Cover Page

BARD Project Number: IS-3947-06

Date of Submission of the report: March 2011

Project Title: Identification of Genetic Determinants that Facilitate Development of *B. cinerea* at Low Temperature and its Postharvest Pathogenicity

Investigators
Principal Investigator (PI): Dr. Amnon Lichter

Institutions
ARO, The Volcani Center

Co-Principal Investigator (Co-PI): Dr. Maria .R. Davis, Univrsity of Alabama

Collaborating Investigators: Prof. Gopi.K. Podila, University of Alabama

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

bR – Bacteriorhodopsin

HP1- Hypothetical protein 1

Abbreviations commonly used in the report, in alphabetical order:

Budget: IS: \$ 165,000

US: \$ 159,000

Total: \$ 324,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	0	1	1	2
Submitted, in review, in preparation			1	1
Invited review papers				
Book chapters			1	1
Books				
Master theses				
Ph.D. theses	1			1
Abstracts	2		6	8
Not refereed (proceedings, reports, etc.)	2			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.

1. Pandey, Ajay K [pandeyak@iastate.edu]
2. Pranjali hazarika [mailto:pranjalhazarika@gmail.com]

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	0	1	2	3
Longer Visits (Sabbaticals)	0	0	0	0

Description Cooperation:

Coordination of expression conditions for proteins and genes

Joint presentations -South Africa

Determination of expression level of genes identified in the US by IS.

Monthly discussions by skype

Patent Summary (numbers)

	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

Abstract

Botrytis cinerea is the postharvest pathogen of many agricultural produce with table grapes, strawberries and tomatoes as major targets. The high efficiency with which *B. cinerea* causes disease on these produce during storage is attributed in part due to its exceptional ability to develop at very low temperature. Our major goal was to understand the genetic determinants which enable it to develop at low temperature. The specific research objectives were: 1. Identify expression pattern of genes in a cold-enriched cDNA library. 2. Identify *B. cinerea* orthologs of cold-induced genes 3. Profile protein expression and secretion at low temperature on strawberry and grape supplemented media. 4. Test novel methods for the functional analysis of cold-responsive genes. Objective 1 was modified during the research because a microarray platform became available and it allowed us to probe the whole set of candidate genes according to the sequence of 2 strains of the fungus, BO5.10 and T4. The results of this experiment allowed us to validate some of our earlier observations which referred to genes which were the product of a SSH suppression-subtraction library. Before the microarray became available during 2008 we also analyzed the expression of 15 orthologs of cold-induced genes and some of these results were also validated by the microarray experiment. One of our goals was also to perform functional analysis of cold-induced genes. This goal was hampered for 3 years because current methodology for transformation with 'protoplasts' failed to deliver knockouts of bacteriorhodopsin-like (bR) gene which was our primary target for functional analysis. Consequently, we developed 2 alternative transformation platforms, one which involves an air-gun based technique and another which involves DNA injection into sclerotia. Both techniques show great promise and have been validated using different constructs. This contribution is likely to serve the scientific community in the near future. Using these technologies we generated gene knockout constructs of 2 genes and have tested their effect on survival of the fungus at low temperature. With reference to the bR genes our results show that it has a significant effect on mycelial growth of the *B. cinerea* and the mutants have retarded development at extreme conditions of ionic stress, osmotic stress and low temperature. Another gene of unknown function, HP1 is still under analysis. An ortholog of the yeast cold-induced gene, CCH1 which encodes a calcium channel and was shown to be cold-induced in *B. cinerea* was recently cloned and used to complement yeast mutants and rescue them from cold-sensitivity. One of the significant findings of the microarray study involves a T2 ribonuclease which was validated to be cold-induced by qPCR analysis. This and other genes will serve for future studies. In the frame of the study we also screened a population of 631 natural *B. cinerea* isolates for development at low temperature and have identified several strains with much higher and lower capacity to develop at low temperature. These strains are likely to be used in the future as candidates for further functional analysis. The major conclusions from the above research point to specific targets of cold-induced genes which are likely to play a role in cold tolerance. One of the most significant observations from the microarray study is that low temperature does not induce 'general stress response in *B. cinerea*', which is in agreement to its exceptional capacity to develop at low temperature. Due to the tragic murder of the Co-PI Maria R. Davis and Gopi Podila on Feb. 2010 it is impossible to deliver their contribution to the research. The information of the PI is that they failed to deliver objective 4 and none of the information which relates to objective 3 has been delivered to the PI before the murder or in a visit to U. Alabama during June, 2010. Therefore, this report is based solely on the IS data.

Achievements

1. Overcoming transformation bottlenecks in *B. cinerea*. The objectives of the study included functional analysis of cold-induced genes. To achieve this goal on candidate genes it is necessary to generate knockout constructs which can take considerable time, to transform the fungus and to select for gene replacement events. The Botrytis community has been using mainly ‘protoplast’ based transformation which requires an enzyme cocktail to remove cell walls and large amounts of construct DNA. Although a routine procedure in few labs, this process often generates a bottleneck in analysis of gene function. In our hands, this procedure often failed to generate transformants and those that were shown to possess the selective marker did not result in gene replacement. To resolve this issue we adopted 2 alternative approaches, the air-gun approach (following a publication for *Sclerotinia sclerotiorum*) and injection of DNA to sclerotia (following an idea of Dr Maggie Levi). Both methods resulted in robust generation of transformants most of which ended up to be gene replacements. The procedure requires much less DNA and does not depend on the variable quality of the enzymatic cocktail. The process has been validated for the bR gene, the HP1 gene and a complementation of bR knockout strains. A short communication describing these results has been prepared and the process is being validated by other labs (Appendix 1).

2. Identification of orthologs of cold-induced genes. We have screened the microbial literature for cold-induced genes which is largely based on studies in yeast, psychrophilic fungi and bacteria. The main focus was based on fatty acid desaturases (FAD) which modify membranes to fit them to low temperature by removing double bonds from the hydrocarbon tail to fatty acids. We identified 2 orthologs of FADS in *B. cinerea* and analyzed their expression by qPCR. The results for strains BO5.10 and T4 (Appendix 2) showed induction of FAD1 after 4 h at 4°C and FAD2 after 1 h but only in strain BO5.10. RNA helicases (RH) are another family of cold-induced genes in bacteria which are supposed to resolve RNA structure which tends to condense at low temperature. Of the 2 *B. cinerea* orthologs, RH1 was shown to be induced in BO5.10 with a maximum at 4 h but similar pattern was not observed in strain T4. In contrast, RH2 was repressed in strain BO5.10 and did not change much in strain T4. Another gene which was shown in yeast to be involved in cold response is the calcium channel CCH1 (Appendix 2.3). As observed in other examples, CCH1 was strongly induced in BO5.10 at 4 h and the response in T4 was attenuated. Hsp104 is a stress induced gene which was previously shown to be strongly induced by heat and ethanol (Lichter et al.,

2003). Both *Botrytis* strains showed about 5-fold induction of the gene at 1 or 4 h which is considered a very mild response for this type of genes.

3. Genes previously shown to be cold induced in *Botrytis*. Of the genes which were previously identified from a SSH library, bR, HP1, HP2 and HP3 show a high scale of induction compared to previous examples (Appendix 3). This result verifies previous data obtained by Northern analysis and suggests that genes identified by SSH are likely to be strongly repressed at regular temperature. Of those genes HP2 (LITAF-like zinc ribbon domain) and HP3 (unique to fungi) do not have an assigned function but have homologs in other fungi while HP1 (surface protein pentapeptide repeat domain) seems to be unique to *Botrytis*. bR is a bacterio-rhodopsin like gene which binds retinal and is believed to be a light induced ion channel in bacteria without a known function in fungi.

4. Microarray analysis of cold response in *B. cinerea*. The results above yield a detailed analysis of particular candidate genes. Microarray analysis should give in principal a complete picture of cold response in *B. cinerea*. After very long debates and unduly stalling in the *Botrytis* community, a microarray platform was finally prepared with over 21,000 of candidate genes from the 2 sequenced strains. The NimbleGen array was composed of 3 probes per gene on a 72,000 spots 4-plex array. The hybridization and readout was performed by NimbleGen with RNA from strain BO5.10 which was exposed to 4°C or 22°C for 1, 4, 10 and 24 h in 3 replications. Some replications had to be discarded due to low quality and the remaining replications were characterized by low dynamic range which means with a maximum of about 20-fold induction compared to the background. This means that the results of the microarray were not to be considered quantitative but to compensate for that fact, the confidence level was increased to a level of 0.01 instead of the standard P value of 0.05. A list of 50 cold-induced genes with putative functions is shown (Appendix 4). It validates results for HP3 and bR. Other genes which were validated by the microarray experiment with lower t-values were: HP2 ($pV \leq 0.1$) and HP1 ($pV \leq 0.8$). One of the prominent genes which were highly expressed following transition to low temperature was a T2 ribonuclease. To date there is no information in the literature with respect to a possible role of this ribonuclease in cold response and it may turn out into a novel discovery once further explored.

5. Functional analysis of bR a cold-induced bacterioRhodopsin gene. The bR gene was shown to have a robust cold-induction in many experiments. To elucidate its potential role in cold-response gene replacement cassette was generated (Appendix 1),

Fig. 1). The cassette was introduced to strain BO5.10 and gene replacement events were screened with external and internal primers. The 4 knockout strains displayed retarded growth and sporulation (Appendix 5) compared to ectopic strain with intact bR gene. Under acidic conditions in liquid medium the strains showed retarded growth which was temperature-independent and on basic medium growth was retarded in a temperature dependent manner (Appendix 5). The effect of the mutation on pathogenicity did not give a clear picture since some knockout mutants were more pathogenic while others were similar in response to the WT (not shown) suggesting that the bR gene is not limiting pathogenicity under the assay conditions.

6. Functional analysis of CCH1. The CCH1 gene was shown to be cold-induced in strain BO5.10. To verify the function of the *B. cinerea* gene it was cloned into a yeast vector (pGML10) and transformed into a yeast strain lacking its native gene as well as a wild type yeast strain (BYa8405). The results (appendix 6.1, appendix 6.2) show that the Botrytis gene (BcCCH1) was able to complement the mutant yeast strain which could not develop in a media contains high level of calcium and that the additional copies of the Botrytis CCH1 enhanced the development of the yeast strain under those conditions. The assay carried out at 12°C and 30°C showed that if the medium contained 0.1 M CaCl₂, the mutant strain could not develop and it began to die while at the higher temperature it developed to a very similar level as did the wild type strain.

7. Exploring cold tolerance in natural isolates of *B. cinerea*.

Although not part of the original objectives of the project, we utilized a collection of natural Botrytis isolates which were collected by Dr Nadia Korolev in the Volcani Center to explore the range of cold tolerance among natural isolates of Botrytis. The collection consisted of 631 strains which was designed to probe fungicide resistance was assayed in 48-well culture plates incubated at 2.5°C or 22°C. After repeated assays we identified 67 isolates with high or low growth at low temperature. These isolates will be used in future studies as genetic resource to explore genetic components which are important for growth at low temperature.

8. Assay of the development of *B. cinerea* on grapes at low temperature by qPCR.

We used the Botrytis-specific primers that we produced to follow the development of *B. cinerea* on grape berries at low temperature (Celik et al., 2010). In this study, we showed that *B. cinerea* could be detected in grape berries to the level of 1-2 cells per berry. The slope of fungal development at 20°C was 0.97 compared to 0.11 at 0°C with a ratio of approximately 10-fold faster development at the higher temperature.

Significance of main achievements

The study removed significant bottleneck of functional analysis in Botrytis by producing a robust transformation method for Botrytis. This achievement which is likely to serve the Botrytis community at large, facilitated detailed functional analysis of 2 knockout constructs which was much less than planned according to the proposal. However, we broadened the scope of the research by including microarray analysis which was not available at the time the proposal was written. We were therefore able to identify, yet uncharacterized targets for cold-induced genes. These targets are likely to impact the field in the near-future. Another major resource obtained by the current research is the collection of natural isolates which show accelerated or reduced capacity to develop at low temperature and are likely to serve for future studies.

Agricultural or economic impacts

As for now, no direct benefit is foreseen to this research. However, the identification of genes induced in cold-response can target future applied research to functions which were not previously thought of. For example, it is shown in our study that bacteriorhodopsin mutants are significantly retarded in development at low temperature and are hypersensitive to osmotic stress. This implied that treatments which will antagonize the function of the light-driven pump may inhibit have significant impact at low temperature.

Details of cooperation (if objectives were promoted by the collaboration)

The tragic death of the US BARD partners Dr Maria Davis and Dr Gopi Podila prohibited chances for transfer of final materials. Materials which were previously transferred are not considered of significance. An attempt was made to retrieve materials by a visit to Alabama in June 2010 but none of the data presented by ex-students could be considered of value to the initial objectives of the proposal.

Publications

1. Celik, M., Kalplunov, T., Zutahy, Y., Ish-shalom, S., Lurie, S., and Lichter, A. (2009). Quantitative and qualitative analysis of *Botrytis* inoculated on table grapes by qPCR and antibodies. *Postharvest Biol. Technol.* **52**, 235-239.
2. Ish-Shalom, S. and Lichter, A. (2009). Analysis of Fungal Gene Expression by Real Time Quantitative PCR In: *Methods in Molecular Biology - Molecular and Cell Biology Methods for Fungi*. Ed. Sharon. A. Humana Press.
3. Celik, M., Kaplunov, T., Zutchi, Y., Ish-Shalom, S., Lurie, S., and Lichter., A. (2007). Detection *Botrytis* infection during cold storage of grapes by qPCR, green fluorescent protein and antibodies. The 5th International Table Grape Symposium, Somerset West, South Africa.
4. Ish-Shalom, S. Kaplunov, T., Zutchi, Y., Lurie, S., Celik, M., Pandey, A.J., Davis, M. R. and Lichter, A. (2007). Monitoring the development of *Botrytis cinerea* at low temperature. The XIV International Botrytis Symposium, Cape Town, South Africa.
5. Lichter, A., Ish-Shalom, S., Kaplunov, T., Zutchi, Y., Lurie, S., Pandey, A.J. and Davis, M. R. (2008). Development of postharvest pathogens at low temperature: the *Botrytis cinerea*-grapes interaction. The 9th International Congress of Plant Pathology, Torino, Italy.
6. Ish Shalom, S., Ophir, R. and Lichter. A. (2009). Global gene expression analysis of the fungus *Botrytis cinerea* at low temperature. Israeli Society of Microbiology.
7. Ish Shalom, S., Ophir, R. and Lichter, A. (2010). Global transcriptome analysis of *Botrytis cinerea* at low temperature. The 31th meeting of the Israeli Society of Plant Pathology, Bet Dagan, Israel.
8. Ish Shalom, S. and Lichter, A. (2010). Changes in expression during growth of *Botrytis cinerea* at low temperature. The XV International Botrytis Symposium, Cadiz, Spain.
9. Ish-Shalom, S. and Lichter, A. (2011). Importance of bacterio-Rhodopsin gene for growth at low temperature in the fungus *Botrytis cinerea*. The 32th meeting of the Israeli Society of Plant Pathology, Bet Dagan, Israel.
10. Ish-Shalom, S. and Lichter, A. (2011). Changes in gene expression of *Botrytis cinerea* at low temperature and functional analysis of a bacteriorhodopsin-like gene. The 26th Fungal Genetics Conference, Asilomar, California.

Bold – peer reviewed publications