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FINAL REPORT

PROJECT NO. US-3094-99

**Phosphorylative Transduction of Developmental
and Pathogenicity-Related Cues in Sclerotinia
Sclerotiorum**

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2004

BARD Final Scientific Report
Cover Page

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Project Title: Phosphorylative Transduction of Developmental and Pathogenicity-Related Cues in Sclerotinia sclerotiorum

Investigators

Institutions

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Collaborating Investigators:

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

fungal signalling, protein phosphatase, protein kinase, sclerotia morphogenesis

Abbreviations commonly used in the report, in alphabetical order:

OA, oxalic acid; MAPK, mitogen activated protein kinase; PKA, cyclic AMP dependent protein kinase; PP2A, protein phosphatase 2A

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Cover Page

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Refereed (published, in press, accepted) BARD support acknowledged	1	3		4
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Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Jeffery Rollins - currently Assistant Professor at the University of Florida
Cooperation Summary (numbers) (Social Security # is confidential)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	2	2	5
Longer Visits (Sabbaticals)	0	0	0	0

Description Cooperation:

The principal investigators have been actively collaborating for the past decade on various aspects of fungal molecular biology. The American group has focused on the MAPK/ROS/RAS and will invest efforts in further characterization of the MAPK pathway and sclerotigenesis. This included microscopic ROS level determination and signaling, expression studies, and two hybrid system screens for identification of MAPK-interacting

proteins. In parallel, the Yarden lab analyzed PKA activity during sclerotigenesis as influenced by environmental factors, inhibitors and activators. The Yarden lab also focused on PKA and PPs inactivation by antisense. The collaborative efforts span all levels defined by BARD (e.g., supportive, complementary and most important- synergistic).

Patent Summary (numbers)

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Submitted	0	0	0	0
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Final Report
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Phosphorylative Transduction of Developmental and Pathogenicity-Related Cues in *Sclerotinia sclerotiorum*

Martin B. Dickman (University of Nebraska)
Oded Yarden (Hebrew University of Jerusalem)

Abstract

Sclerotinia sclerotiorum (Lib.) de Bary is among the world's most successful and omnivorous fungal plant pathogens. Included in the more than 400 species of plants reported as hosts to this fungus are canola, alfalfa, soybean, sunflower, dry bean, and potato. The general inability to develop resistant germplasm with these economically important crops to this pathogen has focused attention on the need for a more detailed examination of the pathogenic determinants involved in disease development. This proposal involved experiments that examined the involvement of protein phosphorylation during morphogenesis (hyphal elongation and sclerotia formation) and pathogenesis (oxalic acid). Data obtained from our laboratories during the course of this project substantiates the fact that kinases and phosphatases are involved and important for these processes. A mechanistic understanding of the successful strategy(ies) used by *S. sclerotiorum* in infecting and proliferating in host plants and this linkage to fungal development will provide targets and/or novel approaches with which to design resistant crop plants including interference with fungal pathogenic development.

The original objectives of this grant included:

- I. Clone the cyclic AMP-dependent protein kinase A (PKA) catalytic subunit gene from *S. sclerotiorum* and determine its role in fungal pathogenicity, OA production (OA) and/or morphogenesis (sclerotia formation).
- II. Clone and characterize the catalytic and regulatory subunits of the protein phosphatase PP2A holoenzyme complex and determine their role in fungal pathogenicity and/or morphogenesis as well as linkage with PKA-regulation of OA production and sclerotia formation.
- III. Clone and characterize the adenylate cyclase-encoding gene from *S. sclerotiorum* and determine its relationship to the PKA/PP2A-regulated pathway.
- IV. Analyze the expression patterns of the above-mentioned genes and their products during pathogenesis and determine their linkage with infection and fungal growth.

We have made progress in all of these objectives. In addition, we have considerably expanded our efforts in two significant areas.

- I. The cloning and characterization of a pH (via oxalic acid) and reactive oxygen induced mitogen activation protein kinase (MAPK) that is required for sclerotial development (Joint Publication MPMI 17:404-413).
- II. Functional genomics – microarray development and EST/genomic sequencing (see later).

In addition, this proposal continued and expanded our efforts and long term goals of developing this important plant pathogen as a viable system for molecular, biochemical and physiological studies. As a result of these efforts, we have not only furthered our understanding of *Sclerotinia sclerotiorum* pathogenic development but also placed *Sclerotinia* in a prominent position in the fungal genomic arena. Dickman has just received funding for the development of 10,000 sclerotinia EST's (Expressed Sequences Tags) and has a \$1,000,000 grant pending (that has been recommended for funding) to obtain the complete genomic sequence for *sclerotinia*.

Summary of Achievements

- Cloned and characterized PKA catalytic subunit gene *Sclerotinia sclerotiorum*. Performed the first successful gene-knockout for this organism. (Manuscript in review)
- Cloned the PP2A holoenzyme genes from *S. sclerotiorum*. (Manuscript in preparation)
- Cloned the adenylate oxclase-encoding gene from *S. sclerotiorum*. (Currently being characterized)
- Cloned an ERK-like MAPK from *S. sclerotiorum* established novel regulation via oxalic acid and reactive oxygen. Antisense inhibition (again, a first for the fungus) demonstrated this MAPK is required for sclerotial formation. (A joint paper detailing this work has recently been published MPMI 17:404-413).
- Developed microarrays and constructed cDNA libraries.
- Obtained funding for an EST project.
- Obtained funding for the complete genomic sequence of *S. sclerotiorum*.

Thus, we have continued to develop experimental tools for this fungus, added new and important information regarding pathogenic development and placed sclerotinia in the genomic era.

Involvement of Ser/Thr kinases phosphatases in sclerotial development of *Sclerotinia sclerotiorum*

Introduction:

Sclerotinia sclerotiorum is a filamentous ascomycete phytopathogen that is able to infect a wide range of cultivated plants resulting in important economic losses (Purdy, 1979; Tu, 1997). The sclerotium, a pigmented, multihyphal structure plays a central role in the life and infection cycles of *S. sclerotiorum* and can serve in dispersal and long term survival of *S. sclerotiorum* (Adams & Ayers, 1979; Chet & Henis, 1975; Tourneau, 1979).

Phosphorylation and dephosphorylation are major post-translational modifications affecting the activity of many regulatory and structural proteins. Type 2A Serine/Threonine Phosphatases (PP2A) have an important part in the regulation of different cellular processes, such as: metabolism, cell division, growth, gene expression and signal transduction (Mayer-Yakel & Hemmings, 1994; Van & Goris, 2003). PP2B (Calcineurin) is a calcium/calmodulin dependent Ser/Thr protein phosphatase that is conserved in all eukaryotes, from *S. cerevisiae* to humans. PP2B plays a central role in the regulation of cation homeostasis, morphogenesis, cell wall integrity, and pathogenesis, in fungi (Dombradi, 1997; Fox & Heitman, 2002).

The analysis of protein kinases and phosphatases in filamentous fungi is generating accumulating evidence for the role of phosphorylative regulation in fungal morphogenesis and pathogenesis (Dickman & Yarden, 1999). However, there is lack of information concerning the role of protein phosphatases in sclerotial morphogenesis in *S. sclerotiorum*.

Dissecting the role of PP2A/PP2B/PP1 in biochemical and physiological pathways may enhance our understanding of morphological and developmental processes in phytopathogenic fungi.

In the following, we briefly describe the following project goals and accomplishments:

- I. Clone and characterize the catalytic subunit of PKA
- II. Clone and characterize MAPK
- III. Characterization of the influence of phosphatase inhibitors, such as Cantharidin a PP2A inhibitor (Li & Casida, 1992; Li *et al.*, 1993); Cyclosporin A and FK506 (PP2B inhibitors) (Fox and Heitman 2002), on the development of sclerotia.
- IV. Finding genes whose expression is altered by changes in PP2A/PP1 activity during sclerotial development.
- V. Characterizing the importance of specific genes whose expression is altered, during phosphatase inhibition of sclerotial development, by altering their expression.

I. Clone and characterize the catalytic subunit of PKA

Increased cAMP levels inhibit sclerotial development and concomitantly raise oxalic acid levels in the necrotrophic phytopathogen *Sclerotinia sclerotiorum*. These effects were hypothesized to be mediated by protein kinase A (PKA), a well characterized eukaryotic cAMP-responsive signaling protein. To test this hypothesis, a *pka* catalytic subunit gene, *pka1*, was cloned and disrupted to create a nonfunctional *pka1* allele in *S. sclerotiorum*. Southern hybridization confirmed targeted gene disruption, and northern blot analysis revealed the absence of wild-type 1.6 kb transcript in *pka1* mutants. Disruptant strains remained cAMP-responsive, exhibited wild-type-levels of PKA activity and retained wild-type pathogenicity. Phylogenetic analyses of *pka1* with various fungal *pka* genes and with the entire annotated serine-threonine kinome of *Magnaporthe grisea* and *Neurospora crassa* suggest that these filamentous fungi have 2 *pka* paralogs. Molecular, biochemical, and phylogenetic data suggest the existence of a second PKA catalytic subunit functioning in *S. sclerotiorum*. (Manuscript in review)

II. Clone and characterize MAPK

Sclerotial development is fundamental to the disease cycle of the omnivorous broad host range fungal phytopathogen *Sclerotinia sclerotiorum*. We isolated a highly conserved homolog of ERK-type Mitogen Activated Protein (MAP) kinases from *S. sclerotiorum* (Smk1) and have demonstrated that Smk1 is required for sclerotial development. *smk1* transcription and MAPK enzyme activity are dramatically induced during sclerotogenesis, especially during the production of sclerotial initials. When PD98059 (a specific inhibitor of the activation of MAPK by MAPK kinase) was applied to differentiating cultures or when antisense expression of *smk1* was induced, sclerotial maturation was impaired. *smk1* transcript levels were highest under acidic pH conditions, suggesting that Smk1 regulates sclerotial development via a pH-dependent signaling pathway, involving the accumulation of oxalic acid, a previously identified pathogenicity factor that functions, at least in part by reducing pH. Addition of cAMP inhibited *smk1* transcription, MAPK activation, and sclerotial development. Thus, *Sclerotinia sclerotiorum* can coordinate environmental signals (such as pH) to trigger a signaling pathway mediated by Smk1 to induce sclerotia formation and that this pathway is negatively regulated by cAMP. (Joint Publication; Chen et. al.; Molecular Plant Microbe Interaction 17:404-413; 2004)

Recently, we have shown the importance of reactive oxygen species (ROS) in sclerotial development (Figs. 1 & 2).

Dose dependent inhibition of paraquat (MV)
in sclerotial maturation

Fig. 1: Effect of paraquat on sclerotial development. Sclerotial agar plugs were inoculated to PDA media supplemented with paraquat at indicated concentrations. The pictures were photographed after 10 days postincubation.

ROS generation is via a Ras-MAPK dependent but
Ras-PI3K independent pathway

Fig. 2: ROS generation in sclerotial initials following different treatments. Protoplasts were generated from sclerotial initials and incubated with 50uM DCFHDA for 20 mins at room temperature prior to fluorescent microscopy. (A) Wild type isolate 1980; (B) Dominant negative Ras mutant (DNRas); (3) Wild type isolate treated with a specific P13K inhibitor; (4) Wild type isolate treated with a specific MAP kinase inhibitor.

III. The effect of phosphatase inhibitors (Cantharidin, Cyclosporin A, FK506) on sclerotial development

Synchronization of sclerotial growth. In order to characterize the effect of Cantharidin on sclerotial development sclerotial formation was synchronized by an induction process. The synchronization was achieved by filtering macerated hyphae over Whatman paper and plating the juphal matt on glass beads that are covered by a liquid medium. In this system, following the desiccation process, the lower section of the hyphae is in contact with the medium and the upper section is in contact with the air, which initialize sclerotia formation.

Cantharidin inhibits sclerotia formation. The concentration of Cantharidin in the medium is in reverse proportion to the number of sclerotia that are formed and in proportion to the size of the sclerotia. The EC_{50} of Cantharidin for sclerotia formation is approximately $70\mu\text{M}$.

Cantharidin influences the morphogenesis of *S. sclerotiorum*. In the presence of Cantharidin there is a decrease in sclerotia size and rigidity. Furthermore there is an increases in melanin production which is proportional to the concentration of Cantharidin (Fig 3).

Fig. 3. Growth of *S. sclerotiorum* in the presence of different concentrations of Cantharidin.

Cyclosporin A (a PP2B inhibitor) inhibits hyphal growth ($EC_{50}=486\text{nM}$). The concentration of Cyclosporin A in the medium is in reverse proportion to area of the colony grown on Joham's minimal solid medium (Fig. 4).

The effect of Cyclosporin A on hyphal growth

Fig. 4. Growth of *S. sclerotiorum* in the presence of different concentrations of Cyclosporin A.

2.40nM of Cyclosporin A inhibited sclerotia formation by approximately 40%, while 90nM of FK506 (a PP2B inhibitor) inhibited sclerotia formation by approximately 60%. Cyclosporin A was also found to induce sclerotia germination. We have tested germination on Joham's solid medium that was pre-incubated for 5 minutes with 0.1% (w/v) Bromocresol Green (Sigma B-6771), as an indicator for changes in pH. Sclerotia were suspended for 1 minutes with 10% sodium hyper chloride, to eliminate the possibility of external hyphal growth, prior to 5 minutes incubation with 4.98 μ M of Cyclosporin A. Sclerotia that acidified the medium surrounding them, identified by yellow haloes of the pH indicator, were considered as germinating. In the presence Cyclosporin A sclerotial germination was increased by approximately 30%. This phenomena did not repeat itself with general inhibitors such as Polyoxin D (a chitin synthase inhibitor) or Hygromycin, indicating that the increase in sclerotia germination We have also found that 3 non-producing sclerotia mutants, (SA-1, SA-3, SA-4) (Godoy et al, 1997) are less susceptible to Cyclosporin A than the WT. Growth of SA-1, SA-3, SA-4 on Joham's solid media containing 190nM Cyclosporin A inhibited hyphal growth by approximately 60%, while the same concentration inhibited hyphal growth in of the WT by 80%.

IV. Identification of genes whose expression is altered during inhibition of sclerotial development by protein phosphatase inhibitors

In order to identify genes whose expression is altered during sclerotia formation we employed a transcriptional profiling approach based on microarray slides spotted with *S. sclerotiorum* cDNA clones.

Process stages:

1. Total RNA was extracted from sclerotia and sclerotia initials.
2. Messenger RNA was extracted from the total RNA and was used in order to produce a CDNA library of *S. sclerotiorum*.
3. PCR products of 3000 inserts of the cDNA library libraries were produced.
4. The products were spotted on glass slides.
5. The microarrays were used in order to identify genes whose expression is altered during sclerotia formation under different conditions.
6. The data obtained in the microarray experiments was further analyzed.

Microarray preparation

1. cDNA library production.

In order to examine the quality of the cDNA library, the inserts of ninety cDNA clones were sequenced and analyzed by BLAST search in NCBI. 16 of the cDNA clones (out of 90) had a sequence with significant similarity to proteins that play a role in a variety of cellular functions.

As part of the collaborative activity, a second *S. sclerotiorum* cDNA library from the Dickman lab was also amplified utilized for the microarray production. This process was employed in order to enrich the variety and number of genes on the microarray slides.

2. Spotting the dsDNA extracted from the cDNA clones by PCR reactions.

Using the OminoGridTM robot (GeneMachines) in Professor D. Galbrath's lab (Arizona), 3000 cDNA clones were spotted on 200 glass slides (Clontech type I).

3. cDNA probe synthesis.

The cDNA probes were synthesized in Robert Fluhr's lab (Weizmann Inst. of Science). The total RNA was amplified by in vitro transcription using the MessageAmp kit (Ambion) in the presence of amino allyl nucleotide (Sigma) to which the Cy dyes (Cy3, Cy5, Amersham) were attached. The probe was then purified using a PCR purification kit (Stratagene) and concentrated to the appropriate volume with a centrifuge based filter device (Amicon). Each probe was analyzed in order to determine the presence of at least 0.2µg cDNA and an incorporation rate of 20-50 labeled nucleotides per 1000 nucleotides.

Results of hybridization experiments

A hydration time of -20 seconds over a 42°C water bath gave the best results by increasing the uniformity of the spots with minimum smear by byproducts.

The control experiment gave hybridization signals with local background in some of the areas and "comet tails" for some of the spots. Blocking procedure with or without 1% SDS produced high background on the microarray slides. Washes with high volume decreased the background phenomena. Treating the slides with 1% SDS prior to hybridization reduced some of the "comet tail" phenomena and improved the signals. The combination of high volume washes after hybridization and treatment with 1% SDS produced the best results so far (Fig. 5).

In further experiments we intend to try different SDS washing conditions in order to achieve even better hybridization signals with lower background and "comet tail" phenomena.

The initiation of the microarray strategy was made possible by the fact that BARD had agreed to divert part of the Yarden lab funding towards supporting Arie Harel's (a graduate student in the Yarden lab) visit to David Galbraith's lab in Tucson, Arizona.

A

B

Fig. 5. Effect of detergent amendment on uniformity and clarity of cDNA spot visualization on glass slides. **A.** Standard large volume (500 ml) wash. **B.** Wash with 1% (w/v) SDS.

The effect of Cantharidin on gene expression in *S. sclerotiorum*

A cDNA probe mixture was synthesized from total RNA isolated from synchronized sclerotia- producing cultures of *S. sclerotiorum* grown in either the presence or absence of Cantharidin (75 μ M). The slides were treated with 1% SDS and subjected to prehybridization. Hybridization was performed at 42°C.

Transcription profile analysis

Two hybridizations were performed. Separate images were acquired using the ScanArray 4000 software (Packard BioScience) for each fluorescence emission at a resolution of 10 μ m per pixel, adjusting the photomultiplier and laser power to achieve an optimal distribution of signals without minimal saturation. Image analysis was performed using QuantArray version 3 software (Perkin-Elmer Life Sciences, Boston, MA). Spots signals were quantified using the histogram method, where the histogram for each pixel in the patch is calculated, producing four parameters (signal low, signal high, background low, background high) that are used to quantify the spots. Spots with visible defects were

omitted from further analysis by flagging. Data analysis was performed with GeneSpring 4.2 software (Silicon Genetics, Redwood City, CA) applying "one-per-chip" normalization where the signal of each gene was divided by the median intensity of the chip.

Computational analysis

In order to further analyze the results from the microarray experiments we utilized a database application (BioCloneDB) developed in our lab. The GeneSpring files containing the data of the different treatments from the microarray results, were automatically inserted into BioCloneDB in a new "project" (*Sclerotinia* Microarray/Cantharidin) using the "array data management" section. The sequence of 200 clones was also automatically inserted into the BioCloneDB using the "import clone" section of the application. The feature of the application that enables to sort out the vector sequence from the sequence of the insert was utilized as well. The sequence of the 200 clones was compared to updated sequence information, from NCBI and EMBL databases using the BLAST annotation section of the application. This section enabled to perform an automatic BLASTn and BLASTx search for all the sequences simultaneously.

The databases combined the integrated information of the microarray result files and the sequence analysis of the 200 clones. The integrated information of the microarray results and sequence information was analyzed by the array data filtering section of the application. The data mining of the integrated information was performed according to the ratio data. Initially we searched for clones that showed at least a 2-fold increase or decrease in the expression intensity in the presence of Cantharidin, in repeated hybridization experiments. This information was automatically connected to the sequence analysis and the BLAST results, thus enabled user friendly and fast automatic analysis.

Fig. 6. Analysis of the transcription profiles produced from *S. sclerotiorum* grown either in the presence or absence of Cantharidin (75 μ M) for 72h. **A.** Distribution of intensity signals. **B.** Comparison of the gene expression profile represented by 3000 cDNA clones. red and blue spots are plotted exterior to the parallel green lines, indicating two folds increase or decrease in intensity level accordingly.

Approximately 4500 spots, representing 1500 cDNA clones, gave a signal significantly higher than the background (figure 3). Among them we identified 144 spotted cDNA clones (in triplicate) that showed at least a 2-fold increase in expression intensity in the presence of Cantharidin, in repeated hybridization experiments. We also identified 49 cDNAs whose signal intensity was reduced by at least 50% in the presence of the inhibitor. Among 45 sequenced clones representing a signal ratio higher than 3-fold or lower then 0.33-fold, 5 were found to encode proteins with similar sequences present in the Swissprot database (Table 1).

Table 1. Examples of *S. sclerotiorum* genes whose expression is altered in the presence of Cantharidin and have matches in Swissprot. **A.** Upregulated genes. **B.** Downregulated genes. The ratio value is a fraction calculated by dividing the intensity level in the presence of Cantharidin by the intensity level in the control media.

A.

Clone	Ratio	BLAST results	Organism	E value	Identity
49 clones	<0.5	No homologue in NCBI			
5h1	0.195	Tetrahydroxynaphthalene (T4HN reductase) red	<i>Magnaporthe grisea</i>	4X10 ⁻⁶⁵	80%
20f8	0.28	Heat shock protein 82 (Hsp82)	<i>Ajellomyces capsulatus</i>	5X10 ⁻²⁰	64%
2d5	0.305	Hypothetical 33.8 kDa protein, belong to sc7/sc14 family	<i>Saccharomyces cerevisiae</i>	3X10 ⁻¹⁰	48%

B.

Clone	Ratio	BLAST results	Organism	E value	Identity
144 clones	>2	No homologue in NCBI			
32e6	3.375	Septin homologue spn2	<i>Schizosaccharomyces pombe</i>	2X10 ⁻⁷⁸	68%
4 clones	7-13	Carboxyvinyl-carboxyphosphonate phosphorylmutase (CPEP)	<i>Streptomyces hygroscopicus</i>	4X10 ⁻²⁵	41-42%

Verification of the microarray results.

A macroarray of 49 genes whose expression was altered by at least 3-fold in the presence of Cantharidin, was utilized in order to verify the expression of those genes in the presence of Cantharidin. The expression pattern of 16 genes was similar to the results from the microarray experiments. For further verification and research, real-time RT-PCR analysis will be utilized for several selected genes. This microarray may be also utilized to examine the expression of those genes during sclerotial development.

V. Genetic analysis of selected genes

In order to further examine the importance of 3 selected genes in sclerotial development, the expression of several selected genes will be suppressed:

1. PP2B. A fragment of ~1000bp of PP2B from *S. sclerotirum* was cloned in the Yarden lab and used for producing the appropriate construct (see below).
2. Clone 31H10, whose expression was shown to increase by 3.82 fold in the presence of Cantharidin. This clone consists of a sequence that did not have any significant similarity to any known gene.
3. Septin homologue spn2, whose expression was shown to be up regulated (~3 fold) in the presence of Cantharidin. A fragment of ~1000bp of Septin homologue spn2 from *S. sclerotirum* was cloned during the preparation of the *S. sclerotirum* cDNA library as described above (section 3).

In order to suppress the expression of the mentioned genes two approaches are being used:

1. Antisense. The selected genes fragments will be inserted in an inverted manner between the terminator and the promoter of a plasmid vector.
2. RNA mediated interference (RNAi) using two possible constructs: A construct containing two *N. crassa* cpc-1 promoters with the desired gene for silencing between them. RNAi silencing based on hairpin loop, created by two uneven fragments of a certain gene inserted into the construct in opposite directions.

Hygromycin-resistance clones that will contain morphological changes interesting for further analysis will be tested for the suppression of the silenced gene by real-time PCR analysis. Further analysis of the transformants may include, testing for susceptibility to phosphatase inhibitors, changes in development, changes in PKA relative activity, changes in phospho-MAPKs (ERK1/2 or HOG homologs) levels, and pathogenesis analysis.

Suppression of PP2B, current status

A 280bp fragment of PP2B was amplified using degenerative primers. This fragment was used as a probe in order to search in *S. sclerotiorum* genomic library. Approximately 87% of the gene was sequenced from cosmid containing the PP2B gene.

A *Sma*I-*Sna*BI fragment of 1000bp was excised and ligated into pSO-1. pSO-1 was originated from pWFas51, a vector described by Fecke et al. (1993). pSO-1 was further manipulated in our lab to carry the hygromycin phosphotransferase gene (*hph*) as a selectable marker. An antisense PP2B construct was made by insertion of the inverted PP2B fragment into the *Sma*I site of pSO-1 between the quinic acid promoter and terminator (Fig. 7).

Fig.7. Plasmid PSB2, containing a 1000bp fragment of PP2B inserted at the *Sma*I site.

Anti-PP2B transformants grown for two weeks on minimal medium containing 15 mM quinic acid, did not produce sclerotia (Fig. 8A). The transformants grown on control medium, containing 15mM glucose, produced sclerotia and had a growth pattern, including the production of initials, which was more similar to WT. Quinic acid did not inhibit sclerotial growth in WT cultures (Fig. 8B).

Fig. 8 A. Growth of an Antisense-PP2B transformant for 14 days, on minimal medium containing 15mM glucose (left) or 15mM quinic acid (right). Medium was also supplemented with 50 ug/ml hygromycin **B.** Growth of the WT on minimal medium containing 15mM glucose (left) or 15mM quinic acid (right).

Further analysis will be performed with this transformant in order to verify that the non-sclerotial phenotype is related to reduction of PP2B in the gene and protein levels. Additional analysis will include testing for susceptibility to phosphatase inhibitors, changes in physiology and morphology, changes in PKA activity, changes in phospho-MAPK (ERK1/2 or HOG homologs) levels, and pathogenesis analysis.

Future Directions

The long-term goals of our research are to understand the regulation of sclerotial development and pathogenicity in *S. Sclerotiorum*. In the future, we will focus on the elucidation of the signaling events and environmental cues involved in the regulation of these processes. To achieve these goals, we will take a "two-pronged" approach which will be based on both a "biased" [selection and analysis of specific candidate genes that are associated with these processes e.g., PKA/MAPK/RAS, as well as phosphoprotein phosphatases (PPs), that counter kinase activity] and an "unbiased" (e.g., seeking new genes based upon analysis of expression patterns during sclerotigenesis) approach. In both cases we will utilize (and continue to develop) tools our research groups have established and/or adapted for analysis of *S. sclerotiorum*.

We will:

1. Follow activities and function of *S. sclerotiorum* PKA.
2. Identify and functionally evaluate effectors of the *S. sclerotiorum* ERK-like MAPK signaling pathway.
3. Perform structural and functional analysis of genes whose expression is altered under conditions affecting either PKA and/or MAPK.
4. Begin functional genomics analysis for *S. sclerotiorum* genome.

Along these lines of primary objectives, emphasis will be put on (i) determining if and how PKA and MAPK act in concert during this developmental phase and what role upstream and downstream elements play in this process. This will include the analysis of Ser/Thr PPs, Ras - an upstream element common to these pathways, and a set of novel genes whose expression is altered during sclerotial development and (ii) Evaluation of the effect of external stimuli (especially pH and ROS) on the activity of the PKA and MAPK pathways and their influence/involvement in sclerotigenesis.

All work performed will be supportive, as we share a common question (understanding sclerotial development) and are studying a common organism (*S. sclerotiorum*) while utilizing shared tools developed in our respective laboratories.

Publications resulting from this work:

Cessna, S.G., Sears, V.E., Dickman, M.B., and Low, P.S. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the host plant oxidative burst. *The Plant Cell* 12:2191-2199.

Chen, C. and Dickman, M.B. 2003. pH regulated MAP kinase signaling pathways in *Sclerotinia sclerotiorum*. US-Israel Binational Agricultural Research and Development Fund (BARD) workshop on molecular perspectives on fungal biology and pathology: current status/future research directions (Lake Tahoe).

Harel, A. and Yarden, O. 2003. Cantharidin, an inhibitor of type 2A phosphatases inhibits hyphal growth and sclerotia formation in *Sclerotinia sclerotiorum*. US-Israel Binational Agricultural Research and Development Fund (BARD) workshop on molecular perspectives on fungal biology and pathology: current status/future research directions (Lake Tahoe).

Rollins, J.A. and Dickman, M.B. 2001. pH signaling in *Sclerotinia sclerotiorum*: Identification of a *pacC/RIMI* homolog. *Applied and Environmental Microbiology* 67:75-81.

Lorang, J.M., Turoi, R.P., Martinez, J.P., Sawyer, T.L., Redman, R.S., Rollins, J.A., Wolpert, T.J., Johnson, K.B., Rodriguez, R.J., Dickman, M.B., and Ciuffetti, L.M. 2001. Green fluorescent protein is lighting up fungal biology. *Applied and Environmental Microbiology* 67: 1987-1994.

Publications in preparation:

Chen, C., Harel, A., Gorovits, R., Yarden, O., and Dickman, M.B. 2004. Regulation of sclerotial development in *Sclerotinia sclerotiorum* is linked with pH and cAMP sensing. *Molecular Plant Microbe Interaction* 17: 404-413.

Harel, A., Gorovits, R., Chen, C., Dickman, M.B., and Yarden, O. 2004. Altered cAMP-dependent kinase and MAPK activity levels accompany sclerotogenesis in *Sclerotinia sclerotiorum*.

Reuveni, E., Leshkowitz, D. and Yarden, O. 2004. BioCloneDB: a database and web interface to manage sequences and gene expression data.

References relevant to the final project report:

Adams, P. B. & Ayers, W. A. (1979). Ecology of *Sclerotinia* species. *Phytopathology* 69, 896-899.

Chet, I. & Henis, Y. (1975). Sclerotial morphogenesis in fungi. *Annual Review Phytopathology* 13, 169-192.

- Dickman, M. B. & Yarden, O. (1999).** Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genet. Biology* **26**, 99-117.
- Dombradi, V. (1997).** Comparative analysis of Ser/Thr protein phosphatases. *Trends Com. Biochem. Physiology* **3**, 23-48.
- Fecke, W., Nehls, U., and Weiss, H. (1993).** An antisense RNA expression vector for *Neurospora crassa*. *Fungal Genet. Newsletter* **40**: 34-35.
- Fox, D. S. & Heitman, J. (2002).** Good fungi gone bad: the corruption of calcineurin. *BioEssays* **24**, 894-903.
- Godoy, G., J. R. Steadman, M. B. Dickman, and R. Dam (1990).** Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiol. Mol. Plant Pathology* **37**:179-91.
- Howe, A. K. & Juliano, R. L. (2000).** Regulation of anchorage-dependent signal transduction by protein kinase A and p21-activated kinase. *Nat. Cell Biology* **2**, 593-600.
- Li, Y. M. & Casida, J. E. (1992).** Cantharidin binding protein: identification as protein phosphatase 2A. *Proc. National Academy Sciences* **89**, 11867-11870.
- Mayer-Yakel, R. & Hemmings, B. (1994).** Protein phosphatase 2A- a 'menage a trois'. *Trends Cell Biology* **4**, 287-291.
- Purdy, L. H. (1979).** *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution and impact. *Phytopathology* **69**, 875-880.
- Tourneau, D. L. (1979).** Morphology, cytology, and physiology of *Sclerotinia* species in culture. *Phytopathology* **69**, 887-890.
- Tu, J. C. (1997).** An integrated control of white mold (*Sclerotinia sclerotiorum*) of beans, with emphasis on recent advances in biological control. *Bot. Bull. Acad. Sin.* **38**, 73-76.
- Van, H., C. & Goris, J. (2003).** Phosphatases in apoptosis: to be or not to be, PP2A is in the heart of the question. *Biochim Biophys Acta* **1640**, 97-104.
- Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J. & Sturgill, T. W. (1993).** Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science* **262**, 1065-1069.
- Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J. & Sturgill, T. W. (1999).** Crosstalk between protein kinase A and growth factor receptor signaling pathways in arterial smooth muscle. *Cell Signal* **11**, 465-477.

BARD Final Scientific Report

Cover Page

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1	11		12
Submitted, in review, in preparation	1	2		3
Invited review papers	1			1
Book chapters				
Books				
Master theses				
Ph.D. theses	1	1		2
Abstracts		3		3
Not refereed (proceedings, reports, etc.)				

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

Dr. Jeffery Rollins – currently Assistant Professor at the University of Florida
Cooperation Summary (numbers) (Social Security # is confidential)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	2	2	5
Longer Visits (Sabbaticals)	0	0	0	0

Description Cooperation:

The principal investigators have been actively collaborating for the past decade on various aspects of fungal molecular biology. The American group has focused on the MAPK/ROS/RAS and will invest efforts in further characterization of the MAPK pathway and sclerotigenesis. This included microscopic ROS level determination and signaling, expression studies, and two hybrid system screens for identification of MAPK-interacting proteins. In parallel, the Yarden lab analyzed PKA activity during sclerotigenesis as influenced by environmental factors, inhibitors and activators. The Yarden lab also focused on PKA and PPs inactivation by antisense. The collaborative efforts span all levels defined by BARD (e.g., supportive, complementary and most important-Patent Summary (numbers) synergistic).

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	0	0	0
Licensed	0	0	0	0