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BARD

FINAL REPORT

PROJECT NO. US-3260-01

**Characterization of Fungal Symbiotic Lifestyle
Expression in *Colletotrichum* and Generating Non-
Pathogenic Mutants that Confer Disease Resistance,
Drought Tolerance, and Growth Enhancement to
Plant Hosts**

R.J. Rodriguez, S. Freeman

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Project Title: Characterization of fungal symbiotic lifestyle expression in *Colletotrichum* and generating non-pathogenic mutants that confer disease resistance, drought tolerance, and growth enhancement to plant hosts

Investigators

Principal Investigator (PI):

Russell J. Rodriguez

Co-Principal Investigator (Co-PI):

Stanley Freeman

Institutions

Univ. of Washington, WA, USA

ARO, Volcani Center, Israel

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

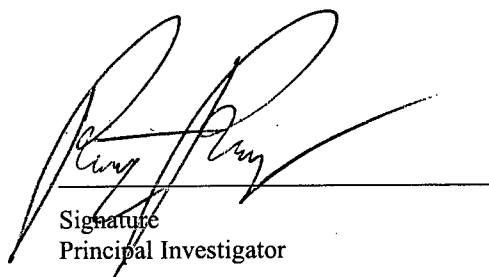
Biological control, fungal disease, fungi, gene expression, genomic libraries, mutualism, pathogenicity, plant protection, symbiosis

Abbreviations commonly used in the report, in alphabetical order:

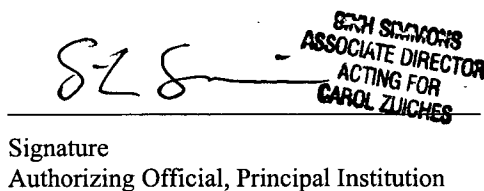
Budget: IS: \$150,000

US: \$150,000

Total: \$300,000

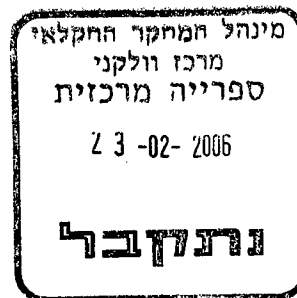


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Principal Investigator



Signature
Authorizing Official, Principal Institution

CAROL ZUCHES
Asst. Vice Provost For Research
Executive Director, Grant and Contract Services



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Publication Summary (numbers)

| | Joint IS/US author | US Authors only | Israeli Authors only | Total |
|---|-----------------------|--------------------|-------------------------|-------|
| Refereed (published, in press, accepted) | 1 | 2 | 1 | 4 |
| Submitted, in review, in preparation | | 1 | 2 | 3 |
| Invited review papers | | 2 | - | 2 |
| Book chapters | | 2 | 1 (in preparation) | 3 |
| Books | | | - | - |
| Master theses | | | - | - |
| Ph.D. theses | | | 1 (in progress) | 1 |
| Abstracts | | 4 | 3 | 7 |
| Not refereed (proceedings, reports, etc.) | | | 2 (submitted) | 2 |

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

| | From US to Israel | From Israel to US | Together, elsewhere | Total |
|--------------------------------|-------------------|-------------------|---------------------|-------|
| Short Visits & Meetings | - | 1 | 1 | 2 |
| Longer Visits (Sabbaticals) | - | - | - | - |

Description of Cooperation:

The US and Israeli cooperators have met twice to discuss experimental results and to plan additional experiments. These meeting have been fruitful and allowed us to coordinate efforts and submit a continuation proposal to BARD.

Patent Summary (numbers)

| | Israeli inventor (s) only | US inventor (s) only | Joint IS/US inventors | Total |
|------------------|------------------------------|-------------------------|--------------------------|-------|
| Submitted | - | - | - | - |
| Issued (allowed) | - | - | - | - |
| Licensed | - | - | - | - |

Abstract

Fungal plant pathogens are responsible for extensive annual crop and revenue losses throughout the world. To better understand why fungi cause diseases, we performed gene-disruption mutagenesis on several pathogenic *Colletotrichum* species and demonstrated that pathogenic isolates can be converted to symbionts (mutualism, commensalism, parasitism) expressing non-pathogenic lifestyles. The objectives of this proposal were to: 1- generate crop-specific mutants by gene disruption that express mutualistic lifestyles, 2- assess the ability of the mutualists to confer disease resistance, drought tolerance, and growth enhancement to host plants, 3- compare *fslm1* sequences and their genomic locations in the different species, and 4- document the colonization process of each *Colletotrichum* species. It was demonstrated that wildtype pathogenic *Colletotrichum* isolates, can be converted by mutation from expressing a pathogenic lifestyle to symbionts expressing non-pathogenic lifestyles. In the US, mutants of *Colletotrichum* were isolated by homologous gene disruption using a vector containing a disrupted *FSLm1* sequence while in Israel, *C. acutatum* mutants were selected by restriction enzyme mediated integration (REMI) transformation. One group (US) of non-pathogenic mutants conferred disease protection against pathogenic species of *Colletotrichum*, *Fusarium*, and *Phytophthora*; drought tolerance; and growth enhancement to host plants. These mutants were defined as mutualists and disease resistance correlated to a decrease in the time required for hosts to activate defense systems when exposed to virulent fungi. The second group (Israel) of non-pathogenic mutants did not confer disease resistance and were classified as commensals. In addition, we demonstrated that wildtype pathogenic *Colletotrichum* species can express non-pathogenic lifestyles, including mutualism, on plants they colonize asymptotically. The expected long term contribution of this research to agriculture in the US and Israel is threefold. Host-specific mutualists will be utilized in the various crops to confer (1) disease resistance to reduce dependence on chemical fungicides; (2) drought tolerance to reduce water consumption for irrigation; (3) growth enhancement to increase yields.

Achievements

Significance of main scientific achievements:

In this research project, we isolated nonpathogenic mutants of *Colletotrichum* species using two strategies: In Israel, *C. acutatum* mutants were selected by REMI transformation, using a rapid, reliable seedling inoculation bioassay (Horowitz et al., 2004). In the US mutants of eight *Colletotrichum* species were isolated by homologous gene disruption using the vector containing a disrupted *fslm1* sequence (Rodriguez et al., in preparation). As a result, two mutant classes were isolated and characterized. One of the mutants from Israel was disrupted in a transcription factor involved in nitrogen metabolism rendering the mutant incapable of host penetration due to developmental changes in hyphal growth and appressorial formation. The *fslm1* mutants generated in the US lab underwent the same development as wildtype isolates but host colonization was asymptomatic and expressed mutualistic lifestyles by conferring disease resistance, drought tolerance and enhanced growth. These results demonstrate the accomplishment of objectives 1 and 2 of this research project.

The integration events in the *fslm1* mutants were analyzed and found to be very similar (objective 3). There appears to be high conservation of the *fslm1* sequence in the genus *Colletotrichum*, however, the sequence does not appear in any of the genome databases currently available for fungi. Translated versions of the *fslm1* sequence also do not occur in any public databases. Therefore, the *fslm1* sequence appears to be highly conserved in *Colletotrichum* but is not common among other fungi.

Colonization of various mutants and wild types were analyzed microscopically (objective 4). For example, the host range and specificity of *C. acutatum* from strawberry was examined on pepper, eggplant, tomato, bean and strawberry. The fungus was recovered from all plant species but caused disease symptoms only on strawberry. Epiphytic and endophytic fungal growth in the different plant species was confirmed by re-isolation and by PCR amplification (Freeman et al., 2001). On strawberry, the germinating conidia formed branched, thick, coiled hyphae with large numbers of appressoria and the pathogen filled the mesophyll with dense mycelium that invaded the cells and caused necrosis. In non-pathogenic interactions, the conidia germinated producing thin, straight

germ tubes while appressoria failed to penetrate leaf tissue, resulting only in epiphytic growth. Non-appressorial penetration occurred several days after inoculation and fungal growth was restricted to the intercellular spaces without causing any visible damage (Horowitz et al., 2002).

The gene disruption mutants (US) have been assessed for the ability to confer drought tolerance and growth enhancement (Rodriguez, unpublished). All of the mutants confer some level of drought tolerance and growth enhancement and we are now quantifying these fitness benefits. The fitness benefits conferred by mutualists is dependent on undefined components of the host genotypes (Redman et al., 2001). Preliminary data suggest that there is a correlation between photosynthetic activity and growth enhancement, and that growth enhancement typically results in increased fruit yields (Rodriguez, unpublished).

In summary:

- 1 - nonpathogenic mutualistic mutants have been generated using two strategies and the genetic basis for one mutant class (*C. acutatum* transcription factor) defined. The second mutant class is based on a DNA sequence that is not represented in any public databases and therefore remains undefined.
- 2 - virulent pathogens can asymptotically colonize some plant hosts and express mutualistic lifestyles.
- 3 - the *fslm1* gene disruption vector can be used to generate nonpathogenic mutants in most if not all *Colletotrichum* species.

In addition, the following hypotheses were tested: 1 - host-specific fungal symbionts can be generated by gene disruption of virulent pathogens that confer disease resistance, drought tolerance, and growth enhancement to plants, and 2 - the genetic basis of symbiotic lifestyle expression is conserved among *Colletotrichum* species. All of these objectives were fulfilled during the period of the proposal resulting in the publication of several manuscripts by both laboratories. In addition, the hypotheses tested appear to be valid.

Agricultural impacts of the research findings

The expected long term impact of this research to agriculture in the US and Israel demonstrates that host-specific mutualists may be utilized in the various crops to confer (1) disease resistance to

reduce dependence on chemical fungicides; (2) drought tolerance to reduce water consumption for irrigation; and (3) growth enhancement to increase yields.

Details of cooperation

All research was performed in close cooperation between the two Institutions in both a complementary and supportive manner. The labs performed similar experiments on different plant/fungal systems to expand the scope of the study, determined the genetically conserved nature of fungal symbiotic lifestyles, and defined genes that were expressed during different host symbiont or pathogenic interactions. Cooperation between SF and RR have been ongoing for approximately 14 years since SF spent a period of 2.5 years doing a postdoctoral training in the laboratory of Rusty Rodriguez at UC Riverside CA, during the years 1991-93. Over this period, at least 8 joint manuscripts were published during and after this fruitful collaboration. Both researchers are continuing their work in the area of proposed research and routinely discuss their mutual ongoing research. All probes, primers, plasmids and cultures are available to both scientists and since their mutual research was commenced in 1991, all data and information regarding the mutant constructs including the fslm1 plasmid and sequence have been shared. Future research is envisaged between the two research teams whereby libraries will be constructed in the lab of SF and hybridization of these products will be conducted on microarray slides to be generated by RR.

List of publications:

- Freeman, S.**, Horowitz, S., and Sharon, A. 2001. Pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. *Phytopathology* 91:986-992.
- Horowitz, S., **Freeman, S.**, and Sharon, A. 2002. Use of GFP-transgenic strains to study pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum*. *Phytopathology* 92:743-749.
- Horowitz, S., Yarden, O., Zveibil, A., and **Freeman, S.** 2004. Development of a robust screening method for pathogenicity of *Colletotrichum* species on strawberry seedlings enabling forward genetic studies. *Plant Dis.* 88:845-851.
- Redman, R.S., Dunigan, D.D., and **Rodriguez, R.J.** 2001. Fungal symbiosis: from mutualism to parasitism, who controls the outcome, host or invader? *New Phytologist*, **151**:705-716.
- Redman, R.S., and **Rodriguez, R.J.** 2003. Characterization and isolation of an extracellular serine protease from the tomato pathogen *Colletotrichum coccodes* (Wallr.), and it's role in

pathogenicity. *Mycol. Res.* 106:1427-1434.

Redman, R.S., Rossinck, M.R., Maher, S., Andrews, Q.C., Schneider, W.L. and **Rodriguez, R.J.**

2002. Field performance of cucurbit and tomato plants infected with a nonpathogenic mutant of *Colletotrichum magna* (teleomorph: *Glomerella magna*; Jenkins and Winstead). *Symbiosis* 32:55-70.

Redman, R.S. and **Rodriguez, R.J.** 2003. Characterization and Isolation of an Extracellular Serine Protease from the Tomato Pathogen *Colletotrichum coccodes* (Wallr.), and it's Role in Pathogenicity. *Mycological Research*, **106**:1427-1434.

Rodriguez, R.J., Cullen, D., Kurtzman, C., Khachatourians G. and Hegedus D. 2004.

Molecular methods for discriminating taxa, monitoring species, and assessing fungal diversity. In "Biodiversity of Fungi: Inventory and Monitoring Methods." Mueller, G. M., G. F. Bills, and M. S. Foster, eds. Elsevier Academic Press, Oxford, U.K. pp77-102.

Rodriguez, R.J., Redman R.S., Henson, J.M. 2004. The Role of Fungal Symbioses in the Adaptation of Plants to High Stress Environments. *Mitigation and Adaptation Strategies for Global Change*, **9**:261-272.

Rodriguez, R.J., Redman R.S., and Henson, J.M. 2004. Symbiotic Lifestyle Expression by Fungal Endophytes and the Adaptation of Plants to Stress: Unraveling the Complexities of Intimacy. In "The Fungal Community: Its Organization And Role In The Ecosystem, Ed. J. Dighton, P Oudemans & J. White. *In Press*.

Yarden, O., Ebbole, D. J., **Freeman, S.**, **Rodriguez, R. J.** and Dickman, M.B. 2003. Fungal biology and agriculture: revisiting the field. *Mol. Plant-Microbe Interact.* 16:859-866.

Appendix

Additional unrefereed publications

Horowitz S., Zveibil, A., Yarden, O., and Freeman, S. (2003). Isolation of REMI mutants of *Colletotrichum* exhibiting impaired pathogenicity on strawberry. *Phytoparasitica* 31:41

Freeman, S., Horowitz, S., Zveibil, A., and Yarden (2004).

Development of a Rapid, Reliable Screening Method for Pathogenicity of *Colletotrichum* Species on Strawberry Seedlings. 5th International Strawberry Symposium Queensland, Australia, 5th - 10th September 2004; pp. 26.

Horowitz, S., **Freeman, S.**, Zveibil, A., and Yarden, O. (2005). A defect in a NirA-like transcription factor confers morphological abnormalities and lack of pathogenicity in *Colletotrichum acutatum*. (submitted) 23rd Fungal Genetics Conference, Asilomar, Pacific Grove CA, USA.

Horowitz, S., **Freeman, S.**, Zveibil, A., and Yarden, O. (2005). A mutation in the NirA-like transcription factor causes lack of pathogenicity in *Colletotrichum acutatum* on strawberry. *Phytoparasitica* (in press).

Freeman, S., Horowitz, S., Zveibil, A., and Yarden, O. (2005). Development of a rapid, reliable screening method for pathogenicity of *Colletotrichum* species on strawberry seedlings. *Acta Hort.* (in press).

Rodriguez, R. J. and Redman R. S. (2003). The Role of Symbiotic Fungi in the Adaptation of Plants to Environmental Stress. *International Symbiosis Society, Halifax, Canada.*

Rodriguez, R. J. and Redman R. S. (2004) Fungal Symbiosis: A Potential Mechanism of Plant Invasiveness. *Third International Spartina Conference, San Francisco, California.*

Unpublished data

Briefly, unpublished data are summarized in the following paragraphs:

Construction of a gene disruption vector for *Colletotrichum* species:

We demonstrated that pGMR1 was capable of inducing gene disruption mutants in *Colletotrichum* species other than *C. magna* resulting in the conversion of pathogens to mutualists. The species were transformed as previously described and homologous integration events occurred at frequencies of 10 - 47% (Redman and Rodriguez, in preparation).

Table 1. Lifestyles expressed by wildtypes and pGMR1 gene disruption mutants

| <i>Colletotrichum</i> species | Squash | Bean | Watermelon |
|-------------------------------|----------------------|-----------------|------------|
| <i>C. magna</i> -wt | Pathogenic (P) | not tested (nt) | P |
| <i>C. magna</i> -gd* | Mutualistic (M) | nt | M |
| <i>C. orbiculare</i> -wt | P | nc | P |
| <i>C. orbiculare</i> -gd | M | nc | M |
| <i>C. lindemuthianum</i> -wt | no colonization (nc) | P | nc |
| <i>C. lindemuthianum</i> -gd | nc | M | nc |

Fungal symbiotic lifestyles were assessed as described (Redman et al., 2001) and mutualism based on symbiotically conferred disease protection. * gd = gene disruption mutant.

Our data indicated that the fungal sequences in pGMR1 were highly conserved and may be useful in defining the genetic basis of symbiosis in other plant-fungal systems. However, pGMR1 contained several duplications and some of the fungal sequence was toxic to *E. coli* making it difficult to subclone and sequence. Therefore, lambda libraries were constructed from several REMI A mutants and pHA1.3 integration sites subcloned and sequenced. The fungal DNA that flanked the pHA1.3 integration site (designated fslm1) was sequenced and does not match any known sequences in the public data bases, including fungal genome databases. Translation of fslm1 in all reading frames also did not show similarity with any known protein sequences. A new gene disruption vector (designated pGD13) was constructed that contained chloramphenicol resistance and the fslm1 sequence disrupted with the hygromycin gene cassette from pHA1.3. Eight *Colletotrichum* species were transformed with pGD13 and transformants screened for symbiotic lifestyle expression

(Redman and Rodriguez, in preparation). Between 25 and 50% of the transformants from each species expressed the same non-pathogenic lifestyle as was expressed by the REMI mutant that was used to isolate the *fslm1* sequence. We are currently analyzing the integration of pGD13 into each of the *Colletotrichum* species.

Comparison of *C. acutatum* Ca-5 mutant and wildtype

A REMI mutant, strain Ca-5 (non-pathogenic on strawberry) is disrupted in a transcription factor (Catf), containing a binuclear zinc finger cluster (Freeman et al., in preparation), similar in homology to Gal4, which binds under nitrogen deprivation to a GATA regulator. In the absence of an external nitrogen source, Ca-5 exhibited extended germ tube growth prior to appressoria formation on solid surface and strawberry leaf. Ca-5 exhibited restricted hyphal growth and did not cause lesions on plants but grew necrotrophically when inoculated directly onto wounded sites (Fig 1). The deduced amino acid sequence of the REMI-impaired gene product, designated Nir1, is highly similar to the *Aspergillus nidulans* NirA protein, a transcriptional regulator of nitrogen metabolism. GATA factors or regulators, activate expression of nitrogen catabolic pathways when a preferred nitrogen source is limited or absent, a phenomenon known as nitrogen catabolic repression. Many pathogenicity genes are induced during nitrogen starvation e.g. *Avr9* of *Cladosporium fulvum*, PSI-9 and PSI-10 of *Cladosporium fulvum*, *Mpg1* of *Magnaporthe grisea*, *pCgCS* of *C. gloeosporioides*, a suppressor of plant defense, GAL4-like protein *CLTA1* of *C. lindemuthianum*, involved in pathogenicity in bean and CLNR1, AREA/NIT2-like nitrogen regulator required for infection of *C. lindemuthianum*.

Inoculation of leaves with wild type or Ca-5 conidia in the presence of a nitrogen source resulted in massive epiphytic hyphal production, appressoria formation and rapid symptom development. The nutritional status of *C. acutatum* at an early stage of colonization and appressoria formation was assessed by following the expression of nitrate reductase (NR) and glutamine synthetase (GS) in different media. Under all growth conditions there was no effect on GS, however, NR was induced by nitrate and repressed by a rich medium. In addition, NR transcription increased at the appressoria stage, indicating that nitrogen starvation constitutes a cue for regulation of appressoria development. Our results suggest that nitrogen starvation stimulates synchronous preinfection development which is lacking in Ca-5.

Summary of achievements:

- The DNA (fslm1) responsible for the REMI A mutation (mutualism) induced gene disruption mutants in other *Colletotrichum* species converting pathogens to mutualists,
- fslm1 has been sequenced, hybridizes to several *Colletotrichum* species and does not match any known DNA or translated sequences in public data bases.
- Mutants of *C. magna* expressing a mutualistic lifestyle confer disease resistance against several fungal taxa.
- The nonpathogenic *C. acutatum* mutant (Ca-5) is disrupted in Catf, does not produce appressoria under nitrogen starvation conditions and has an identical phenotype to the wildtype infecting asymptotically on certain hosts.