its natural environment. UV causes dimerization between adjacent pyrimidines. It was shown that *Halobacterium* is extremely efficiently photoreactivated, and survival is restored to near 100% after relatively high UV doses. The principal protective enzyme is a photolyase





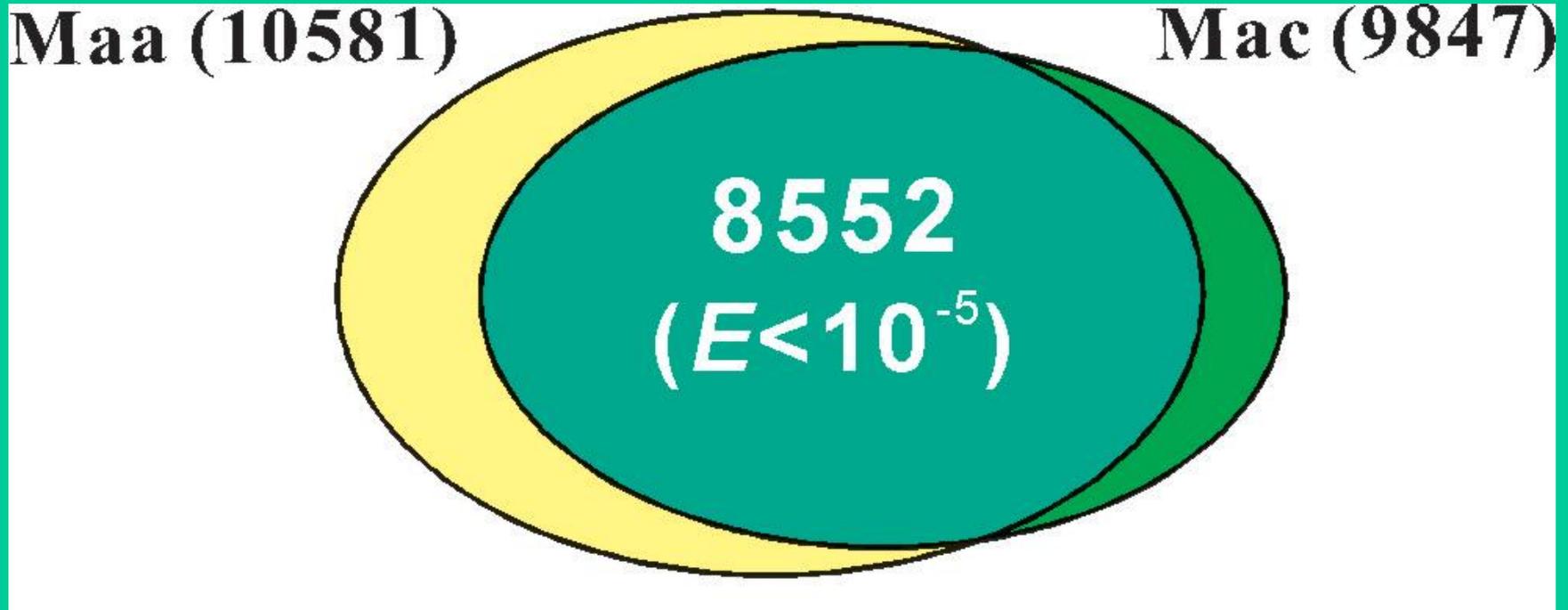
It will soon be possible to engineer microbes that show narrow specificity for target pests and that persist in the environment, providing sustainable cheap control for much longer periods than existing chemicals.

From the point of view of risk assessment, the issue of strain mutability and stability will be crucial to these endeavors

A science based regulatory system currently has three requirements for enhanced biocontrol agents:

- limited off site dispersal,
- poor long term persistence,
- limited possibility of recombination between pathogens (Gressel, 2007).

Metarhizium homologous gene overlap



Multitudes of mutational differences related to virulence or host range are found in *Metarhizium* species adapted to different hosts. The number of mutations required for a non pathogenic fungi to attain virulence or for a pathogen to change host range may make it almost impossible to achieve in human times, and such changes are only likely on an evolutionary time scale (50,000 years in *F. oxysporum*)

RNA binding proteins mediate the ability of a fungus to adapt to the cold

Weiguo Fang* and Raymond J. St. Leger

University of Maryland, Department of Entomology, 4112
Plant Sciences Building, College Park, MD 20742, USA.

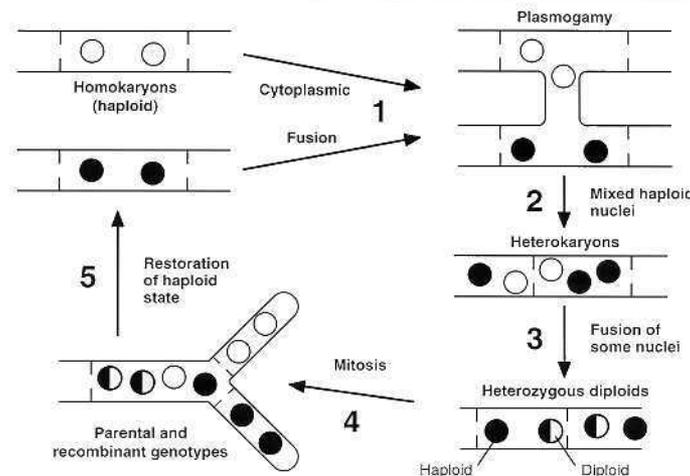
Summary

Little is known about how fungi adapt to chilling. In eubacteria, cold shock proteins (CSPs) facilitate translation by destabilizing RNA secondary structure. Animals and plants have homologous cold shock domains within proteins, and additional glycine-rich RNA binding proteins (GRPs), but their role in stress resistance is poorly understood. In this study, we identified GRP homologues in diverse fungi. However, only *Aspergillus clavatus* and *Metarhizium anisopliae* possessed cold shock domains. Both

biology, they cannot elucidate the mechanisms involved in major fungal lifestyles they do not share. This matters because the fungal kingdom, with an estimated 1.5 million different species, displays extraordinary evolutionary diversity. This is reflected in different life histories, developmental processes and ecological niches that make them central to every terrestrial ecosystem on our planet.

Many fungi have the capacity to adapt to cold temperatures and become freeze-tolerant and these are very important physiological factors affecting their overall distribution (Robinson, 2001). The ecological consequences of cold-adapted mycelia include continuing symbiotic and pathogenic relationships, and rapid exploitation of nutrients made available in spring (Tibbett *et al.*, 2002). To date, however, very little attention has been paid to the molecular mechanisms by which fungi adapt to

The possibility that transgenes will move by horizontal transfer into fungi in other families or kingdoms is remote. *Metarhizium* CYP is 86–92% similar to *Pseudomonas* CYP proteins.



Emergence of a new disease as a result of interspecific virulence gene transfer

Timothy L Friesen¹, Eva H Stukenbrock², Zhaohui Liu³, Steven Meinhardt³, Hua Ling⁴, Justin D Faris¹, Jack B Rasmussen³, Peter S Solomon⁵, Bruce A McDonald² & Richard P Oliver⁵

New diseases of humans, animals and plants emerge regularly. Enhanced virulence on a new host can be facilitated by the acquisition of novel virulence factors. Interspecific gene transfer is known to be a source of such virulence factors in bacterial pathogens (often manifested as pathogenicity islands in the recipient organism¹) and it has been speculated that interspecific transfer of virulence factors may occur in fungal pathogens². Until now, no direct support has been available for this hypothesis. Here we present evidence that a gene encoding a critical virulence factor was transferred from one species of fungal pathogen to another. This gene transfer probably occurred just before 1941, creating a pathogen population with significantly enhanced virulence and leading to the emergence of a new damaging disease of wheat.

Stagonospora (syn. *Septoria*, teleomorph *Phaeosphaeria*) *nodorum* is a major wheat pathogen in many parts of the world causing *Stagonospora nodorum* blotch of wheat⁹. Quantitative trait loci (QTLs) for resistance to *S. nodorum* have been found on many chromosomes in different wheat genotypes. Recent evidence has shown that proteinaceous toxins contribute to pathogenicity^{10,11}. Semipurified preparations of culture filtrates induced necrosis in appropriate wheat cultivars, and genetic analysis of the reaction indicated collocation of toxin-insensitivity loci with QTL for disease resistance.

A homolog of *P. tritici-repentis* *ToxA* in the *S. nodorum* genome

A search of the recently acquired genomic sequence of *S. nodorum* identified a predicted gene (SNU16571.1) with 99.7% similarity to *P. tritici-repentis* *ToxA*. For comparison, the genes for glyceraldehyde

The parasexual cycle is a mechanism for hybridization. The barriers blocking vegetative fusion of different fungi include vegetative incompatibility, which results from heterokaryon incompatibility proteins that block exchange of DNA.

M. acridum has fewer (25 genes) heterokaryon incompatibility proteins than *M. robertsii* strain 23 (35 genes), so *M. acridum* may be less reproductively isolated than *M. robertsii*.

However,

it is likely that *M. acridum* with its more specialized lifestyle and narrow environmental range encounters fewer genetically distinct individuals than the more opportunistic *M. robertsii*.

Gene functional description	<i>A. nidulans</i>	<i>N. crassa</i>	<i>M. anisopliae</i>	<i>M. acridum</i>
ABC transporter required for a-factor transport	AN2300	NCU07546	MAA_05864	MAC_03697
Amino acid permease involved in sexual differentiation	AN5678	NCU05830	MAA_04108	MAC_08710
APC component	AN0905	NCU00494	MAA_03573	MAC_08351
APC component	AN8002	NCU01377	MAA_01547	MAC_02072
APC component	AN2772	NCU05901	MAA_04652	MAC_09483
APC component	AN4735	NCU01963	MAA_03722	MAC_05347
APC component	AN8013	NCU01174	MAA_09174	MAC_05975
APC regulator	AN2965	NCU01269	MAA_03661	MAC_06443
APC regulator	AN2965	NCU01269	MAA_03661	MAC_06443
APC regulator	AN0814	NCU02616	MAA_00735	MAC_06221
Ascospore lethal-1	AN2911	NCU01345	MAA_02972	MAC_03844
Ascospore maturation-1	AN5836	NCU01414	MAA_02988	MAC_03829
Ascus development; rhamnogalacturonase B	AN7135	NCU05598	—	—
Ascus development-4	AN6221	NCU07039	MAA_06419	MAC_07065
ATP-dependent efflux pump for a-factor like pheromone	AN2300	NCU07546	MAA_05864	MAC_03697
Binuclear zinc transcription factor	AN5170	—	MAA_05237	MAC_05412
B-type cyclin	AN2137	NCU01242	MAA_07313	MAC_00492
CAAX prenyl protease a-factor C-terminal processing	AN6528	NCU11314	—	MAC_01887
Chromosome segregation, kinetochore-associated Nd80 complex	AN4969	NCU03899	MAA_02015	MAC_03415
Dipeptidyl aminopeptidase alpha-factor processing STE13	AN2946	NCU02515	MAA_03364	MAC_07820
Dipeptidyl aminopeptidase for a-factor processing STE23	AN8044	NCU00481	MAA_05780	MAC_03112
DNA-binding helix-hairpin-helix protein, DNA strand exchange	AN0992	—	—	MAC_01106
Endoprotease for alpha-factor processing	AN3583	NCU03219	MAA_05263	MAC_02797
Fork head domain TF, meiotic regulator	AN8858	NCU06173	MAA_05810	MAC_03141
GATA-transcription factor	AN3152	NCU01154	MAA_05601	MAC_01133
GEF involved in conjugation; related to Cdc25	AN2130	NCU06500	MAA_03615	MAC_06489
GTP binding (alpha-1 subunit) involved in conjugation	AN3090	NCU06729	MAA_05603	MAC_01131
HMG-box TF, target of pheromone signaling	AN3667	NCU09387	MAA_00248	MAC_04576
Inducer of meiosis, S/T kinase	AN6243	NCU01498	MAA_05403	MAC_01790
Kinesin-like motor required for karyogamy	AN6340	NCU04581	MAA_04756	MAC_02000
MADS-box domain TF, pheromone receptor activator	AN8676	NCU07430	MAA_07379	MAC_00861
Mating response protein POI2	—	NCU05768.4	MAA_06024	—
Mating type mat A-2	—	NCU01959	—	—
Mating type mat A-3	—	NCU01960	—	—
Mating-type mat A	AN4734	NCU01960	MAA_03719	MAC_05350
Mating-type mat B (mat A-1)	AN2755	NCU01958	MAA_03718	—
Mating-type M-specific polypeptide Mc, HMG-box TF	AN1962	NCU03481	—	MAC_07229
Mating-type switching protein swi10	AN4331	NCU07066	MAA_02005	MAC_03408
Meiosis-specific transcriptional activator	AN6015	NCU09915	MAA_06439	MAC_08428
Meiotic B-type cyclin	AN2137	NCU01242	MAA_07313	MAC_00492
M-factor farnesyl cysteine carboxyl methyltransferase	AN6162	NCU00034	MAA_07087	MAC_07177
Microtubule-binding protein	AN2862	NCU00243	MAA_09680	MAC_09803
Modulator of pheromone-inducible gene expression	AN8676	NCU07430	MAA_07379	MAC_00861
Mutase, cell wall turnover during sexual development	AN7349	—	—	—
Pheromone adaptation feedback response	AN7252	NCU00455	MAA_02467	MAC_01635
Pheromone precursor (alpha-factor like)	AN5791	—	—	—
Pheromone Receptor (for a-factor like pheromone)	AN7743	NCU00138	MAA_05941	MAC_00610
Pheromone Receptor (for alpha-factor like pheromone)	AN2520	NCU05758	MAA_00341	MAC_02467
Repressor of b mating type regulated genes	AN8211	NCU01238	MAA_01434	MAC_04053
Required for synaptonemal complex formation	AN8259	NCU01120	—	—
RNA binding protein required for meiotic recombination	AN0900	NCU00768	MAA_07476	MAC_07476
RNA-binding protein involved in meiosis	AN6494	NCU00118	MAA_03639	MAC_06466
RNA-binding protein involved in meiosis, SpMei4 target	AN7700	NCU00556	MAA_03790	MAC_00971
SAM domain, similar to Sc STE50	AN7252	NCU00455	MAA_02467	MAC_01635
Serine carboxypeptidase, degrades extracellular P-factor	AN2555	NCU06720	MAA_09950	MAC_02763
Serine/threonine protein kinase, negative regulator of meiosis	AN4935	NCU04990	MAA_09884	MAC_02282
Signalosome subunit 4, regulation of sexual development CsnD	AN1539	NCU07361	MAA_04103	MAC_03079
Signalosome subunit 5, regulation of sexual development CsnE	AN2129	NCU00467	MAA_08893	MAC_05426
Spindle pole body component	AN5618	NCU09871	MAA_02280	MAC_03427
Sporulation regulated septin	AN4667	NCU02464	MAA_10305	MAC_04367
Sporulation-specific enzyme required for spore wall maturation	AN2705	—	MAA_09988	—
Velvet activator VeA	AN1052	NCU01731	MAA_01811	MAC_00039

Sexuality-related genes in *Aspergillus nidulans* and *Neurospora crassa* are retained in *Metarhizium robertsii* and *M. acridum*. These include putative α -mating type genes and genes similar to a high mobility group (HMG) mating type gene, suggesting a potential to be either self (homothallic) or non-self (heterothallic) fertile.

The number of putative transposases in the *M. acridum* genome is 5-fold less than *M. robertsii*. This could result from RIP introducing CpG to TpA transitions in duplicated sequences during the sexual cycle. A RIP bias was observed in *M. acridum* (RIP index, 2.17) but not in *M. robertsii* (1.09)

One Fungus, Two Lifestyles: Generalist pathogen and plant symbiont

Generalist strains of *Metarhizium* spp infect a wide range of insects such as grubs, crickets, termites, beetles and caterpillars

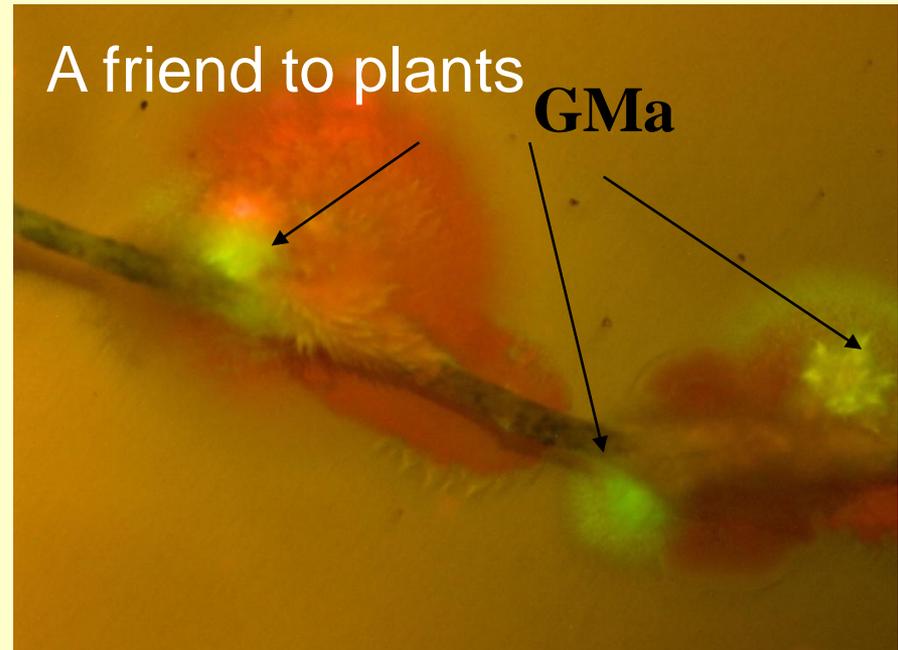
Unlike specialists, they also live at the rhizosphere of plants including **grasses**, beans, corn, onions, trees and cabbage

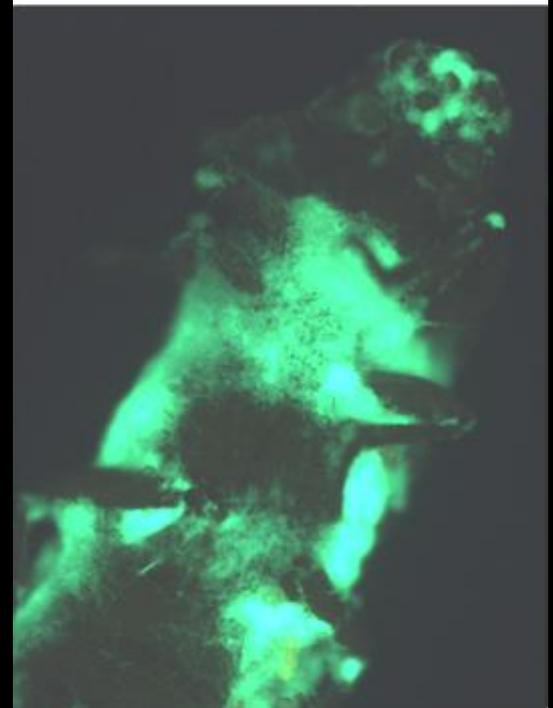
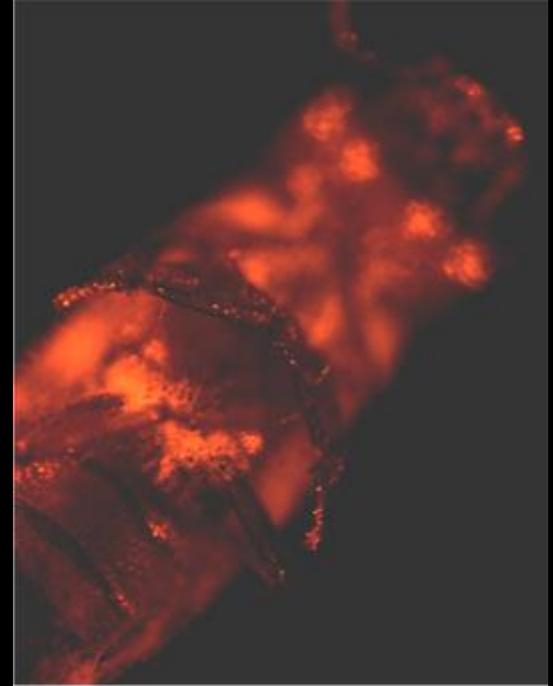
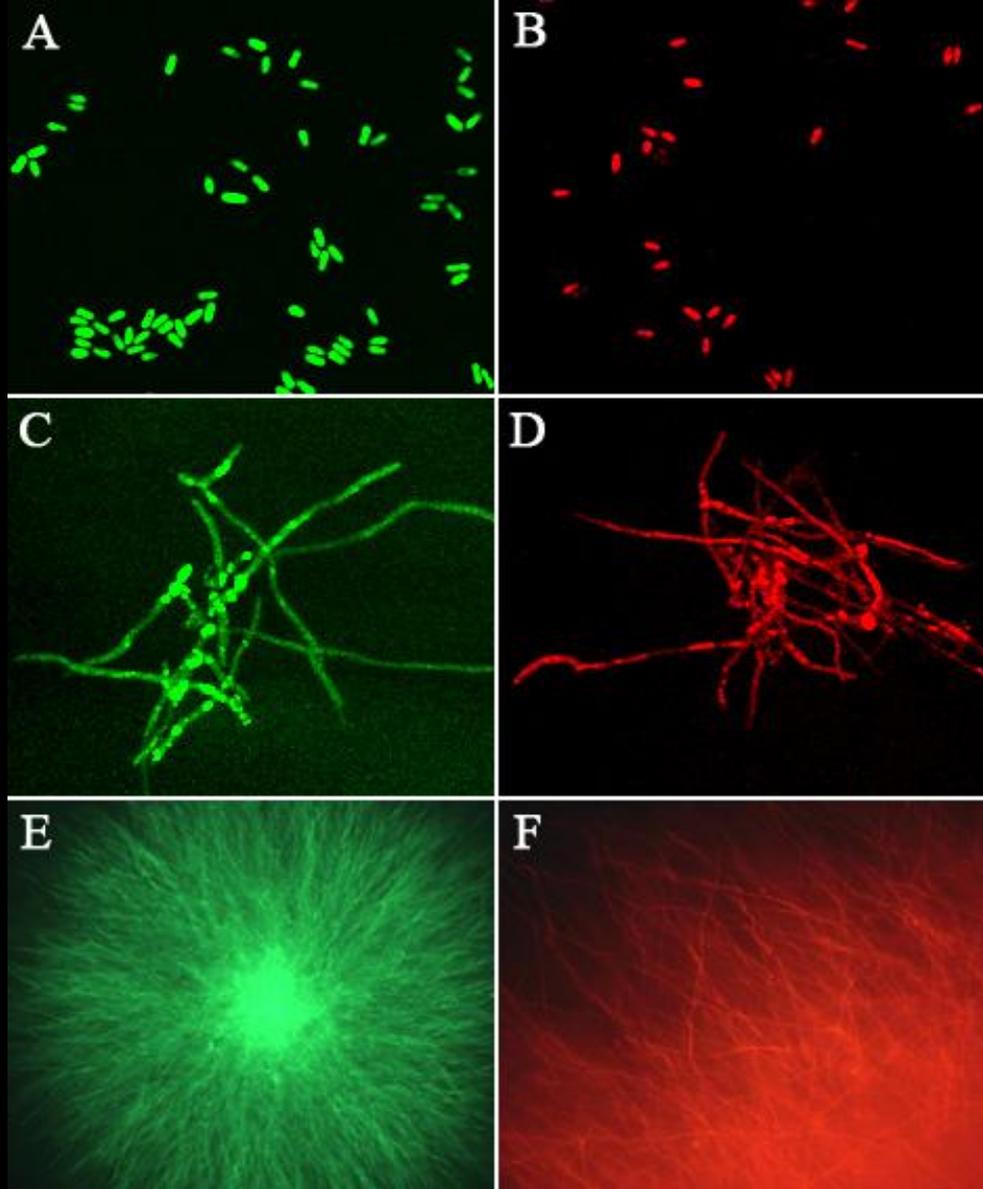
A foe to insects



<http://spatafora.science.oregonstate.edu>

A friend to plants





Tribolium infected in the laboratory with RFP-2575 and GFP-2575 showing how this technique enables us to detect infections by different strains



Field study to compare the biology of wild type *M. robertsii* strain 2575 with transgenic strains

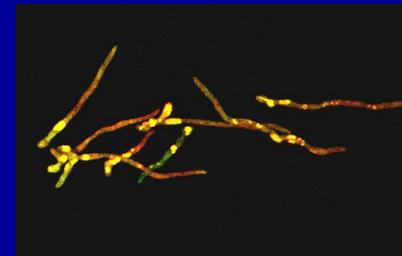
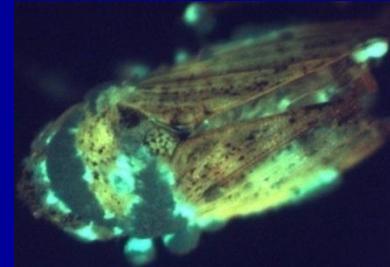
a) Δ Mcl1-reduced virulence

- co-expressing rfp



b) Δ MAD2-reduced ability to adhere to plant surfaces.

- co-expressing gfp

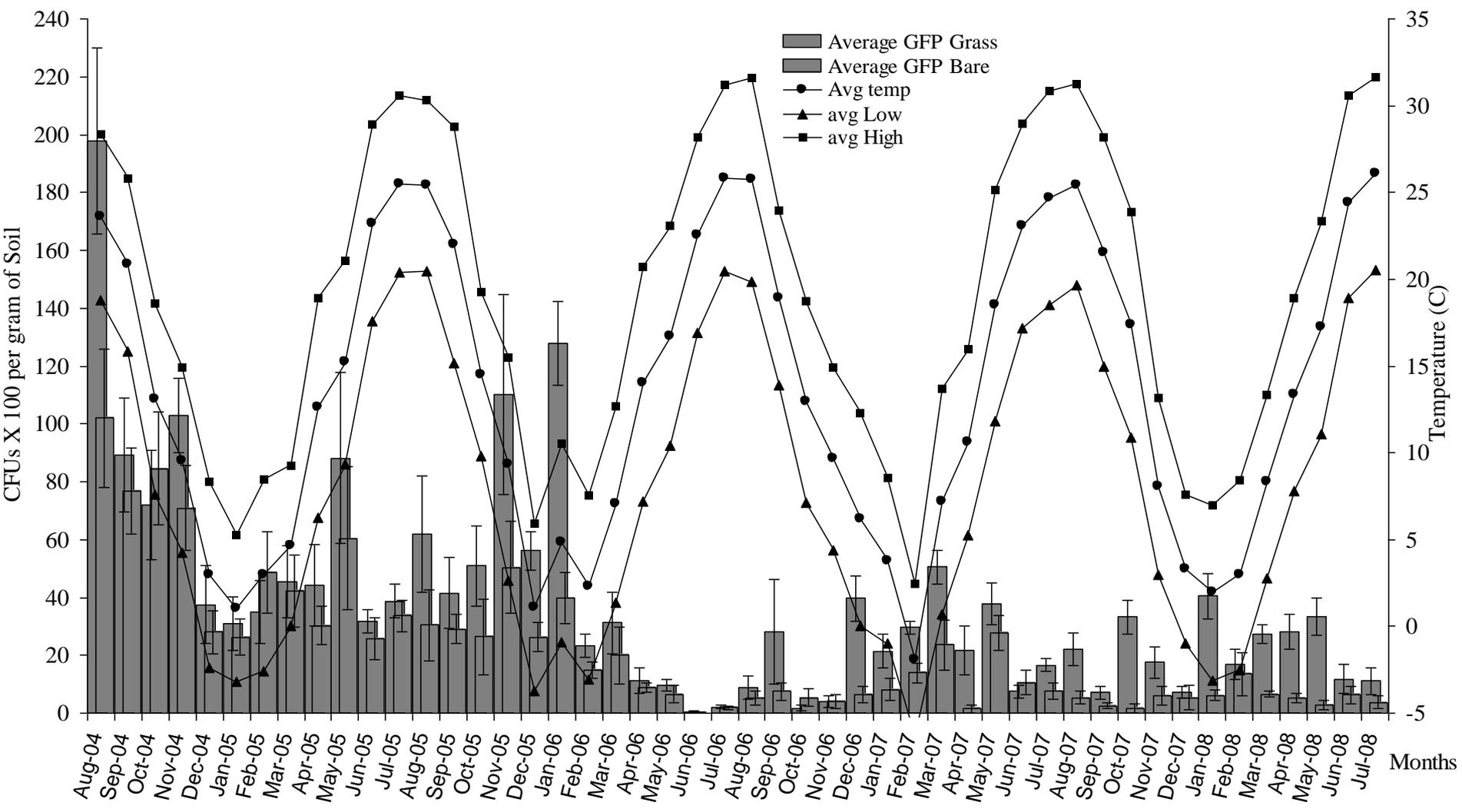


c) co-expressing GFP/RFP

- to identify colonies that have lost a marker

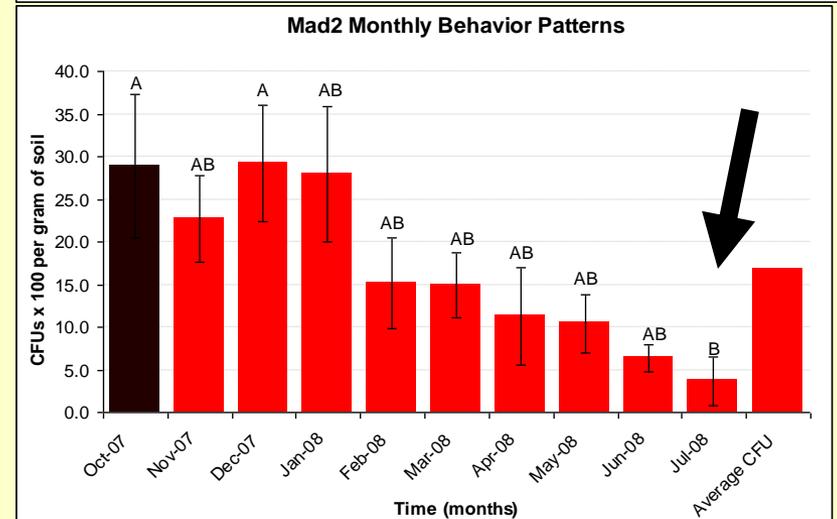
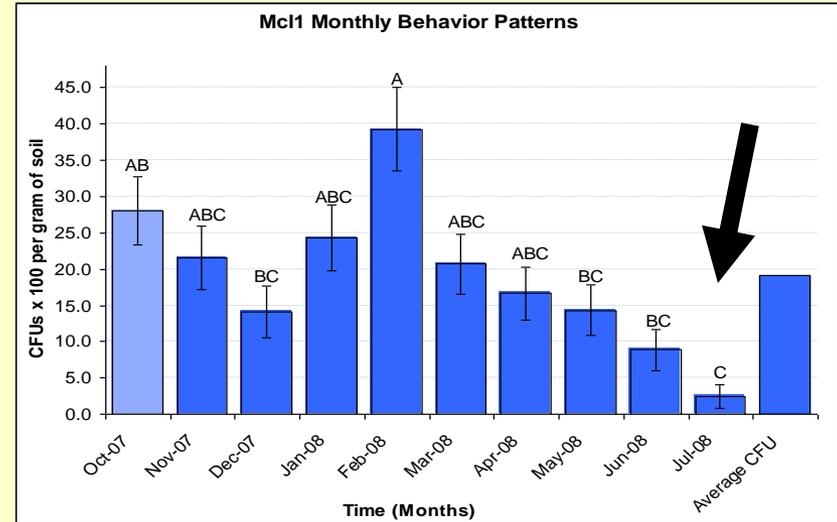
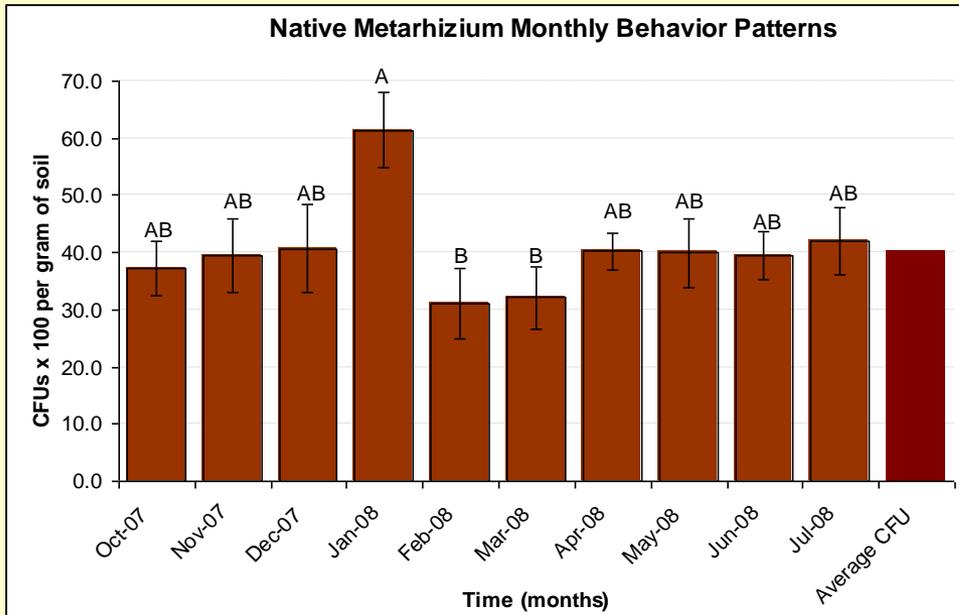
2) Monitor and compare indigenous *M. anisopliae* population structure to introduced populations

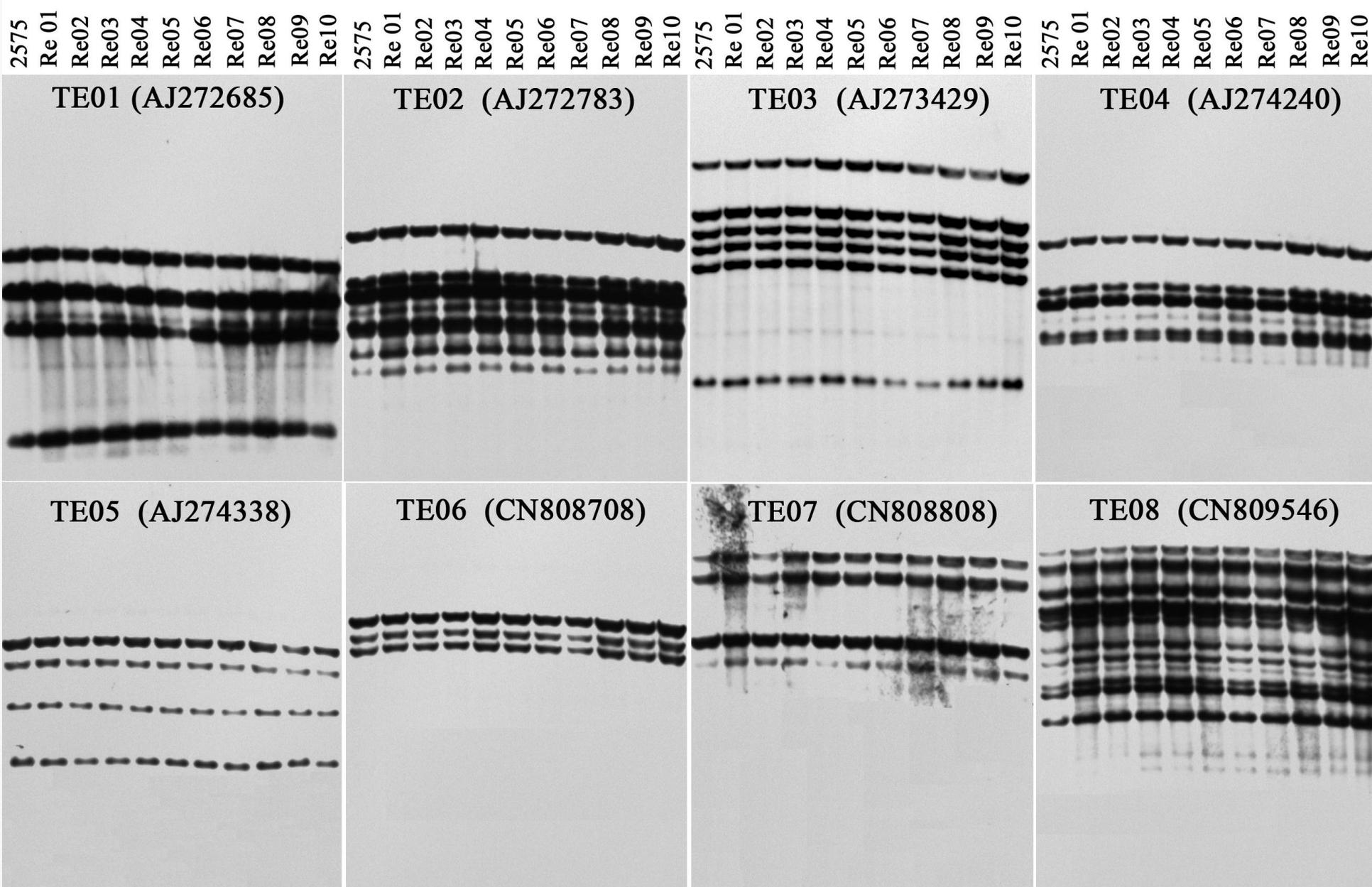




Cycling Patterns

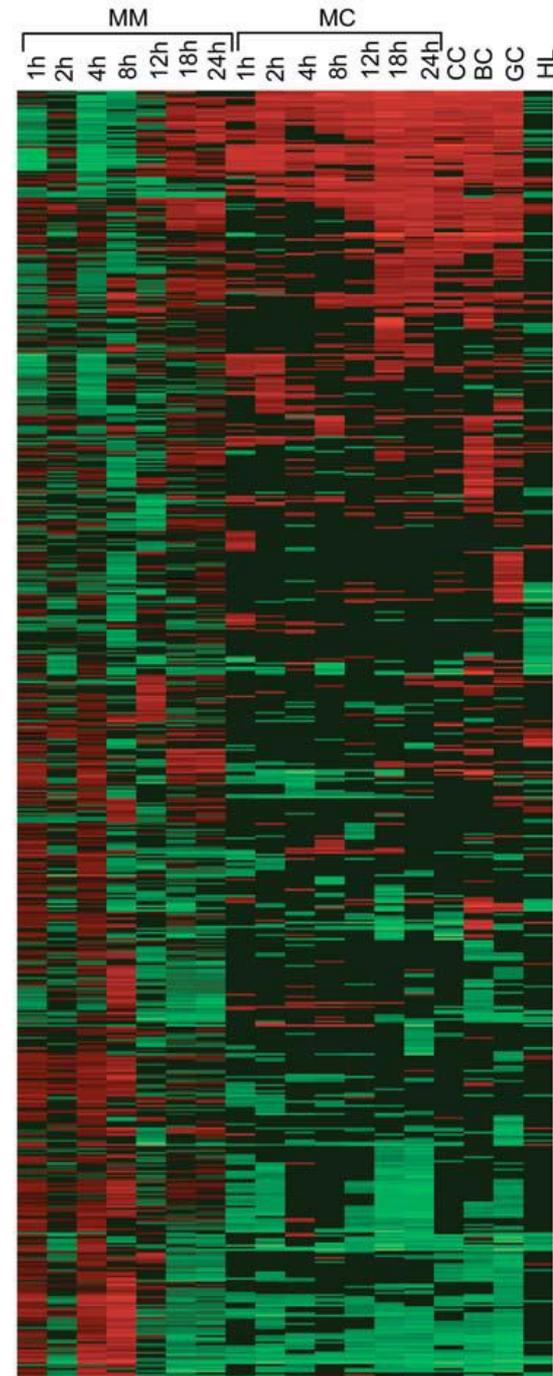
Rhizosphere competence helps account for *Metarhizium* being so common



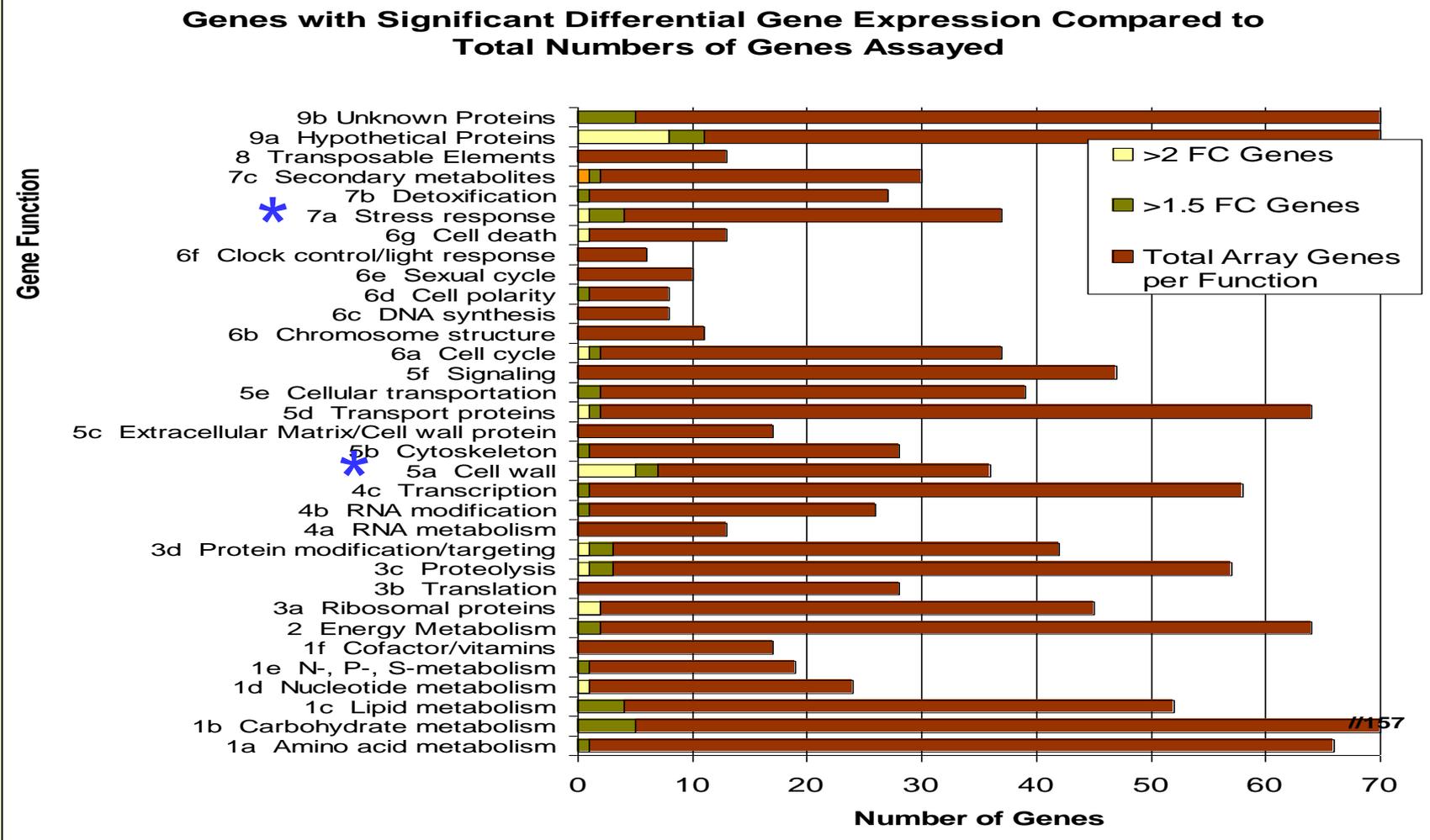


Southern Blot Images for Ten Recovered Isolates using eight transposable elements

Several fundamental issues regarding selection are poorly understood. When organisms adapt by natural selection to “new environments” do they do so because of changes in few genes or many? Can these genes be identified? Are the same genes involved in independent cases of adaptation to the same environment? We are attempting to picture the distribution and timescale over which genetic variation in traits can occur in a microbe in field conditions. This knowledge is fundamental to our understanding of how large the mutational and selective effects can be on an introduced or transgenic biocontrol agent.

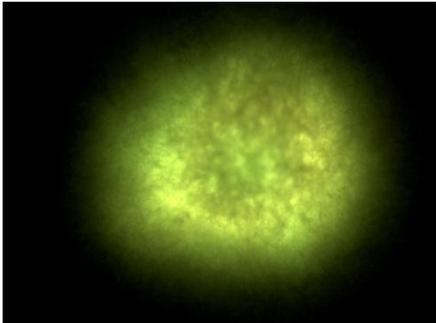


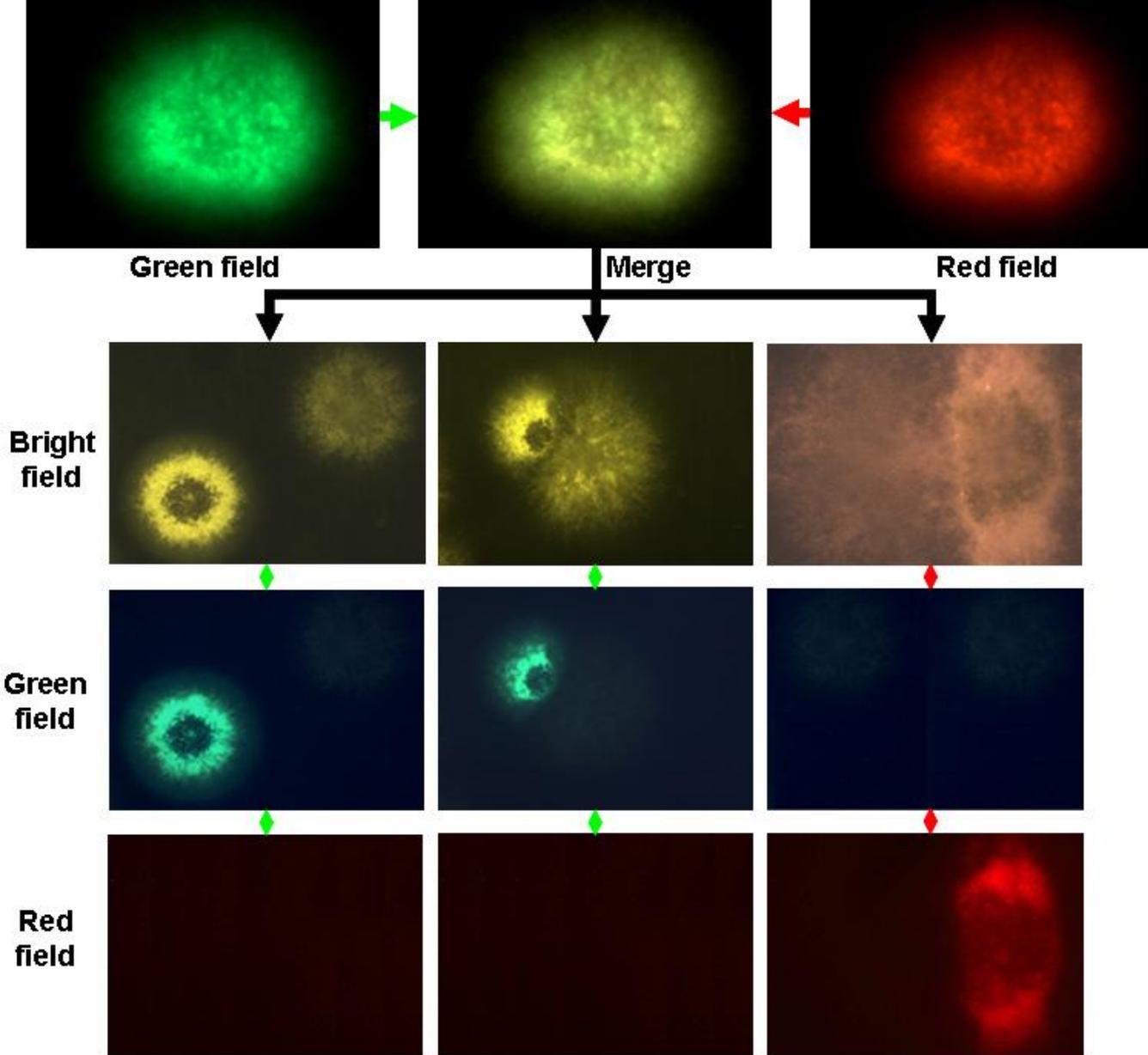
Microarrays were used to measure genetic changes (mutability) after 3.5 years in field conditions-mutations in many different loci can effect abundance of any one transcript and a single mutation in a regulatory loci can effect many genes. Recovered isolates differed from the input strain by an average of 0.28% of the genes. Stress and cell wall genes were disproportionately altered in expression. Genes effecting pathogenicity were highly conserved.



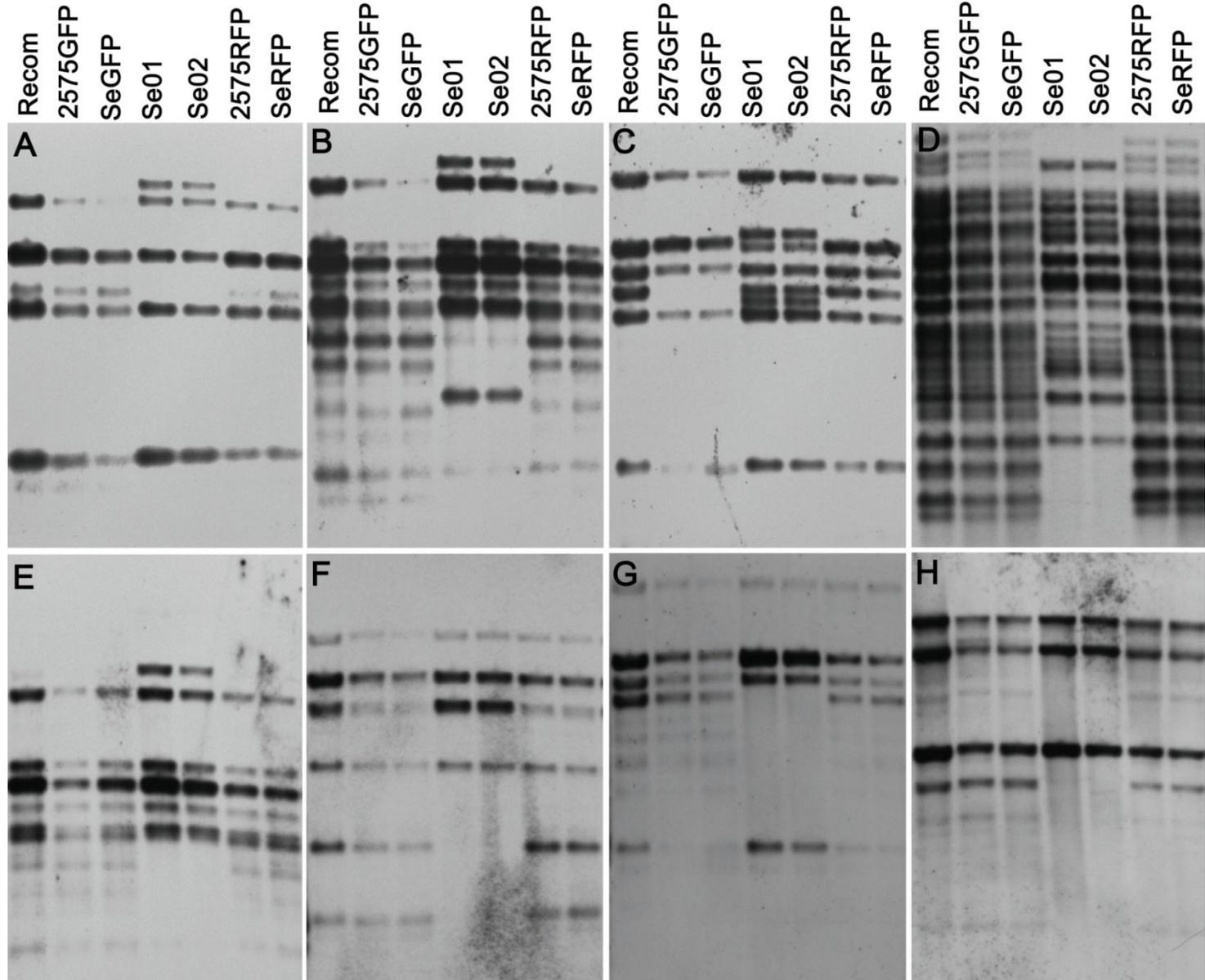
Genetic recombination of *Metarhizium anisopliae* in turfgrass field site

Treatments	Isolates assayed	Percentage of recombinants
Ma2575GFP×Ma2575RFP	8091	1.2 %
Ma2575GFP+RFP that have lost RFP or GFP	12682	0





Colonies expressing both *gfp* and *rfp* fluoresce in both red and green field, and appear yellow when the pictures are merged. This figure shows an unstable diploid recombinant from GFP-Mad2 RFP-Mcl1 cross (isolated from the field) giving rise to parasexual segregants



Heterokaryon formation with latter nuclear and then parasexual recombinations; parasexual recombinations and/or replication of transposable elements could have produced the extra bands in segregants

Genetic exchange in *Metarhizium anisopliae* strains co-infecting *Phaedon cochleariae* is revealed by molecular markers

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² EMBRAPA/CENARGEN, SAIN Parque Rural, CP 02372, Brasília, DF, Brazil.

³ School of Biological Sciences, University Wales, Swansea, Singleton Park, Swansea SA2 8PP, UK.

⁴ School of Biological Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK.

Accepted 18 June 1999.

Recombination between two strains of *Metarhizium anisopliae*, subcultured on artificial medium and passed through an insect host was investigated. When grown on agar culture, or passed through a host, strains did not show detectable genetic changes. When passed through an insect in the presence of the other strain, genotypic changes in the isolates, as detected by esterase electrophoresis, restriction digestion of total genomic DNA and RAPD-PCR, were apparent. The instability of molecular markers suggests recombination between strains.

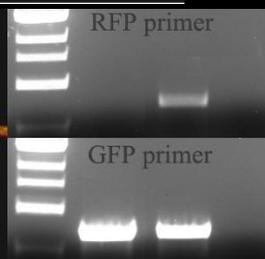
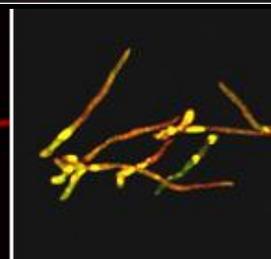
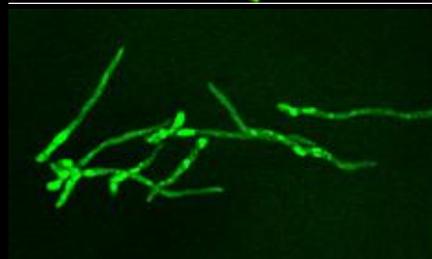
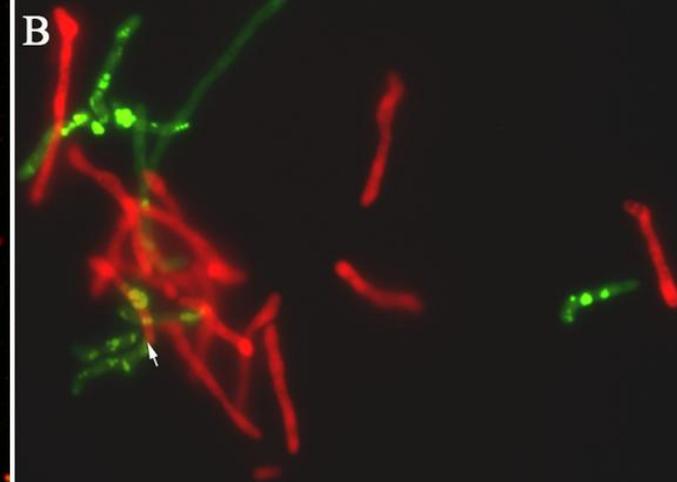
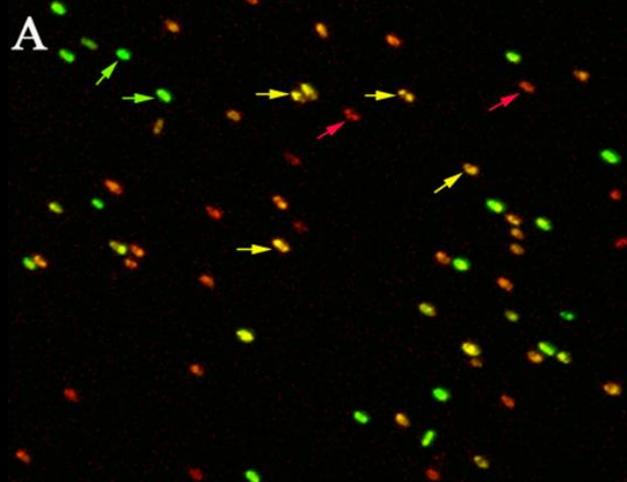
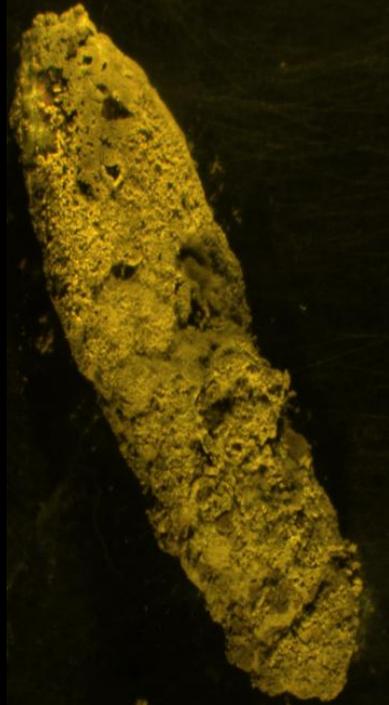
INTRODUCTION

Wild isolates of *Metarhizium anisopliae* exhibit significant genetic variability (St Leger *et al.*, 1992; Fegan *et al.*, 1993; Cobb & Clarkson, 1993; Bidochka & Khachatourians, 1994; Leal *et al.*, 1994, 1997). The cause for this is largely unknown, but the parasexual cycle (only observed *in vitro*) is thought to be a major source of genetic variability in this species (Al-Aidroos, 1980; Messias & Azevedo, 1980). Diversity may also result from mutations, deletions, translocations, or the flow of extranuclear elements (Bainbridge, 1987; Kistler & Miao, 1992; St Leger *et al.*, 1992). We have previously

Because the profile generated by the DNA of a population of conidia from a co-infected insect could be confusing, and because two co-infecting fungal strains, both growing within the confines of the same insect body, may conceivably undergo some type of genetic exchange.

The main objective of this study was to evaluate the rate of co-infection, the stability of molecular markers and genetic exchange between two wild, genetically divergent strains, when inoculated on the same group of insects. We also examined the phenotypic and genetic stability of strains passed through a susceptible host and on agar culture.

The likelihood of gene transfer increases when two organisms are attacking the same target e.g., *Staganospora* and *Pyrenophora* co-infecting wheat



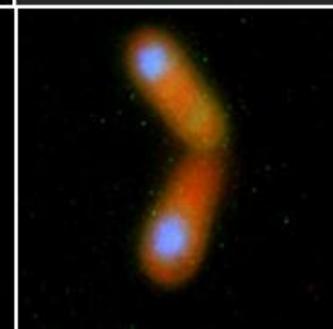
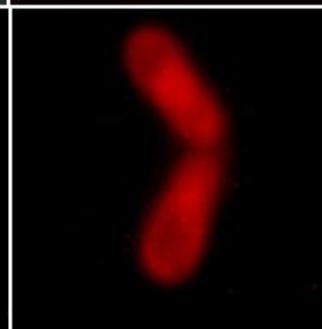
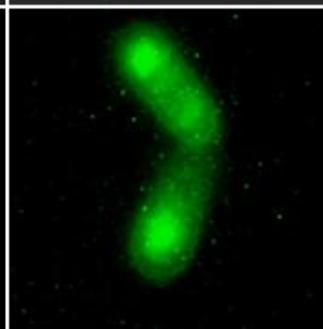
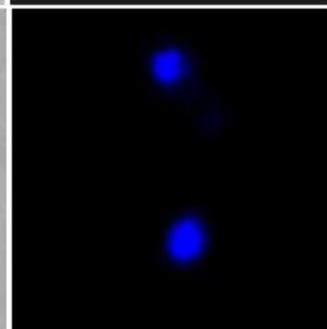
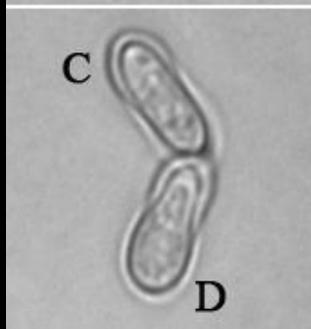
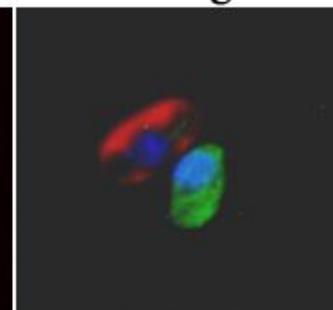
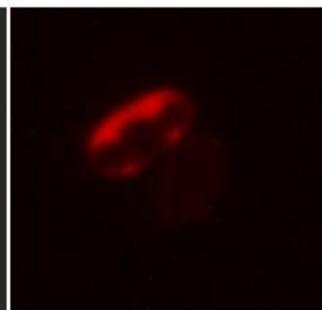
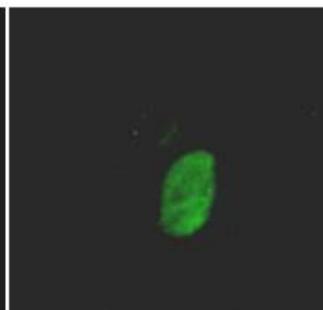
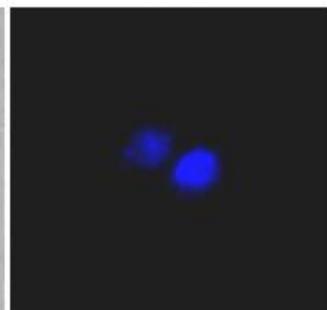
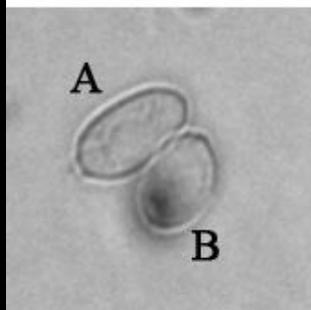
BR

UV

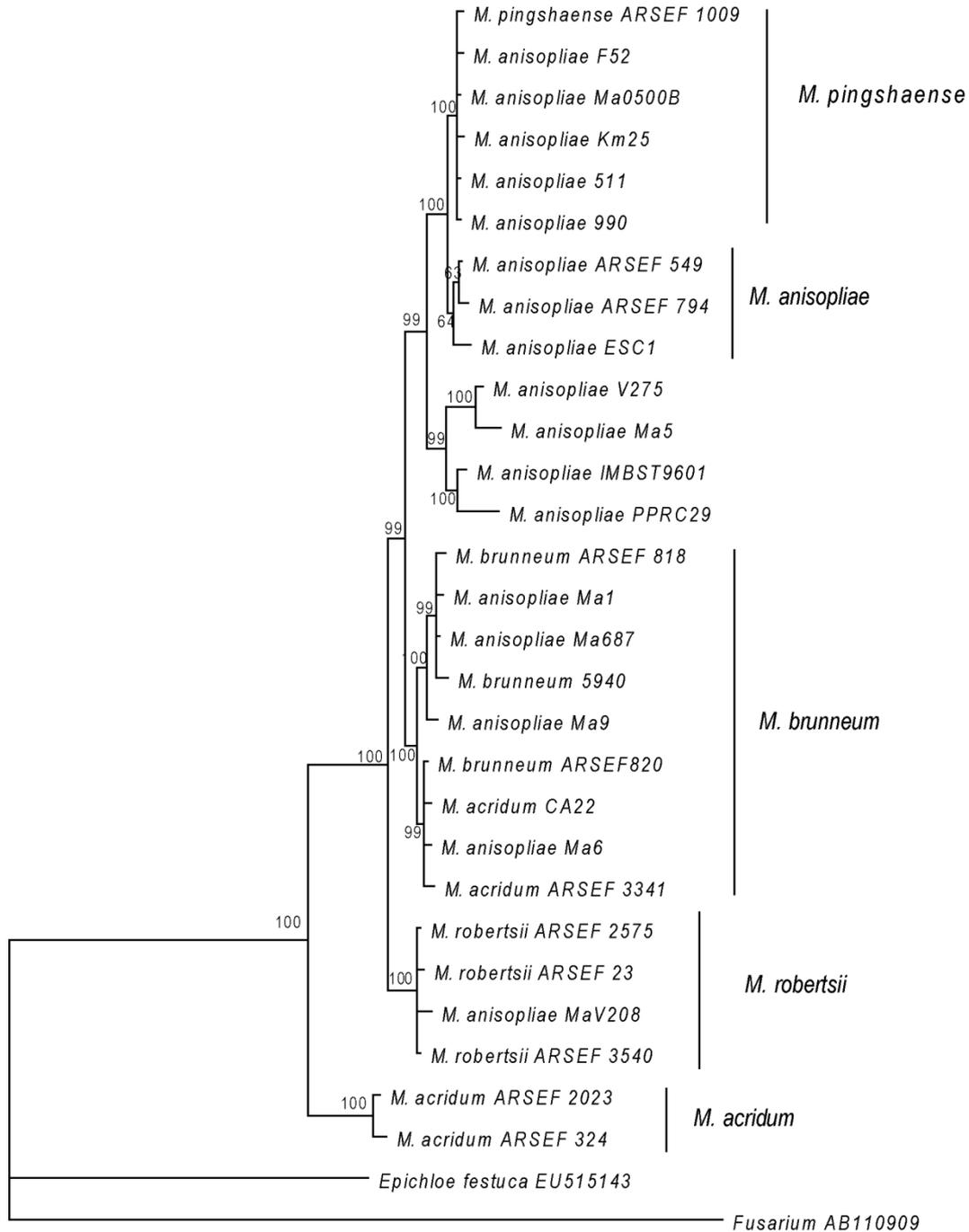
GF

RF

Merge

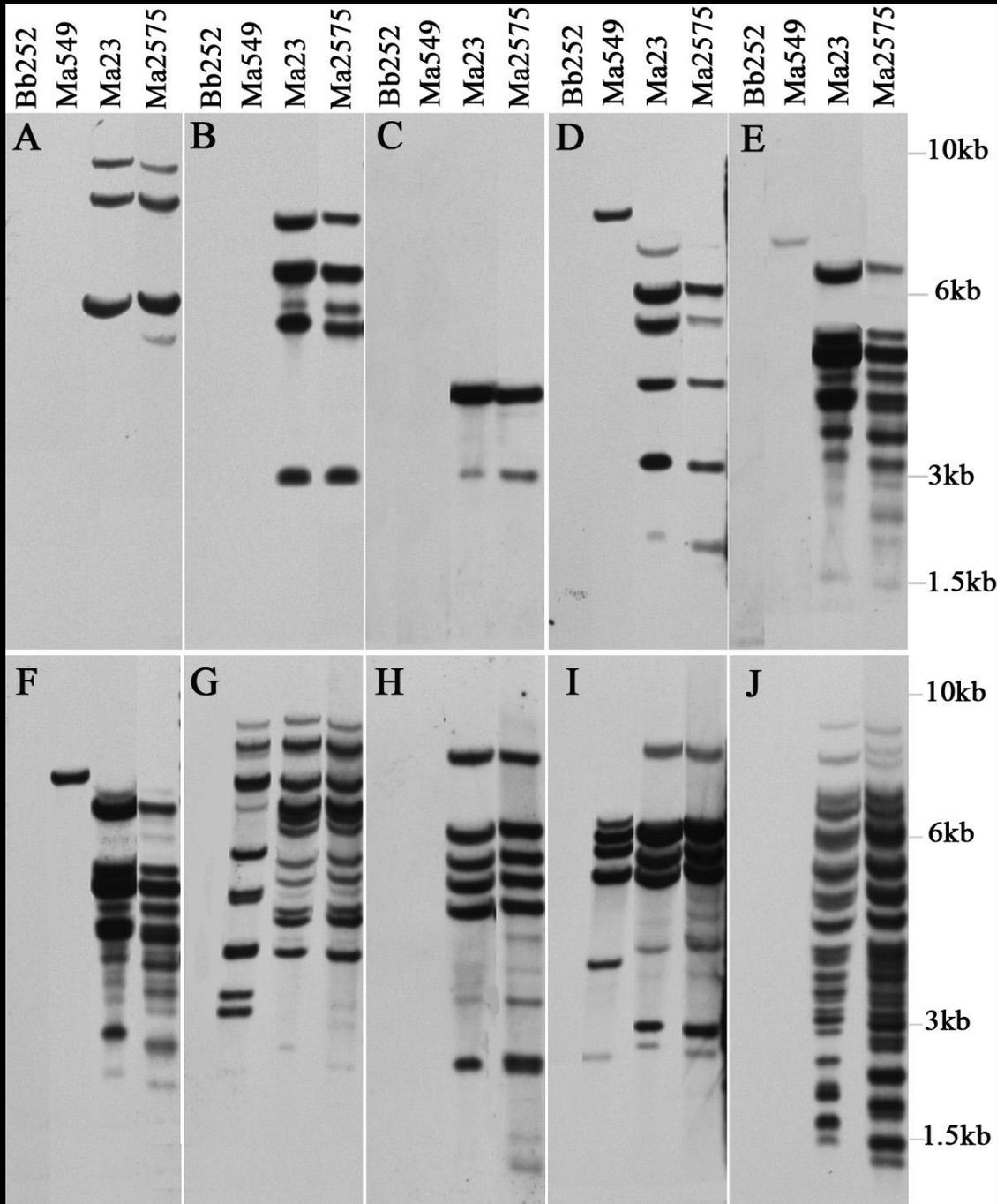


Galleria
coinfectd
by
Ma2575GFP
×
Ma2575RFP



Co-infecting *Galleria* with spores expressing either GFP or RFP.
 2575 vs 2575, 2575 vs 23, 2575 vs 1080, 2575 vs *B. bassiana*

Crosses		Conidia analysed ^a	Recombinant conidia ^b	Percentage of recombinants (%)
Mr2575RFP	×	2634	624	23.7
Mr2575GFP				
Mr2575RFP	×	>10000	0	0
Mr23GFP				
Mr2575RFP	×	>10000	0	0
Ma549GFP				
Mr2575RFP	×	>10000	0	0
Ma1080GFP				
Mr2575RFP	×	>10000	0	0
Bb252GFP				
SeGFP × SeRFP ^c		2436	989	40.6



Southern hybridization analysis of *Metarhizium anisopliae* Ma2575, Ma23, Ma549 and *Beauveria bassiana* Bb252 genomic DNA with ORF sequences of ten Ma2575 transposable elements.

Washes were conducted at low stringency (65°C in 0.5 × SSC).

Failsafe mechanisms could be used, depending on the ability of a pathogen to sexually or asexually conjugate with related organisms-what is possibility of mutation to a broader host range, and the means by which the pathogen is applied

Strains 2575 and 23 have strong barriers that prevent recombination

Fusarium oxysporum is subdivided into hundreds of *forma speciales* each with its own host range-diverged from each other at least 50,000 years ago

Under severe selection pressure, chromosomes transferred between strains converting a non-pathogen into a pathogen of tomatoes. If this happened in nature it could explain the polyphyletic origin of host specificity in *F. oxysporum* (Ma *et al.*, 2010 Nature 464: 367-).

M. majus and *M. acridum* are monophyletic

'GM-gene-deletor': fused *loxP-FRT* recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants

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¶Visiting scientist from Hubei University, Hubei, China

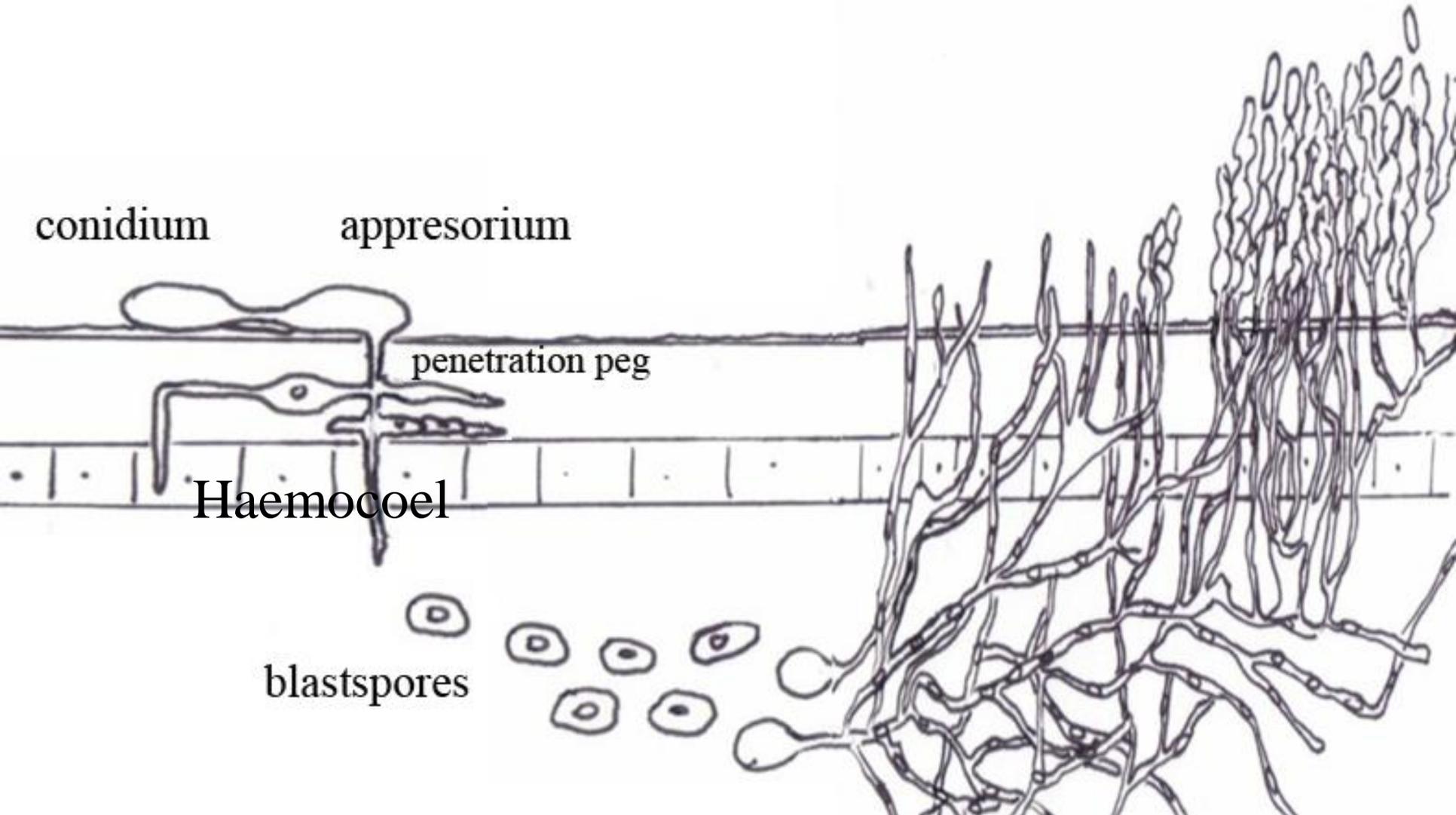
**Visiting scientist from Beijing Forestry University, Beijing, China

Keywords: biosafety, *CRE/loxP*, *FLP/FRT*, *loxP-FRT* fusion recognition site, non-transgenic pollen and seed, transgene flow.

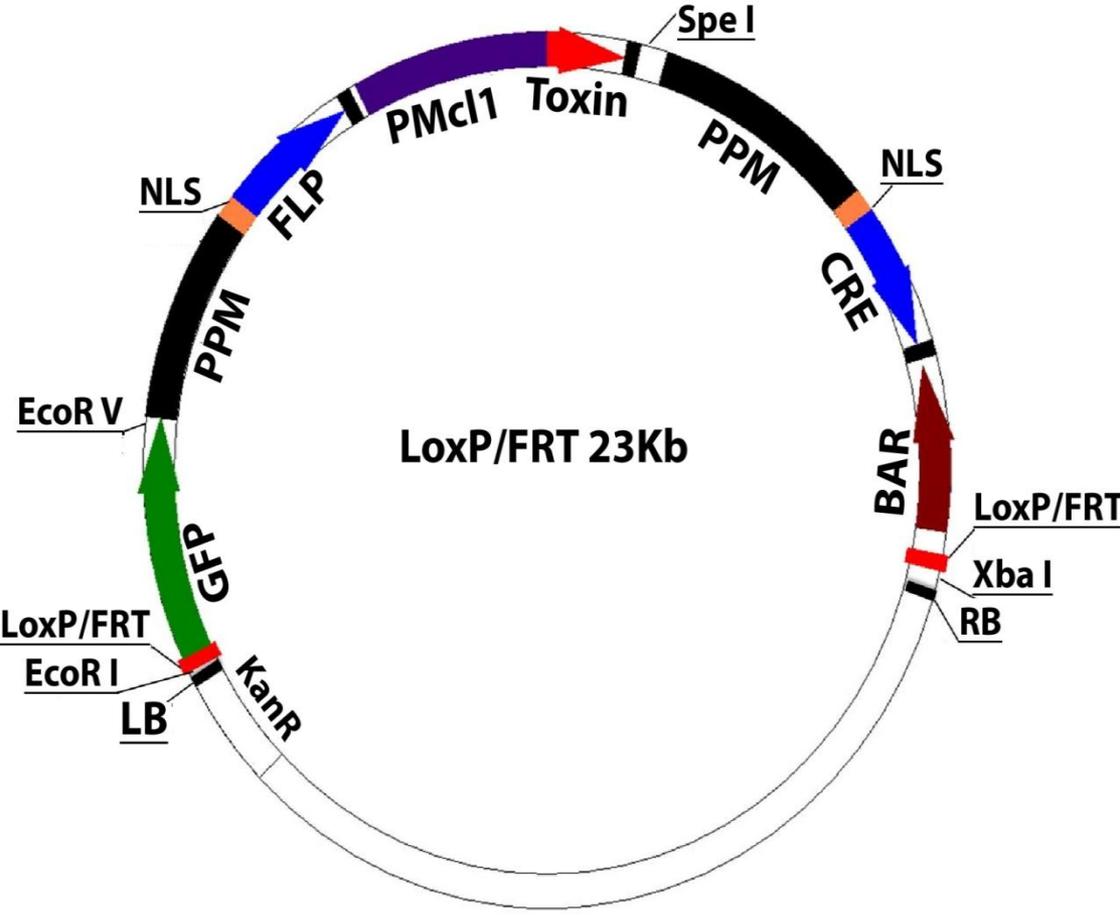
Summary

Pollen- and seed-mediated transgene flow is a concern in plant biotechnology. We report here a highly efficient 'genetically modified (GM)-gene-deletor' system to remove all functional transgenes from pollen, seed or both. With the three pollen- and/or seed-specific gene promoters tested, the phage *CRE/loxP* or yeast *FLP/FRT* system alone was inefficient in excising transgenes from tobacco pollen and/or seed, with no transgenic event having 100% efficiency. When *loxP-FRT* fusion sequences were used as recognition sites, simultaneous expression of both FLP and CRE reduced the average excision efficiency, but the expression of FLP or CRE alone increased the average excision efficiency, with many transgenic events being 100% efficient based on more than 25 000 T₁ progeny examined per event. The 'GM-gene-deletor' reported here may be used to produce 'non-transgenic' pollen and/or seed from transgenic plants and to provide a bioconfinement tool for transgenic crops and perennials, with special applicability towards vegetatively propagated plants and trees.

Insect fungal pathogenesis

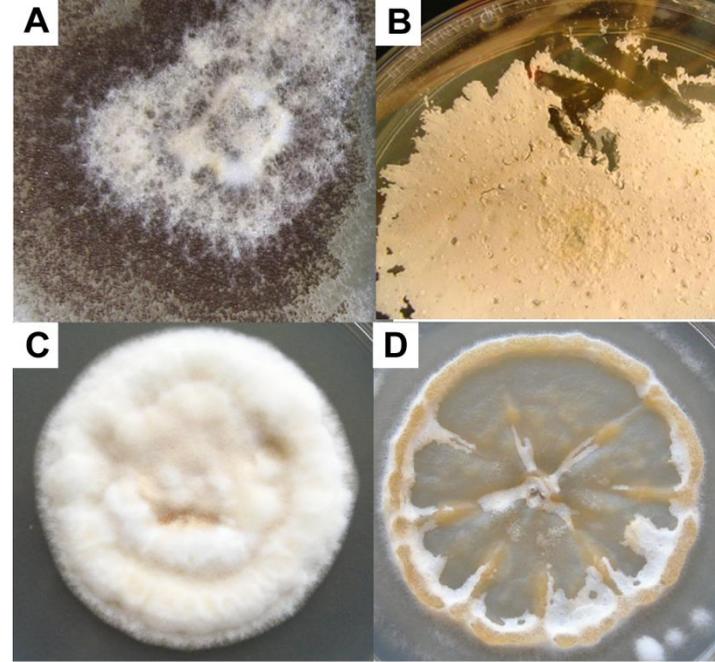
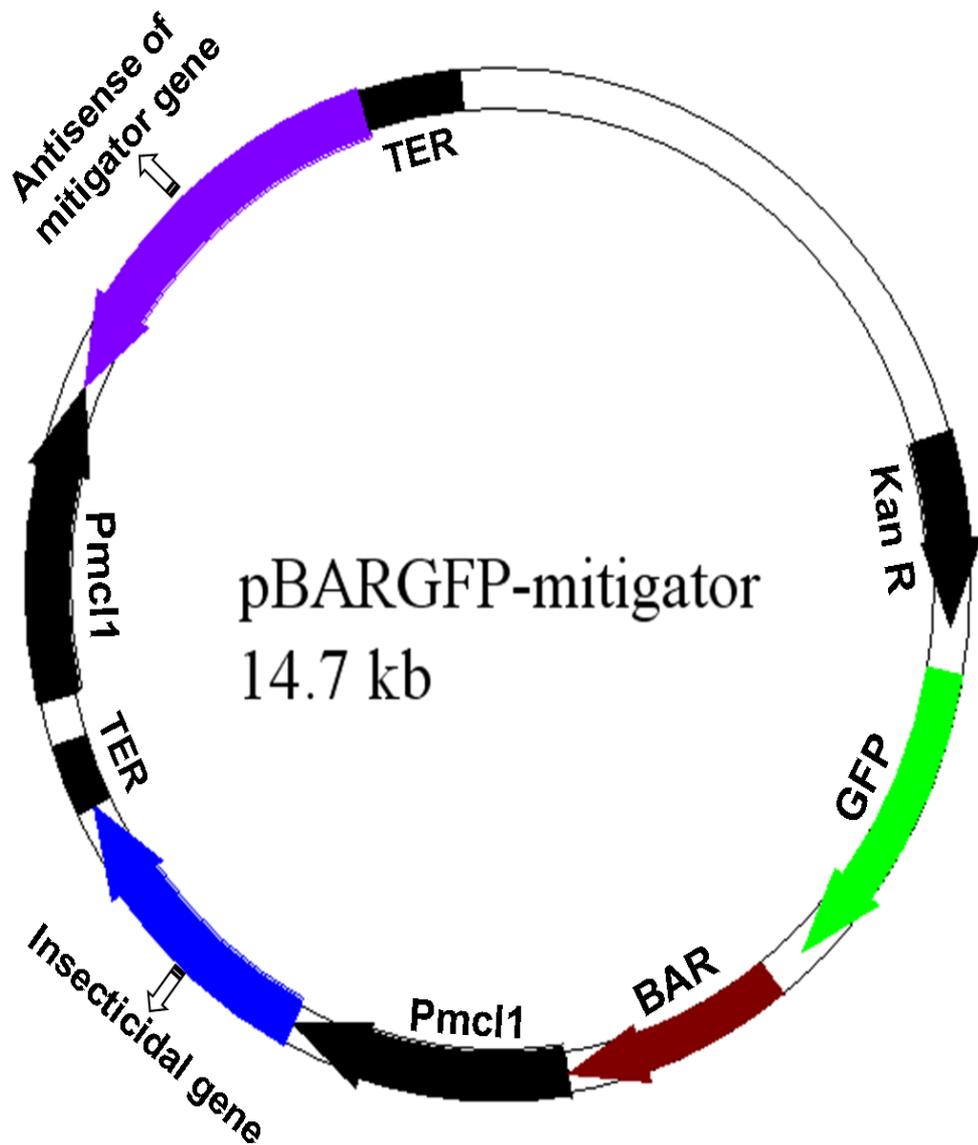


Small C. & Bidochka J. M., 1999



During *Agrobacterium* mediated transformation, the transferring region between the right (RB) and left (LB) borders is excised and inserted into the fungal genome. LoxP/FRT: the hybrid recognition site of recombinases CRE and FLP. NLS: nuclear localization sequence (from a *Metarhizium* transcription factor) targets recombinases to the nucleus. PPM: promoter of the late expressing pheromone processing metallopeptidase. PMcI1: the *Mcl1* promoter.





A. Wild type *M. anisopliae*; B, white spored mutant; C, asporogenic “fluffy” mutant, and D, spontaneous autolysis

pBARGFP-mitigator. AaIT (insecticidal gene) flanked by an antisense mitigating gene under control of an infection specific promoter (Mcl1) to suppress sporulation and block gene flow.

Conclusions

Spread of genes between fungal species is very rare-only one reported case

The risk of transgene introgression between strains of the same species needs to be considered on a case-by-case basis, depending on sexuality and heterokaryon compatibility.

- insects are hot spots for recombination for at least some *Metarhizium* strains (how common are co-infections?)

Where natural barriers exist, anti-introgressional failsafe mechanisms could lower the risk to an infinitesimal level, providing a level of containment and safety greater than that of the inefficient wild type.

However-what about long term goal of producing specific pathogens that recycle through pest populations suppressing them more cheaply than chemical insecticides?

Brown Marmorated Stink Bug



Metarhizium as a plant growth stimulating agent

Metarhizium anisopliae Seed Treatment Increases Yield of Field Corn When Applied for Wireworm Control

J. Todd Kabaluk* and Jerry D. Ericsson

ABSTRACT

In an effort to protect field corn (*Zea mays* L.) from wireworm (*Agriotes obscurus* L.) herbivory and yield loss, seeds were treated with conidia of *Metarhizium anisopliae* strain F52 alone or in combination with clothianidin or spinosad before planting at three farm fields in south coastal British Columbia, Canada. Corn seed treated with *M. anisopliae* conidia (main effect) resulted in significant increases in stand density (78% *M. anisopliae* treated vs. 67% no *M. anisopliae*) and stock and foliage area fresh wt. yield (9.6 Mg ha⁻¹ *M. anisopliae* treated vs. 7.6 Mg ha⁻¹ no *M. anisopliae*), and significantly increased plant (stock and foliage) fresh wt. when it was applied together with spinosad or with no additional agrichemical at one location. Spinosad had no effect on corn yield, whereas clothianidin caused a significant increase in plant stand density and yield. Wireworm cadavers showing *M. anisopliae* strain F52 growth were retrieved from treated plots, suggesting that the increase in yield may have been due to wireworm control. Laboratory experiments provided no evidence that the increase in stand density and yield from the *M. anisopliae*-treated corn seed was attributable to an increase in germination rate or root growth. We concluded that seed treatment with this fungus may be a novel method to increase stand density and yield of corn.

phate insecticides applied as granules with the seed or by using insecticide-treated seeds (Kuhar et al., 2003). Although the insecticide treatments are efficacious, concerns associated with human and environmental health have caused attention to be focused on the development of reduced-risk compounds and practices. Furthermore, while the majority of corn growers currently have access to agrichemicals for wireworm control, many compounds are unacceptable for organic corn production.

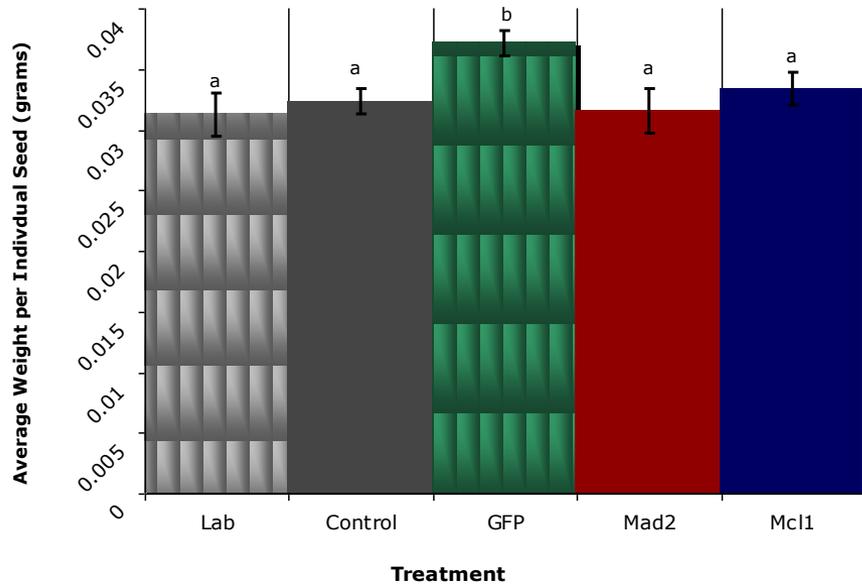
Microbial pesticides have been developed that capitalize on their antagonistic interaction with plant diseases and pathogenic interactions with pest plants and insects (Vakili, 1992). For insect pests however, there have been no reports of using microorganisms as seed treatments for crop protection. *Metarhizium anisopliae* Sorokin (Hypocreales: Clavicipitaceae) can infect wire-

Published in *Agron. J.* 99:1377–1381 (2007).
Notes & Unique Phenomena
doi:10.2134/agronj2007.0017N

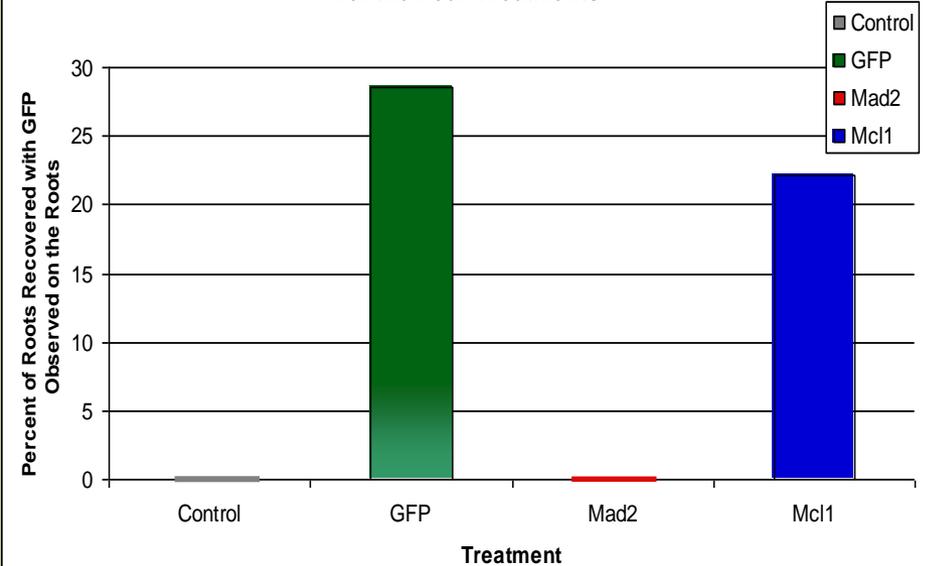
Rhizosphere competence places sharp focus on the biology of the soil root interface as a site where plants, insects and pathogens interact.

Applying *Metarhizium* conidia to seeds **increased** mass of recovered winter wheat seeds

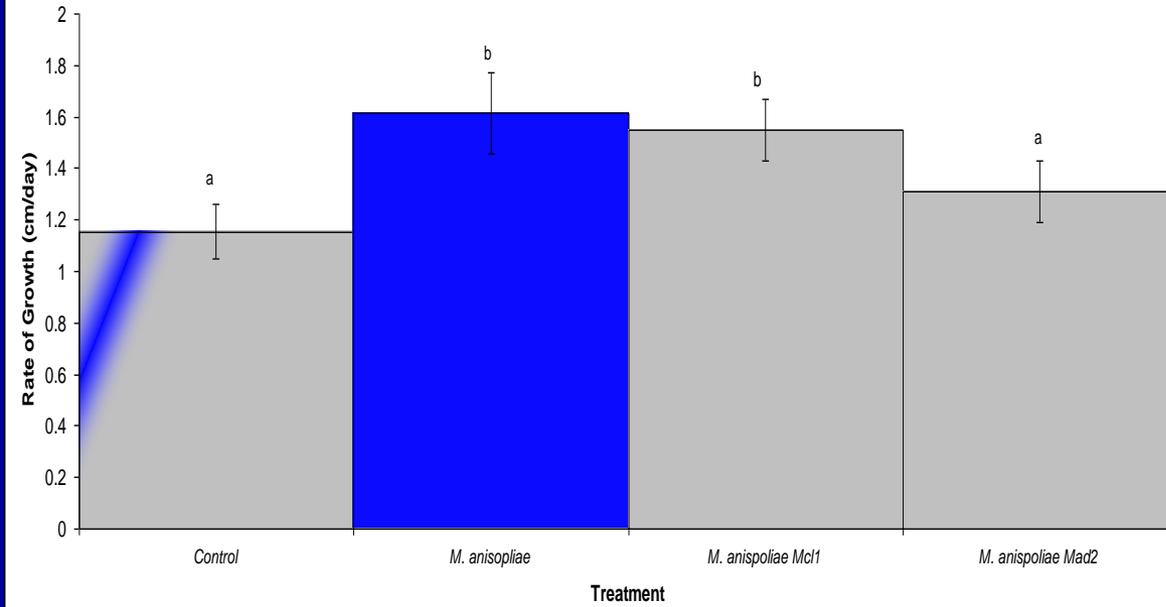
Average Seed Weight for Each Treatment



Percent of Plants with *M. anisopliae*-GFP Observed on the Roots for the Four Treatments



Average Growth Rate of Winter Wheat



This strain was not specifically selected for its rhizosphere competence. *Metarhizium* has a multifactorial influence on plant growth that could potentially be modified and improved. A deeper understanding of the mechanistic basis of rhizosphere competency could enable us to identify genes that we could use to develop *Metarhizium* as a comprehensive plant symbiont

Selected or improved strains of *M. anisopliae* that were comprehensive plant symbionts could be implemented as part of an IPM approach to reduce use of:



<http://www.superway.com.au/Images/Products/Dimethoate300.jpg>



<http://www.orau.org/ptp/collection/consumer%20products/fertilizer.jpg>

Mrt, a Gene Unique to Fungi, Encodes an Oligosaccharide Transporter and Facilitates Rhizosphere Competency in *Metarhizium robertsii*^{[C][W]}

Weiguo Fang* and Raymond J. St. Leger

Department of Entomology, University of Maryland, College Park, Maryland 20742

The symbiotic associations between rhizospheric fungi and plants have enormous environmental impact. Fungi are crucial to plant health as antagonists of pathogens and herbivores and facilitate the uptake of soil nutrients. However, little is known about the plant products obtained by fungi in exchange or how they are transported through the symbiotic interface. Here, we demonstrate that sucrose and raffinose family oligosaccharides in root exudates are important for rhizosphere competence in the insect pathogen *Metarhizium robertsii* (formerly known as *Metarhizium anisopliae*). We identified mutants in the *Metarhizium raffinosae transporter (Mrt)* gene of *M. robertsii* that grew poorly in root exudate and were greatly reduced in rhizosphere competence on grass roots. Studies on sugar uptake, including competition assays, revealed that MRT was a sucrose and galactoside transporter. Disrupting MRT resulted in greatly reduced or no growth on sucrose and galactosides but did not affect growth on monosaccharides or oligosaccharides composed entirely of glucose subunits. Consistent with this, expression of *Mrt* is exclusively up-regulated by galactosides and sucrose. Expressing a green fluorescent protein gene under the control of the *Mrt* promoter confirmed that MRT was expressed by germlings in the vicinity of grass roots but not in surrounding bulk soil. Disrupting *Mrt* did not reduce virulence to insects, demonstrating that *Mrt* is exclusively involved in *M. robertsii*'s interactions with plants. To our knowledge, MRT is the first oligosaccharide transporter identified and characterized in a fungus and is unique to filamentous fungi, but homologous genes in *Magnaporthe*, *Ustilago*, *Aspergillus*, *Fusarium*, *Epichloe*, and *Penicillium* species indicate that oligosaccharide transport is of widespread significance.

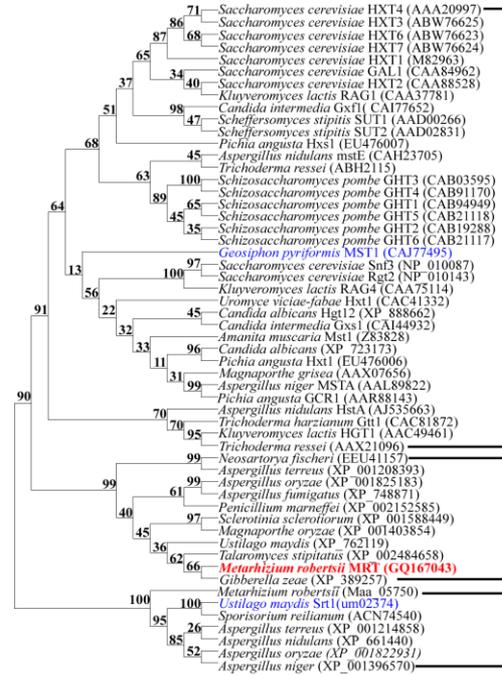
The rhizosphere is the narrow zone of soil directly influenced by root secretions. It is the site of complex interactions between plants, bacteria, fungi, protists, nematodes, and insects (Bais et al., 2006) that are important for nutrient cycling, ecosystem functioning, and carbon sequestration (Singh et al., 2004). Fungi in particular are crucial to plant growth and health as nutrient solubilizers, phytase producers, and antagonists of plant pathogens and insects (Bridge and Spooner, 2001; Hu and St. Leger, 2002; Marx, 2004; Harman and Shores, 2007).

It is generally accepted that the large microbial population in the rhizosphere is supported by a very complex mixture of relatively labile organic compounds (amino acids, organic acids, sugars, phenolics, and various secondary metabolites) in the root exudate (Walker et al., 2003). These compounds can have negative as well as positive interactions with the

microbial community and influence the relationship between microbes and insects (Li and Holdom, 1995; Ganade and Brown, 1997). However, due to the complexity of root exudates, identifying the roles of different components in these rhizospheric processes has been highly problematic (Walker et al., 2003).

The ascomycete *Metarhizium robertsii* ARSEF2575 (formerly known as *Metarhizium anisopliae* var *anisopliae*; Bischoff et al., 2009) is ubiquitous in the soil community, where it establishes mutualistic interactions with plants as a rhizospheric fungus (Hu and St. Leger, 2002) and is a potent insect pathogen (Prior, 1992; Roberts and St. Leger, 2004). The distribution of genetic groups of *M. robertsii* depends on their adaptations to specific soils and plant types rather than their pathogenicity to insects (Bidochka et al., 1998), but applying *Metarhizium* to seed increases the yield of field corn (*Zea mays*), possibly in part by killing soil insects (Kabaluk and Ericsson, 2007). *Metarhizium* also increases plant growth in insect-free microcosms in a multifactorial manner that involves mobilizing nutrients (O'Brien, 2009) and inhibition of plant pathogens (Kang et al., 1996; Ownley et al., 2010). *M. robertsii*, therefore, provides an unusually versatile model system for studying complicated root-insect-fungus interactions.

The most informative approach for evaluating the importance of root exudate production in establishing rhizosphere competence will involve comparisons with mutant fungi that cannot colonize the rhizo-



¹ This work was supported by the U.S. Department of Agriculture Biotechnology Risk Assessment Research Grants Program (award no. 2006-0692).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Weiguo Fang (wfang1@umd.edu).

^[C] Some figures in this article are displayed in color online but in black and white in the print edition.

^[W] The online version of this article contains Web-only data.

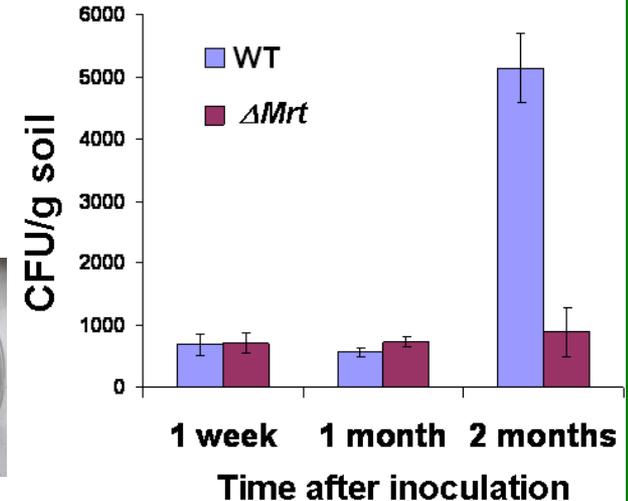
www.plantphysiol.org/cgi/doi/10.1104/pp.110.163014

Rhizosphere competency assay



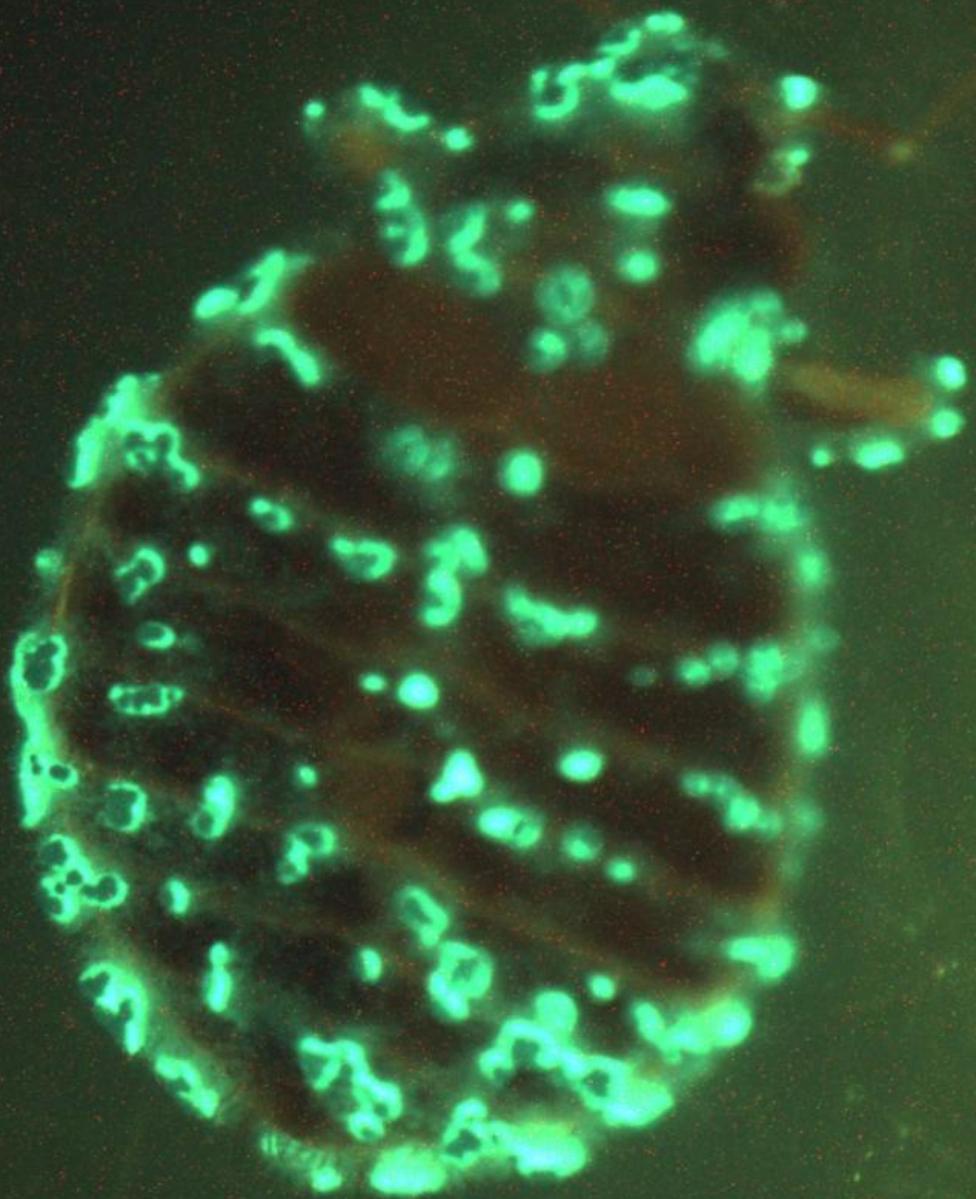
Wild type

ΔMrt

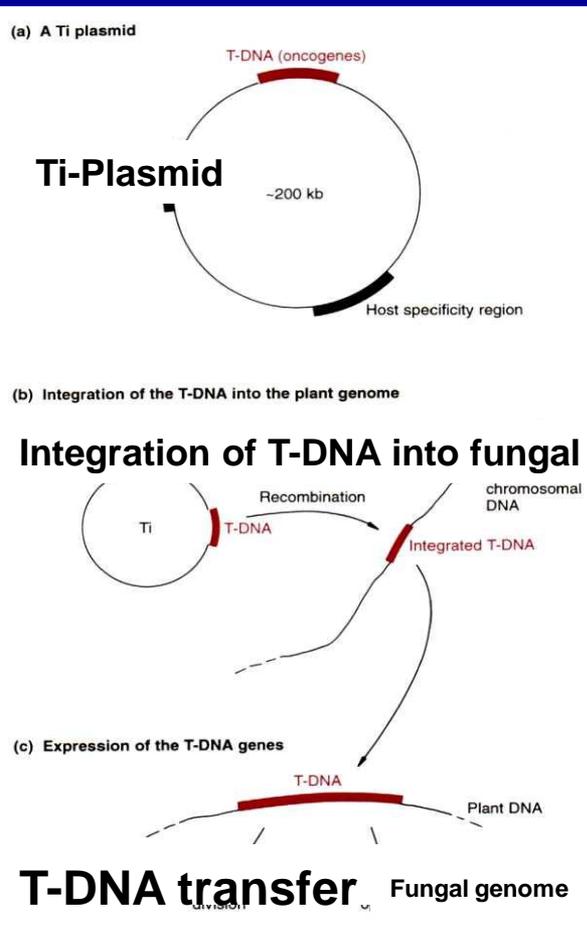


Wild type

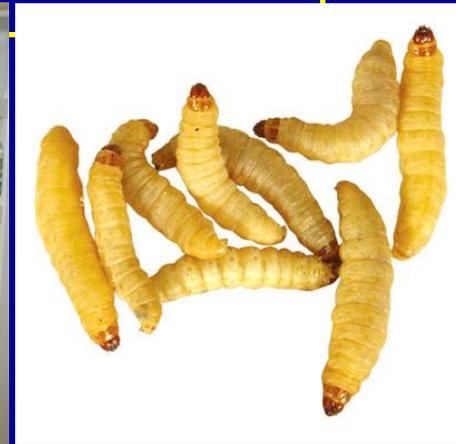
ΔMrt



Construction of *Metarhizium anisopliae* T-DNA Library using *Agrobacterium tumefaciens*-mediated transformation



- ◆ Conidiation mutants
- ◆ Virulence mutants (decreased or increased) adhesins, immune evasion, osmosensor, CDE's
- ◆ Mutants related to other fungal traits, in particular rhizosphere competence



The genome is linked with the mutant library by extensive sequencing of the insertion sites. It is thus a seamless process to go from identifying a novel gene sequence to finding the corresponding insertion sequence.

Genes and infection

MPL1, turgor pressure and penetration, 16-20 hrs pi



JBC, 2007. 282: 21110.

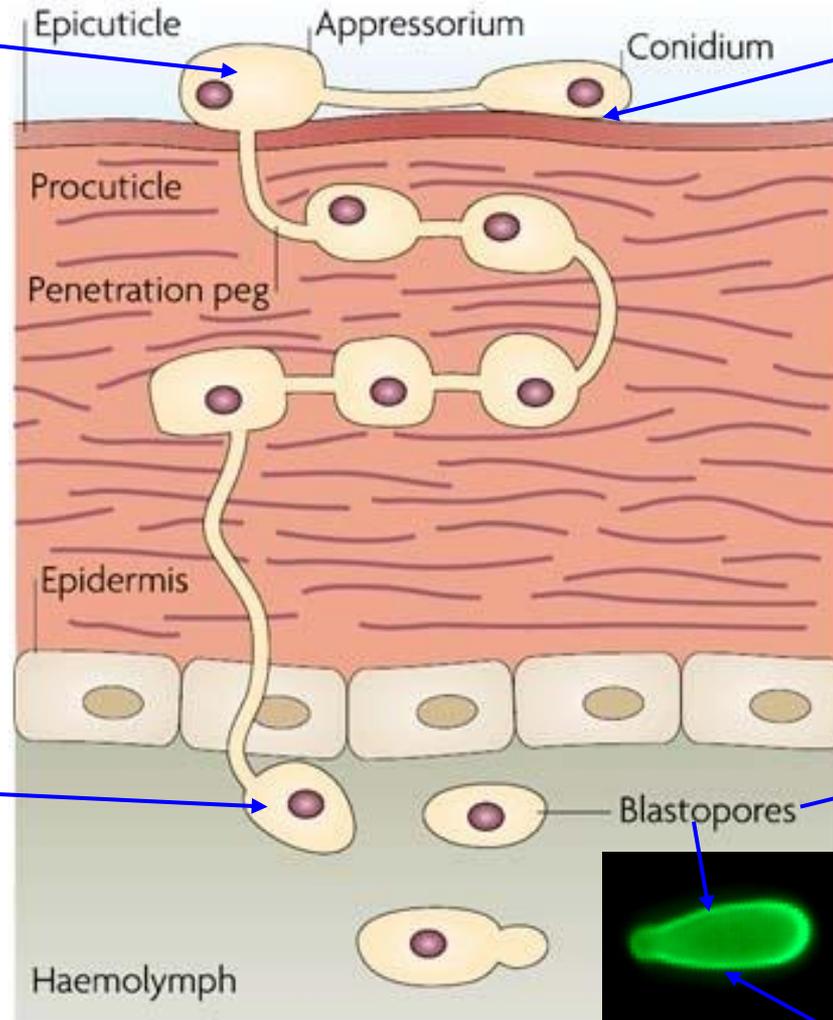
MAD1 adhesin, spore adhesion, 8-12hrs pi



Eukaryot. Cell, 2007.6:808.

MOS1, osmosensor, 24-30 hrs pi

Eukaryot. Cell, 2008. 7: 302.



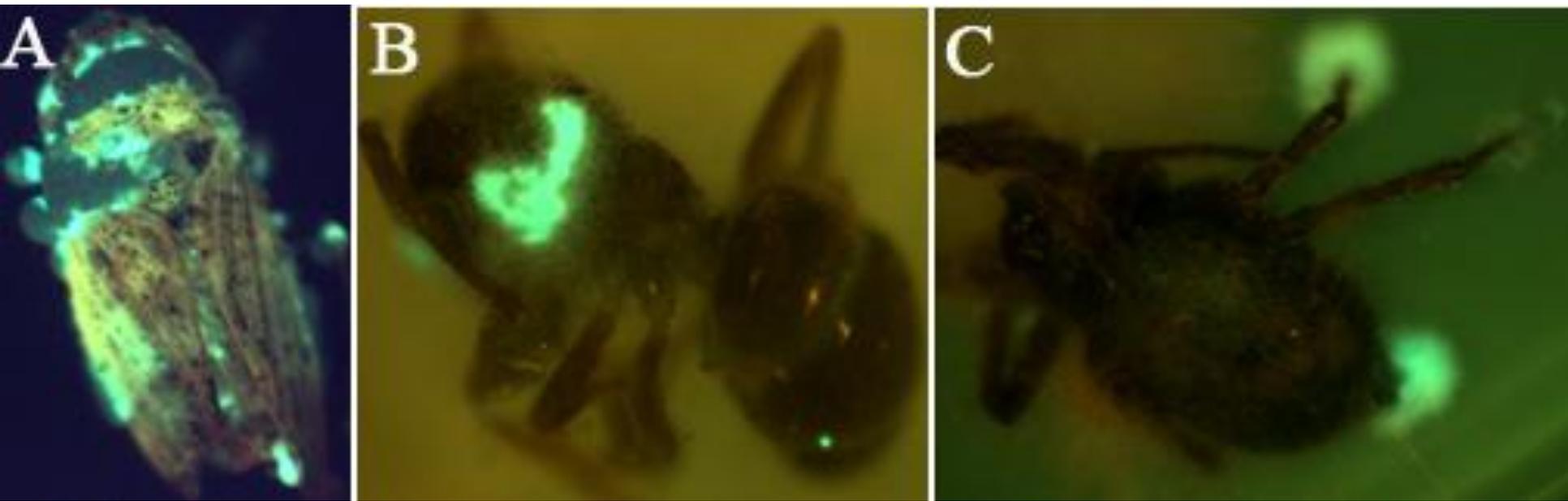
MCL1, Immune protective coat, 30-72 hrs pi



PNAS, 2006. 103: 6647.



Blue Mountains funnel-web spider *Hadronyche versuta*
being milked for venom

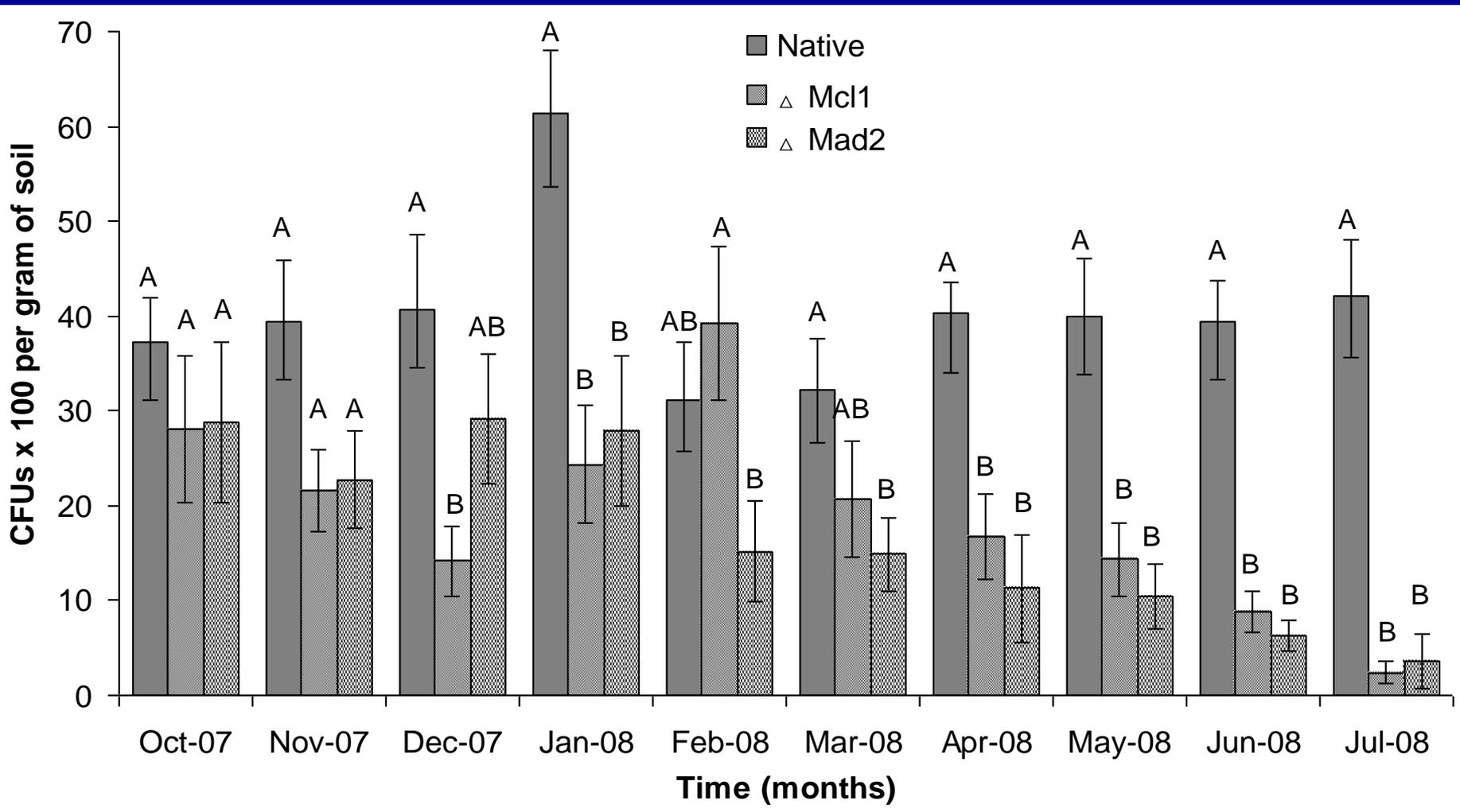


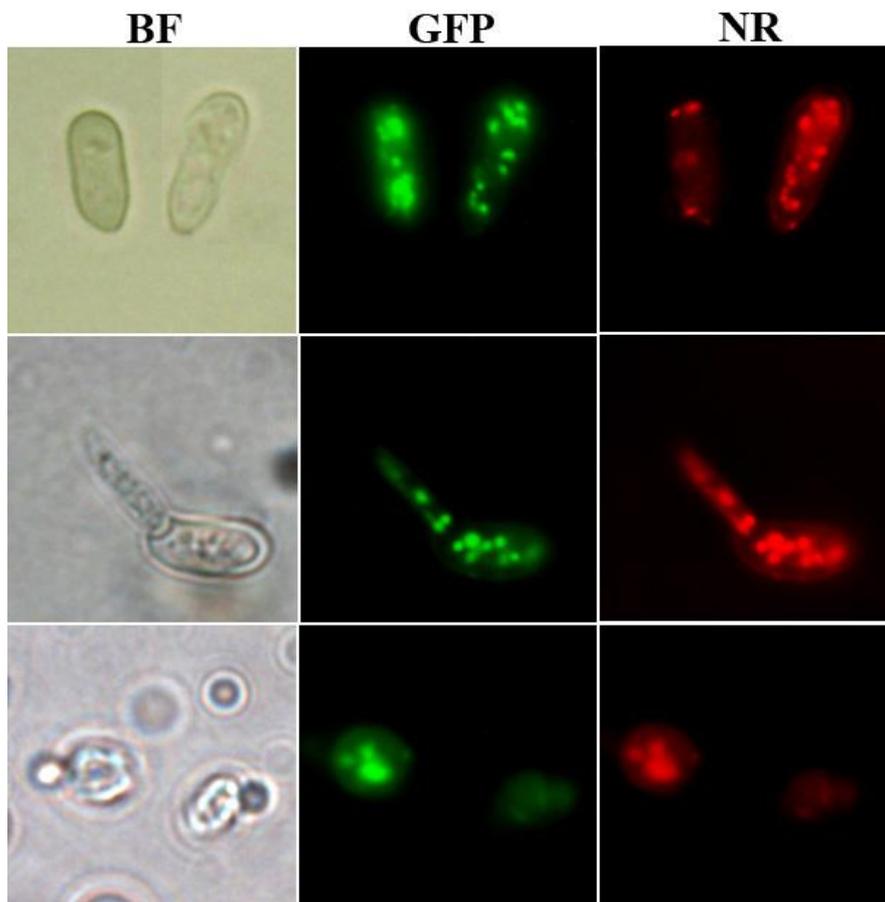
A), Cercopid bug and B), ant collected from pit fall traps and maintained in the lab. Both insects died within two days and were placed on selective medium. Fluorescent hyphae (sporulating in A) confirm infection with Δ Mad2. Red fluorescent hyphae were never observed emerging from cadavers confirming low infectivity of Δ Mcl1. C) Spider from a pit fall trap plated onto selective medium was carrying Δ Mad2. Spiders were not hosts for *M. robertsii*, but dispersed it for several weeks after inoculation.

Antibodies that target, for example, hormone receptors would allow construction of very effective, highly specific, biopesticides with minimal negative environmental impact

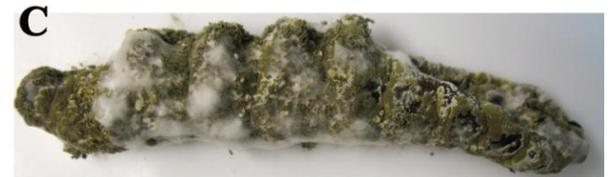
Parasexual segregants derived from the first haploidization process of a Ma2575GFP× Ma2575RFP recombinant

<i>Segregants</i>	<i>Phenotype of segregant</i>	<i>Percentage (%)</i>
SeGFP	Green florescence	17.14
SeRFP	Red florescence	49.79
SeUnGR	No florescence	32.93
SeGFPRFP	Green and Red florescence	0.134





Co-localization of Mest1 and neutral lipids demonstrated by Nile red staining of GFP-MEST1 expressing cells in conidia (E), germinated spores of *M. robertsii* (F) and transgenic yeast (*S. cerevisiae*) cells (G), respectively. BR, bright field microscopy. GFP, fluorescent filter. NR, Nile red staining.



Grasshoppers infected with wild type *M. acridum* Ma324. (B) *Galleria* infected with Ma324-Mest1. (C) *Manduca sexta* infected with wild type *M. robertsii* Mr2575 and (D) transgenic *M. acridum* Ma324-Mest1.

