BARD

FINAL REPORT

PROJECT NO. IS-3499-03

**Identification and Characterization of Genes Involved
in the Acquisition of Chilling Tolerance in Citrus Fruit**

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Cover Page**BARD Project Number:** IS-3499-03R**Date of Submission of the report:** December 30, 2007**Project Title:** Identification and characterization of genes involved in the acquisition of chilling tolerance in citrus fruit**Investigators****Principal Investigator (PI):**

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Grapefruit, conditioning, cold stress, transcript profiling, cDNA libraries.

Abbreviations commonly used in the report, in alphabetical order:

CD, conditioning; CI, chilling injury; ESTs, expressed sequence tags, HWB, hot water brushing;

Budget: IS: \$150,000

US: \$150,000

Total: \$300,000

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	2			2
Submitted, in review, in preparation	1			1
Invited review papers	1			1
Book chapters				
Books				
Master theses			1	1
Ph.D. theses				
Abstracts	1			1
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.

Pilar Maul

Cooperation Summary (numbers)

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

Description Cooperation:

The US and Israeli partners were in continuous cooperation by e-mails to discuss the research plan and share data. As part of the research, Porat's lab constructed cDNA libraries from grapefruit and shipped them to McCollum's lab in Florida to conduct the EST sequencing project. Consequently, McCollum's lab sent requested cDNA clones to Portat's lab in Israel for further research and characterization. At last, and most important, Dr. Porat spent an entire year (sabbatical stay) at Prof. Charles Guy lab in the University of Florida, and during that time performed regular working visits to McCollum's lab at the USDA, Fort Pierce, FL.

Patent Summary (numbers)

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

Abstract

Citrus, like many other tropical and subtropical fruit are sensitive to chilling temperatures. However, application of a pre-storage temperature conditioning (CD) treatment at 16°C for 7 d or of a hot water brushing (HWB) treatment at 60°C for 20 sec remarkably enhances chilling tolerance and reduces the development of chilling injuries (CI) upon storage at 5°C.

In the current research, we proposed to identify and characterize grapefruit genes that are induced by CD, and may contribute to the acquisition of fruit chilling tolerance, by two different molecular approaches: cDNA array analysis and PCR cDNA subtraction. In addition, following the recent development and commercialization of the new Affymetrix Citrus Genome Array, we further performed genome-wide transcript profiling analysis following exposure to CD and chilling treatments.

To conduct the cDNA array analysis, we constructed cDNA libraries from the peel tissue of CD- and HWB-treated grapefruit, and performed an EST sequencing project including sequencing of 3,456 cDNAs from each library. Based on the obtained sequence information, we chose 70 stress-responsive and chilling-related genes and spotted them on nylon membranes. Following hybridization the constructed cDNA arrays with RNA probes from control and CD-treated fruit and detailed confirmations by RT-PCR analysis, we found that six genes: *lipid-transfer protein*, *metallothionein-like protein*, *catalase*, *GTP-binding protein*, *Lea5*, and *stress-responsive zinc finger protein*, showed higher transcript levels in flavedo of conditioned than in non-conditioned fruit stored at 5 °C. The transcript levels of another four genes: *galactinol synthase*, *ACC oxidase*, *temperature-induced lipocalin*, and *chilling-inducible oxygenase*, increased only in control untreated fruit but not in chilling-tolerant CD-treated fruit.

By PCR cDNA subtraction analysis we identified 17 new chilling-responsive and HWB- and CD-induced genes. Overall, characterization of the expression patterns of these genes as well as of 11 more stress-related genes by RNA gel blot hybridizations revealed that the HWB treatment activated mainly the expression of stress-related genes (*HSP19-I*, *HSP19-II*, *dehydrin*, *universal stress protein*, *EIN2*, *1,3;4-β-D-glucanase*, and *SOD*), whereas the CD treatment activated mainly the expression of lipid modification enzymes, including *fatty acid disaturase2 (FAD2)* and *lipid transfer protein (LTP)*.

Genome wide transcriptional profiling analysis using the newly developed Affymetrix Citrus GeneChip® microarray (including 30,171 citrus probe sets) revealed the identification of three different chilling-related regulons: 1,345 probe sets were significantly affected by chilling in both control and CD-treated fruits (chilling-response regulon), 509 probe sets were unique to the CD-treated fruits (chilling tolerance regulon), and 417 probe sets were unique to the chilling-sensitive control fruits (chilling stress regulon). Overall, exposure to chilling led to expression governed arrest of general cellular metabolic activity, including concrete down-regulation of cell wall, pathogen defense, photosynthesis, respiration, and protein, nucleic acid and secondary metabolism. On the other hand, chilling enhanced various adaptation processes, such as changes in the expression levels of transcripts related to membranes, lipid, sterol and carbohydrate metabolism, stress stimuli, hormone biosynthesis, and modifications in DNA binding and transcription factors.

Achievements

Significance of main scientific achievements and innovations

In order to gain a better understanding of the molecular mechanisms involved in the responses of citrus fruit to low temperatures and in acquisition of chilling tolerance, we aimed to isolate and characterize chilling-responsive and HWB- and CD-induced genes from grapefruit peel tissue.

To fulfill this task, we first conducted two cDNA libraries from grapefruit flavedo tissue after exposure to HWB and CD treatments and performed an EST sequencing project including sequencing of 3,456 cDNAs from each library (Table 1). All of the cDNA sequences were submitted to the GeneBank database, and the obtained data was used to choose selected clones for further expression analysis studies.

Table 1. Summary of EST sequencing projects of grapefruit flavedo cDNA libraries.

	HWB cDNA library	CD cDNA library	Total
# ESTs	3,456	3,456	6,912
# good sequences	2,930	2,101	5,031
# contigs	325	197	522
# singlets	1,705	1,447	3,152
# unique sequences	2,030	1,644	3,674

Overall, we identified in this research chilling-responsive and HWB- and CD-induced genes by three different approaches: 1) PCR cDNA subtraction, 2) self constructed cDNA arrays, and 3) transcript profiling using the commercial Affymetrix Citrus GeneChip[®] microarray.

The data achieved from the PCR cDNA subtraction analysis was published in J. Exp. Bot. (2006) 57: 2943-2953. In this work we identified 17 new HWB- and CD-induced genes and characterized the expression patterns of 11 more stress-related genes, and concluded that HWB activated mainly the expression of stress-related genes(*HSP19-I*, *HSP19-II*, *dehydrin*, *universal stress protein*, *EIN2*, *1,3;4-β-D-glucanase*, and *SOD*), whereas CD activated mainly the expression of lipid modification enzymes (*FAD2*, *lipid transfer protein*).

The data achieved from the self constructed cDNA arrays study was summarized in the manuscript of Maul et al. (submitted). Overall, based on the EST sequencing project (Table 1), we constructed nylon arrays including 70 grapefruit stress-responsive and chilling-related cDNAs, and among them identification six genes (*lipid-transfer protein*, *metallothionein*, *catalase*, *GTP-binding protein*, *Lea5*, and *zinc finger protein*) that showed higher transcript levels in flavedo of conditioned fruit, whereas the transcript levels of four

genes (*galactinol synthase*, *ACC oxidase*, *lipocalin*, and *chilling-inducible oxygenase*) increased in control untreated fruit but not in chilling-tolerant CD-treated fruit.

The data achieved from the genome-wide transcript profiling analysis using the commercial Affymetrix Citrus GeneChip[®] microarray was summarized in the manuscript of Maul et al., (2008) recently accepted for publication in *Plant, Cell & Environment* (in press). In this study, we identified 9,127 probe sets whose expression was significantly ($P \leq 0.001$) affected by either the conditioning or the chilling treatment. Among them, 1,762 probe sets (505 up-regulated and 1,260 down-regulated) were differentially expressed by at least 4-fold in chilling-sensitive control fruits, and 1,854 probe sets (549 up-regulated and 1,305 down-regulated) were differentially expressed in CD-treated chilling-tolerant fruits. Further analysis revealed that 1,345 probe sets were affected by chilling in both control and CD-treated fruits (this group of genes was termed chilling-response regulon), 509 probe sets were unique to the CD-treated fruits (chilling tolerance regulon), and 417 probe sets were unique to the chilling-sensitive control fruits (chilling stress regulon). Overall, global transcript profiling studies revealed that exposure to chilling led to expression arrest of general cellular metabolic activity, including concrete down-regulation of cell wall, pathogen defence, photosynthesis, respiration, and protein, nucleic acid and secondary metabolism. On the other hand, chilling enhanced adaptation processes that involve changes in the expression patterns of transcripts related to membranes, lipid, sterol and carbohydrate metabolism, stress stimuli, hormone biosynthesis, and modifications in DNA binding and transcription factors (Fig. 1).

Fig. 1. A model describing the molecular mechanisms involved in the responses of grapefruit to chilling.

Agricultural impacts of the research findings

The current study provides the first ever genome-wide transcriptional profiling analysis performed to identify and characterize the molecular components involved in the acquisition of chilling tolerance in citrus fruits, an horticultural important chilling-sensitive commodity. The results suggest that acquisition of chilling tolerance is a multi trait phenomenon, involving modification in various cellular aspects and biochemical pathways, such as lipid, sterol and carbohydrate metabolism, stress stimuli, hormone biosynthesis, and DNA binding and transcription factors. This study further resulted in identification of key genes that may be of crucial agricultural importance and contribute to elevation of chilling tolerance.

Details of cooperation

The US and Israeli partners were in tight and continuous cooperation throughout the entire research project. First, Porat's lab constructed cDNA libraries from grapefruit and shipped them to McCollum's lab in Florida to conduct the EST sequencing project. Consequently, McCollum's lab sent requested cDNA clones to Portat's lab in Israel for further research and characterization. At last, and most important, Dr. Porat spent an entire year (sabbatical stay) at Prof. Charles Guy lab in the University of Florida, and during that time performed regular working visits to McCollum's lab at the USDA, Fort Pierce, FL. Porat (PI) and McCollum (Co-PI) also met at the Gordon postharvest research conference held in the US at July 2006.

List of publications

- Sapitnitskaya, M., Maul, P., McCollum, G.T., Guy, C.L., Weiss, B., Samach, A., Porat, R. (2006). Postharvest heat and conditioning treatments activate different molecular responses and reduce chilling injuries in grapefruit. *J. Exp. Bot.* 57: 2943-2953.
- Porat, R., Guy, C.L. (2007). Arabidopsis as a model system to study chilling tolerance mechanisms in plants. *Plant Stress* 1: 85-92.
- Maul, P., McCollum, G.T., Popp, M., Guy, C.L., Porat, R. (2008a) Transcriptome profiling of grapefruit flavedo following exposure to low temperature and conditioning treatments uncovers principal molecular components involved in chilling tolerance and susceptibility. *Plant Cell Environ.* (In press).
- Maul, P., McCollum, G.T., Guy, C.L., Porat, R. (2008b). Temperature conditioning alters transcript abundance of genes related to chilling stress in 'Marsh' grapefruit flavedo. *J. Amer. Soc. Hort. Sci.* (Submitted).

Appendix

1. Published papers:

Sapitnitskaya et al (2006). *J. Exp. Bot.* 57: 2943-2953.

Porat, R., Guy, C.L. (2007). *Plant Stress* 1: 85-92.

2. Accepted papers:

Maul et al. (2008a). *Plant Cell Environ.* (In press).

3. Submitted papers:

Maul et al. (2008b). *J. Amer. Soc. Hort. Sci.* (Submitted).

4. Poster presentations:

McCollum et al. (2007). ASHS conference.