BARD

FINAL REPORT

PROJECT NO. US-2814-96

**Regulation of Early Events in Hyphal
Elongation, Branching and Differentiation
of Filamentous Fungi**

M.B. Dickman, O. Yarden

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BARD Final Scientific Report
(Cover Page)

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Address & e-mail of Investigators and Institutions:

Investigators

Institutions

Principal Investigator:

Martin B. Dickman

Department of Plant Pathology

University of Nebraska - Lincoln

Lincoln, Nebraska 68583, U.S.A.

mdickman@unlnotes.unl.edu

University of Nebraska

Cooperating Investigator:

Oded Yarden

Department of Plant Pathology and Microbiology

Faculty of Agricultural, Food and Environmental

Quality Sciences

The Hebrew University of Jerusalem

Rehovot 76100, Israel

yardeno@agri3.huji.ac.il

The Hebrew University of
Jerusalem

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☐ Yes

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations. **Fungal development, kinase, phosphatase, Neurospora, Colletotrichum?????**

Abbreviations used in the report, in a order:

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Signature

Principal Investigator

Signature

Research Authority, Principal Institution

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	Joint IS/US authors	US Autho only	Israeli Aut only	Total
Refereed (published, in press, accepte		6	7	13
Submitted, in review, in preparation			1	1
Invited review papers	1			1
Book chapters		2		2
Books		1		1
Master theses				
Ph.D. theses		2		2
Abstracts				
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Patent Summary (numbers)

	Israeli inve only	US invent only	Joint IS/US inventor	Total
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Issued (allowe				
Licensed				

Cooperation Summary (numbers)

	From US t Israel	From Isr to US	Together elsewher	Total
Visits/Meetings	2	1	2	5
Sabbaticals				
Postdoctorates				

1 **Cooperation**, briefly explain whether synergistic, complementary or supportive.

We feel that the cooperation between the two investigators fits all of the descriptors mentioned above. We have collaborated on all aspects of anti COT1/TB3 antibody preparation. The design, production and analysis of the antibodies were carried out simultaneously in both labs, which are under constant contact with each other. In addition, we constantly communicated and exchanged all relevant ideas and reagents.

Apart from the constant interactions concerning both conceptual and technical aspects of the project, the two PIs prepared a joint refereed review on fungal kinases and phosphatases published in Fungal Genetics and Biology.

During the project the PIs met several times. Apart from BARD-sponsored reciprocal visits, the investigators made it a point to meet at appropriate scientific meetings (e.g. 1999 - Fungal Genetics Conference - U.S.; Crop Protection Congress - Israel). The visits were instrumental for the advance of the project and included meeting and discussions with the students involved as well as with additional colleagues who provide their professional input to the project.

Abstract

In filamentous fungi, hyphal elongation, branching and morphogenesis are in many cases, the key to successful saprophytic and pathogenic fungal proliferation. The understanding of the fungal morphogenetic response to environmental cues is in its infancy. Studies concerning the regulation of fungal growth and development (some of which have been obtained by the participating collaborators in this project) point to the fact that ser/thr protein kinases and phosphatases are (i) involved in the regulation of such processes and (ii) share common structural and functional features between saprophytes and pathogens. It is our objective to combine a pharmaceutical and a genetic approach in order to identify, characterize and functionally dissect some of the regulatory factors involved in hyphal growth, branching and differentiation. Using an immunohistochemical approach, a ser/thr protein kinase involved in hyphal elongation in both *Neurospora crassa* and *Colletotrichum trifolii* has been localized in order to identify the physical arena of regulation of hyphal elongation. The analysis of additional kinases and phosphatases (e.g., Protein kinase C, cAMP-dependent kinase, lipid-activated protein kinase, components of the type 2A protein phosphatase) as well as a RAS-related gene (an additional key participant in signal transduction) has been performed. In order to succeed in advancing the goals of this project, we have taken advantage of available elongation/branching mutants in *N. crassa* and continuously combined the accumulated information obtained while studying the two systems in order to dissect the elements involved in these processes. The various inhibitors/effectors analyzed can serve as a basis for modification to be used as anti-fungal compounds. Understanding the regulation of hyphal proliferation is a key requirement for identifying novel target points for either curbing fungal growth (as in the case of pathogenesis) or affecting growth patterns in various biotechnological processes.

The major objective of our joint project was to advance our understanding of regulation of hyphal growth, especially during early events of fungal germination. Towards achieving this goal, we have coupled the analysis of a genetically tractable organism (*N. crassa*) with a plant pathogen of economic importance (*C. trifolii*). As the project progressed we believe that the results obtained have provided a reinforcement to our basic approach which called for combining the two fungal systems for a joint research project. On the one hand, we feel that much of the advance made was possible due to the amenability of *N. crassa* to genetic manipulations. The relevance of some of the initial findings obtained in *Neurospora* have been proven to be relevant to the plant pathogen, while unique features of the pathogen have been identified in *Colletotrichum*.

Most of the results obtained from this research project have been published. Thus, the main volume of this report is comprised of the relevant publications describing the research and results obtained.

Achievements

The main achievements of this BARD-supported project are:

1. COT1 kinase: Detection, subcellular localization and immunohistochemical detection of COT1 kinase in *N. crassa* and immunodetection of the homologous TB3 kinase of *C. trifolii*.
2. Identification of the *cot-1* mutation. This was carried out by the cloning and comparative sequencing of both wild type and mutant *cot-1* cDNAs. We have identified a single base change conferring a His->Arg change within catalytic domain VII, in the vicinity of the highly conserved DFG motif.
3. Photoregulation and alternative transcription of *cot-1*. Blue light plays a key role as an environmental signal in the regulation of growth and development of fungi and plants. We have demonstrated that the *cot-1* gene encodes two transcript species of about 2100 nt (*cot-1* (s)) and about 2400 nt (*cot-1* (l)) in length, and that the ratio of both transcript species' abundance is photoregulated. The origin of the difference between *cot-1* (l) and *cot-1* (s) was localized to the 5' end of the *cot-1* transcripts, suggesting that two COT1 isoforms, with different activities may be formed. Both light effects, on branching and on *cot-1* expression, were dependent on functional *wc-1* and *wc-2* gene products.
4. Phylogenetic analysis of *C. trifolii* PKC1. Blast searches indicated that PKC1 was most similar to mammalian PKCs. However, known inducers of PKC (DAG, Phorbol ester) were ineffective in activating PKC1. A more robust analysis was undertaken in order to more precisely determine the phylogenetic placement of the enzyme. Interestingly, PAUP analysis clearly indicated PKC1 was more similar to PKA than to PKC. Thus, based on mode of regulation (see below), we have renamed this gene - LAPK (Lipid Activated Protein Kinase).
5. PKC inhibitor studies. A potent mammalian PKC inhibitor, RO-31-8220, at 10 M, completely inhibited *C. trifolii* appressorium formation. Overexpression of LAPK resulted in abnormally high numbers of appressoria formed. Specifically, we notice that in contrast to the single appressorium produced per germinating conidium of the wild type, a large majority (>75%) of the LAPK-overexpressing transformants produced 2 appressoria per conidium.
6. Analysis of LAPK induction. During the examination of LAPK regulation, we noticed that host cutin specifically and rapidly activated transcription of this gene. Transcriptional activation of LAPK occurred within 15 minutes when *C. trifolii* conidia were treated with apple cutin. Moreover, fatty acids which are abundant in plant cutin (e.g., 9,10, 16 trihydroxy palmitic acid) appear to be responsible. Thus, plant host cutin constituents activate a *C. trifolii* gene involved in appressorial formation.
7. Development of gene targeting in *C. trifolii*. Inhibitor studies also indicated an important role for cAMP-dependent protein kinase (PKA) in *C. trifolii* growth and development. We have cloned both the regulatory and catalytic subunit genes and have been successful in directing homologous recombination for the first time in *C. trifolii*. Importantly, we were able to determine that this gene encoded a functional regulatory subunit by complementation of the

Neurospora crassa mcb mutant, which has a temperature sensitive mutation in the regulatory subunit of PKA. These data further our overall approach of exploiting the sophisticated genetics of *N. crassa* to address questions in a less treatable plant pathogenic fungus (*C. trifolii*). Our results demonstrate that PKA has an important role in regulating the transition between vegetative growth and conidiation, and is essential for pathogenic development in *C. trifolii*.

8. *C. trifolii* Ras. Ras is a low molecular weight guanine nucleotide- (GDP/GTP) binding protein that transduces signals for growth and differentiation in eukaryotes. In mammals, the importance of Ras in regulating growth is underscored by the observation that activating mutations in *ras* genes are found in many animal tumors. *Colletotrichum trifolii* is a filamentous fungal pathogen of alfalfa which causes anthracnose disease. To investigate signaling pathways regulating growth and development in this fungus, a gene encoding a Ras homolog (*CT-Ras*) was cloned. CT-Ras exhibited extensive amino acid similarity to Ras proteins from higher and lower eukaryotes. A single amino acid change resulting in mutationally active CT-Ras induced cellular transformation of mouse fibroblasts (NIH 3T3) and tumor formation in *nu/nu* mice. In *Colletotrichum*, mutationally active CT-RAs induced abnormal hyphal proliferation, defects in polarized growth and significantly reduced differentiation in a nutrient-dependent manner. These results show that *C. trifolii* Ras is a functional growth regulator in both mammals and fungi and demonstrate that proper regulation of Ras is required for normal fungal growth and development.
9. Type 2A phosphoprotein phosphatase (PP2A). The major focus of this project involves analysis of the phosphorylation machinery and hyphal growth. The advances in analysis of the PKA components and the findings establishing the high significance of PKA to hyphal growth and development have convinced us to increase the emphasis on proteins which may be involved with the PKA-regulated cascade. The type 2A phosphatase complex is one such example. Linking the findings concerning PKA in *C. trifolii* with previous results demonstrating that the PP2A catalytic subunit is essential in *N. crassa* has brought us to further our analysis of the catalytic subunit and also isolate and characterize (structurally and functionally) the B regulatory subunit of this phosphatase complex. It is now obvious that the PP2A complex is important for hyphal elongation. Furthermore, it is also now very clear that this complex is involved in many developmental processes. During the course of this study and additional, structurally-related phosphatase (*pzl-1*) has also been isolated.
10. *C. trifolii* cAMP dependent protein kinase (PKA). As previous inhibitor data indicated an important role for PKA in *C. trifolii* we have cloned both the regulatory and catalytic subunit genes. When the catalytic subunit gene was insertionally inactivated by gene replacement, the transformants showed a small reduction in growth and conidiation patterns were altered relative to the wild type. Importantly, PKA deficient strains were non-pathogenic, although conidial germination and appressorium formation took place. These mutants were able to colonize host

tissue following artificial wounding. Coupled with microscopy, these data indicate that the defects in pathogenicity are likely due to a failure in penetration. The regulatory subunit gene was also characterized.

11. The Dickman lab has constructed a *C. trifolii* yeast - two hybrid library for the purpose of identifying interacting partners with TB3 (COT-1) and Ras. The TB3 "bait" constructs have been made in three ways; (i) the entire coding sequence (ii) - the catalytic domain, and (iii) the amino terminal domain. This was done to help facilitate identification of bona fide interacting proteins. These studies are ongoing.

The following is a list of publications, which have been prepared on the basis of this research project. The publications themselves appear in the following pages. As is evident from the different publications, most of the results are of fundamental nature. As mentioned in our grant application, our intent was to elucidate the basic processes involved in hyphal elongation/branching. While performing the research it has become evident that protein phosphorylation plays a key role in regulation of such processes. We believe that our results have assisted in advancing our understanding of hyphal growth in filamentous fungi, and will be assist, in the long term, to devise means for interfering with this essential phase of fungal development. Furthermore, we believe that our results will prove to have implications on additional eukaryotes. The cooperation between the collaborators has been instrumental in advancing the project (details are mentioned in the initial part of this report). The combination of two different fungal systems and the continuous and free flow of information between the research groups have been a key component for the success of this project.

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