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**Role of Phosphorylation in Fungal Spore
Germination**

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Role of phosphorylation in fungal spore germination

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II. ABSTRACT

Title: Role of Phosphorylation in Fungal Spore Germination

Spore germination is a common and fundamental event in fungal development and in many instances an essential phase of fungal infection and dissemination. Spore germination is also critical for hyperparasites to function as biocontrol agents as well as in fermentation processes. Our common objective is to understand the mechanisms which regulated spore germination and identify factors involved in pathogenicity related prepenetration development. Our approach is to exploit the overall similarity among filamentous fungi using both a plant pathogen (*Colletotrichum trifolii*) and a model system that is genetically sophisticated (*Neurospora crassa*). The simultaneous use of two organisms has the advantage of the available tools in *Neurospora* to rapidly advance the functional analysis of genes involved in spore germination and development of an economically important fungal phytopathogen. Towards this we have isolated a protein kinase gene from *C. trifolii* (*TB3*) that is maximally expressed during the first hour of conidial germination and prior to any visible gene tube formation. Based on sequence similarities with other organisms, this gene is likely to be involved in the proliferative response in the fungus. In addition, *TB3* was able to functionally complement a *N. crassa* mutant (*COT-1*). Pharmacological studies indicated the importance of calmodulin in both germination and appressorium differentiation. Using an antisense vector from *N. crassa*, direct inhibition of calmodulin results in prevention of differentiation as well as pathogenicity. Both cAMP dependent protein kinase (PKA) and protein kinase C (PKC) like genes have been cloned from *C. trifolii*. Biochemical inhibition of PKA prevents germination; biochemical inhibitors of PKC prevents appressorium differentiation. In order to analyze reversible phosphorylation as a regulatory mechanism, some

ser/thr dephosphorylative events have also been analyzed. Type 2A and Type 2B (calcineurin) phosphatases have been identified and structurally and functionally analyzed in *N. crassa* during this project. Both phosphatases are essential for hyphal growth and maintenance of proper hyphal architecture. In addition, a first novel-type (PPT/PP5-like) ser/thr phosphatase has been identified in a filamentous fungus.

The highly collaborative project has improved our understanding of a fundamental process in fungi, and has identified targets which can be used to develop new approaches for control of fungal plant pathogens as well as improve the performance of beneficial fungi in the field and in industry. In addition, the feasibility of molecular technology transfer in comparative mycology has been demonstrated.

III. ORIGINAL OBJECTIVES

- To identify, isolate and characterize protein kinases expressed in conjunction with fungal spore germination.

- To determine the role of such genes in the germination process.

The broad objectives are:

- To study the regulation of conidial dormancy/germination by analyzing a spore germination specific protein kinase in *C. trifolii*.

- To exploit *N. crassa* for complementing studies not currently feasible in *C. trifolii*.

- Verifying (to the possible extent) the information obtained in *N. crassa* in *C. trifolii*.

- To test the possibility of adopting technology available for *N. crassa* for studies in *C. trifolii* and determine the limits in the transfer of current technology (to be used

in determining feasibility of future pathogen-model co-studies).

IV. OVERVIEW OF ACHIEVEMENTS

In the original proposal, our objectives were to isolate genes involved in spore germination, evaluate the role of phosphorylation during this process, and compare a genetically well-developed model organism (*N. crassa*) with an important plant pathogen (*C. trifolii*), to examine whether parallels exist and whether the sophisticated genetic development of *N. crassa* could be used to study *C. trifolii*. All of these objectives have been addressed and our working hypotheses appear to be valid. For example, we have isolated a number of genes from *Colletotrichum trifolii* involved with "prepenetration development" (spore germination-appressorium maturation).

Table 1 - Genes Isolated from *Colletotrichum trifolii* and *Neurospora crassa*^a with support from BARD

Gene	Comment	Ref.
1. Cyclic AMP dependent protein kinase (PKA)	Both the regulatory and catalytic subunit genes has been cloned and sequenced. Pharmacological data indicates functional involvement with prepenetration morphogenesis.	Yang and Dickman (in preparation) Yang and Dickman (in press)
2. TB3-protein kinase	Functionally complements the <i>N. crassa cot-1</i> mutant. Most highly expressed during spore gemination.	Buhr, <i>et al</i> , 1996.

3. G-protein	G α subunit gene most highly expressed during prepenetration development.	Truesdell and Dickman (Unpublished)
4. Calmodulin	Pharmacological and antisense data indicates functional involvement with prepenetration morphogenesis.	Warwar and Dickman, 1996; Warwar and Dickman (submitted)
5. Histidine kinase	Sequence similarity to bacterial (and <i>Neurospora crassa</i>) two component system protein kinases, most highly expressed during prepenetration morphogenesis.	Huang and Dickman, (unpublished)
6. PKC	Inhibitor studies strongly suggest importance for appressorium development and germination Expression induced by plant cutin.	Huang, Yang and Dickman (submitted)
7. RAS	Expressed during fungal proliferation	Truesdell, Jones, Holt and Dickman (in preparation)
8. Protein phosphatase (pph-1)	PP2A catalytic subunit. Essential for hyphal growth.	Yatzkan and Yarden (1995)
9. Protein phosphatase (rgb-2)	PP2A regulatory component. Essential for hyphal growth.	Yatzkan and Yarden (unpublished)

10. Protein phosphatase (ppt-1)	PPT/PP5 like phosphatase. Transcripts present in dormant conidia and early phases of germination. Function unknown.	Yatzkan and Yarden (in press)
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^a 1-7 from *C. trifolii*

8-10 from *N. crassa*

Details

More specific details, at least in part, can be found in the publications. Of particular note are (i):

PKC

Inhibitor studies using staurosporine treated fungal spores showed that both *C. trifolii* conidial germination and appressorial differentiation were inhibited (Huang *et al*, submitted). Staurosporine completely inhibited appressorial formation at 100nM, while at 1 uM staurosporin completely prevented conidial germination.

In a study designed to establish conditions for synchronizing large scale *C. trifolii* populations for developmental studies (Buhr and Dickman, in press; supported by BARD; see publications), we found that cutin influenced germination. Interestingly, addition of cutin to an aqueous spore suspension, prior to placing on a contact surface, (which is required for appressorial differentiation) significantly enhanced PKC expression. Northern analysis of spores in various nutritional regimes confirmed that PKC is rapidly induced (30 minutes) by plant cutin. Thus, plant cutin specifically activates PKC gene expression (Huang, et al., submitted). The fact

that transcription of *pkc1* is rapidly induced by plant cutin and inhibition of this protein prevents appressorial differentiation, encourages further analysis.

TB3

Using a sequential degenerate primer approach in library screening, a ser/thr kinase from *C. trifolii* was isolated and designated *tb3*. The deduced amino acid sequence of TB3 showed high similarity to the *N. crassa*, serine/threonine protein kinase, COT1, required for hyphal elongation. The carboxyl terminal catalytic domains of TB3 and COT1 are highly conserved but the amino terminal regions are divergent, particularly in the homopolymeric glutamine repeats of TB3. Northern analysis indicated that *tb3* expression was highest one hour after inducing conidial germination and two hours before germ tubes were observed. *Tb3* transcripts were at constitutive levels by 3 hours after inducing germination. Even though some structural divergence was identified between the predicted *cot-1* and *tb3* gene products, *tb3* complemented the *cot-1* mutant of *N. crassa* demonstrating the functional conservation of this kinase between a pathogenic and a saprophytic fungus (see publications).

Other members of the serine/threonine kinase family that are TB3 related are encoded by the *warts/lats* gene of *Drosophila* and *DM* gene of human. The *warts* gene is required for the control of the amount and direction of cell proliferation as well as for normal morphogenesis. Mutations in *DM* cause myotonic cystrophy. Therefore, functional expression of those genes is required for normal cell differentiation in different organisms. TB3 is down regulated during appressorium differentiation; thus TB3 may be involved cell proliferation.

Thus, *N. crassa* was successfully used to functionally study TB3. A joint paper detailing these studies is in the publication list. In addition, an *N. crassa* vector (pSO-1) was successfully

used to examine calmodulin function in *C. trifolii* by an antisense approach. Thus, not only have we identified genes involved in spore germination and appressorium development, but we have successfully exploited *N. crassa* to facilitate such analysis, and then have demonstrated that a model organism can be used to study genetically loss tractable plant pathogens.

In summary, we have achieved our stated objectives; and hope to continue our collaborative efforts in this area.

V. COLLABORATION

Overall, both partners have experienced this project as a highly collaborative close contact effort. Dickman has visited the Yarden lab on two separate occasions, Yarden has visited the Dickman lab once. In addition, both P.I.'s have met at the Fungal Genetics Conference (Asilomar, California, US) in the spring of 1995 and 1997. Thus in addition to E-mail, FAX and "regular" mail, the P.I.'s have physically interacted on a number of occasions, which has proven most beneficial. For example, when Dickman initially visited the Yarden lab in Israel, experiments were performed that led to the Buhr et al manuscript. In addition, sharing of clones, antibodies, primers, vectors and strains have occurred throughout the duration of this proposal. Research was also greatly expedited by the elimination of redundancy and exploitation of the unique traits of different (but linked) experimental systems. Both groups have benefitted by the routine transfer of methodology and tools from *N. crassa* to *C. trifolii* and from clones and primers from *C. trifolii* to *N. crassa*. Besides the physical exchanges, the exchange of ideas and approaches greatly enhanced the spirit of experimentation and the advancement of the systems.

VI. LIST OF PUBLICATIONS

1. Dickman, M.B., Buhr, T.L., Warwar, V., Truesdell, G., and Huang, C. 1995. Molecular signals during the early stages of alfalfa anthracnose. *Canadian Journal of Botany* 73:1169-1177.
2. Warwar, V., and Dickman, M.B. 1996. Calcium-calmodulin mediated appressorium development in *Colletotrichum trifolii*. *Applied & Environmental Microbiology* 62:74-79.
3. Buhr, T.L., Oved, S., Truesdell, G.M., Huang, C., Yarden, O., and Dickman, M.B. 1996. A kinase-encoding gene from *Colletotrichum trifolii* complements a colonial growth mutant of *Neurospora crassa*. *Molecular and General Genetics* 251:565-572.
4. Yang, Z., and Dickman, M.B. 1997. Regulation of cAMP and cAMP dependent protein kinase during conidial germination and appressorium formation in *Colletotrichum trifolii*. *Physiological and Molecular Plant Pathology* (in press).
5. Buhr, T.L., and Dickman, M.B. 1997. Gene expression analysis during conidial germ tube and appressorium development in *Colletotrichum trifolii*. *Applied and Environmental Microbiology* (in press).
6. Warwar, V., and Dickman, M.B. 1997. Antisense expression of the calmodulin gene from *Colletotrichum trifolii* impairs appressorium development. *Mol. Plant-Microbe Inter.* (Submitted).
7. Yatzkan, E., and Yarden, O. 1995. Inactivation of a single type-2A phosphoprotein phosphatase is lethal in *Neurospora crassa*. *Curr. Genet.* 28:458-466.
8. Yatzkan, E., and Yarden, O. 1997. A *Neurospora crassa* PPT/PP5 subfamily serine/threonine protein phosphatase. *Biochim. Biophys. Acta* (in press).
9. Prokisch, H., Yarden, O., Mischler, M., Tropschug, M., and Barthelmiers, I.b. 1997. Impairing

calcineurin of *Neurospora crassa* reveals its essential role for hyphal growth, morphology, and maintenance of the apical Ca^{2+} - gradient. Mol. Gen. Genet. (Submitted)