Final Scientific Report

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BARD Project Number: US-3575-04 R

Date of Submission of the report: October 9, 2007

Project Title: Citric Acid Metabolism and mobilization in citrus fruit

Investigators

Principal Investigator (PI): Eduardo Blumwald

Co-Principal Investigator (Co-PI): Avi Sadka

Collaborating Investigators:

Institutions

Dept. Plant Sciences, University
of California, Davis

Dept of Fruit Trees, Volcani
Institute, ARO.

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
ion homeostasis; juice sac cell; vacuole; proteomics; sugar to acid ratio

Abbreviations commonly used in the report, in alphabetical order:

Budget: IS: \$162,000

US: \$163,000

Total: \$325,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Final Scientific Report

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	3			3
Submitted, in review, in preparation	2			2
Invited review papers				
Book chapters				
Books				
Master theses			1	1
Ph.D. theses			1	1
Abstracts	9			9
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. Ehud Katz SIN#619-49-9279

Cooperation Summary (numbers)

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

Description Cooperation:

During the last three years, Sadka and Blumwald groups have maintained a tight and dynamic collaboration. Dr. Katz (working in Blumwald's lab) has periodically visited Sadka's lab in Israel, and Dr. Sadka have visited Blumwald's lab at UC Davis. The treatment and collection of plant material is done in parallel at the Volcani Center and the University of California, ensuring common methodologies and identical treatments, strengthening the significance of the data obtained.

Patent Summary (numbers)

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

ABSTRACT

Accumulation of citric acid is a major determinant of maturity and fruit quality in citrus. Many citrus varieties accumulate citric acid in concentrations that exceed market desires, reducing grower income and consumer satisfaction. Citrate is accumulated in the vacuole of the juice sac cell, a process that requires both metabolic changes and transport across cellular membranes, in particular, the mitochondrial and the vacuolar (tonoplast) membranes. Although the accumulation of citrate in the vacuoles of juice cells has been clearly demonstrated, the mechanisms for vacuolar citrate homeostasis and the components controlling citrate metabolism and transport are still unknown. Previous results in the PIs' laboratories have indicated that the expression of a large number of proteins is enhanced during fruit development, and that the regulation of sugar and acid content in fruits is correlated with the differential expression of a large number of proteins that could play significant roles in fruit acid accumulation and/or regulation of acid content. The objectives of this proposal are: i) the characterization of transporters that mediate the transport of citrate and determine their role in uptake/retrieval in juice sac cells; ii) the study of citric acid metabolism, in particular the effect of arsenical compounds affecting citric acid levels and mobilization; and iii) the development of a citrus fruit proteomics platform to identify and characterize key processes associated with fruit development in general and sugar and acid accumulation in particular. The understanding of the cellular processes that determine the citrate content in citrus fruits will contribute to the development of tools aimed at the enhancement of citrus fruit quality.

Our efforts resulted in the identification, cloning and characterization of CsCit1 (*Citrus sinensis* citrate transporter 1) from Navel oranges (*Citrus sinensis* cv Washington). Higher levels of *CsCit1* transcripts were detected at later stages of fruit development that coincided with the decrease in the juice cell citrate concentrations (Shimada et al., 2006). Our functional analysis revealed that CsCit1 mediates the vacuolar efflux of citrate and that the CsCit1 operates as an electroneutral 1CitrateH²⁻/2H⁺ symporter. Our results supported the notion that it is the low permeable citrateH₂⁻ the anion that establishes the buffer capacity of the fruit and determines its overall acidity. On the other hand, it is the more permeable form, CitrateH²⁻, which is being exported into the cytosol during maturation and controls the citrate catabolism in the juice cells. Our Mass-Spectrometry-based proteomics efforts (using MALDI-TOF-TOF and LC²-MS-MS) identified a large number of fruit juice sac cell proteins and established comparisons of protein synthesis patterns during fruit development. So far, we have identified over 1,500 fruit specific proteins that play roles in sugar metabolism, citric acid cycle, signaling, transport, processing, etc., and organized these proteins into 84 known biosynthetic pathways (Katz et al. 2007). This data is now being integrated in a public database and will serve as a valuable tool for the scientific community in general and fruit scientists in particular. Using molecular, biochemical and physiological approaches we have identified factors affecting the activity of aconitase, which catalyze the first step of citrate catabolism (Shlizerman et al., 2007). Iron limitation specifically reduced the activity of the cytosolic, but not the mitochondrial, aconitase, increasing the acid level in the fruit. Citramalate (a natural compound in the juice) also inhibits the activity of aconitase, and it plays a major role in acid accumulation during the first half of fruit development. On the other hand, arsenite induced increased levels of aconitase, decreasing fruit acidity. We have initiated studies aimed at the identification of the citramalate biosynthetic pathway and the role(s) of isopropylmalate synthase in this pathway. These studies, especially those involved aconitase inhibition by citramalate, are aimed at the development of tools to control fruit acidity, particularly in those cases where acid level declines below the desired threshold. Our work has significant implications both scientifically and practically and is directly aimed at the improvement of fruit quality through the improvement of existing pre- and post-harvest fruit treatments.

ACHIEVEMENTS

Significance of main scientific achievements and innovations

Citrate homeostasis in the fruit juice cell: Our efforts resulted in a better understanding of citrate metabolism and transport across the tonoplasts. We cloned and characterized a citrus vacuolar citrate transporter, *CsCit1*, from Washington navel juice vesicles (Shimada et al., 2006). Higher levels of *CsCit1* transcript were detected at later stages of fruit development that coincided with the decrease in the juice cell citrate concentrations and the raise in the juice pH (Shimada et al., 2006). *CsCit1* mediates the electroneutral vacuolar efflux of citrate and suggested that *CsCit1* operates as an electroneutral 1CitrateH²⁻/2H⁺ symporter. In addition to characterize vacuolar citrate homeostasis, we also identified key steps of citrate catabolism in fruits. Aconitase, which catalyzes the first step of acid catabolism, is a key regulatory step of acid metabolism and accumulation. While inhibition of the mitochondrial form plays a role in acid accumulation early in fruit development, induction of the cytosolic enzyme is important for acid decline during the second half of fruit development. Our studies have identified key regulatory elements of aconitase and showed that the cytosolic form, and not the mitochondrial form of aconitase, that is sensitive to iron limitation and may induce acid level at fruit maturation (Schlizerman et al., 2007). Arsenite, which reduces acid level, induce the expression and activity of aconitase. Citramalate, a natural compound in the juice, inhibit the activity of aconitase, and probably plays a role in acid accumulation.

The citrus proteome: Our original objective was to identify changes occurring in the juice sac cell vacuoles during development. We isolated juice sacs from fruits at different growth states (50-60, 60-70, 70-80 and >80 mm diameter) representing stages II and III in fruit development. We isolated the vacuolar membranes and identified tonoplast proteins using Two-Dimensional PAGE (2D gels) and Coomassie-blue staining. We identified 290 spots which displayed significant changes in their expression during fruit development. The gel spots were analyzed using Mass Spectrometry (MALDI-TOF-TOF, ABI 4700), and the identified spectra were used to search the NCBI non-redundant (green plants) and the citrus ESTs databases (<http://cgf.ucdavis.edu/home/>) using a MASCOT algorithm (<http://www.matrixscience.com/>). Hundred-sixty five high scored proteins expression changes were identified between the four developmental stages such as the down regulation of 14-3-3, V-ATPases, glutathione S-transferases, etc., and the up-regulation of calnexins, Rab1C, ABA stress ripening-like protein, isoflavone reductases, SEC12, etc (Katz, E; Fon, MG; Sadka, A.; Phinney, B; Blumwald, E. unpublished results). In addition to the 2D gels, we used a complementary approach for the high throughput identification of juice sac proteins using LC-MS/MS (LTQ-FT). Citrus fruit juice sacs were collected and fractionated in order to isolate the soluble proteins and tonoplast-, plasma

membrane-, ER/Golgi- , and mitochondria-enriched fractions. The different protein fractions were digested by trypsin and analyzed by LC-MS/MS. The peptide sequences were identified by database searching of uninterpreted fragment ion mass spectra using MASCOT. We have identified 1,394 fruit proteins and assigned function to 1,247 proteins, and integrated these proteins in 84 biosynthetic known pathways. Our results clearly show that in during the acid decline of fruits there is a significant amount of citrate used for the synthesis of proteins. In addition, our results show that the sucrose in the fruits is not only delivered by the leaves, but there is also sucrose synthesis in the leaves. These results are of high relevance since they provide support to the notion of regulation of sucrose content by synthesis in the fruit. These processes could provide important practical applications not only for the regulation of sugar content, but also to the development of treatments that could change the fruit sugar and acid balance and as a consequence represent targets for the manipulation of abscission processes, control of fruit size, fruit quality, etc (Katz et al., 2007). We have made available our results in our website and are now building an interactive website for the use of the research and user community.

Agricultural and/or economic impacts of the research findings

The immediate benefits are two-fold: 1) the enhancement of fruit quality during pre-harvest (treatment of trees and fruit); 2) The development of new post-harvest practices (handling, packaging and storage) aimed at optimizing these processes to obtain fruits with enhanced sweetness and optimal acidity. In addition, the identification of the fruit-specific protein determinants of fruit quality could facilitate the development of fast and reliable diagnostic kits for the assessment of quality (composition, disorders, etc) in the field; and 3) The understanding of the biochemical processes involved in the establishment of the citrus fruit sugars and the TSS/TA ratio will enable the development of criteria to be used by growers and breeders to optimize overall fruit quality. The long-term benefits are: 1) The identification of key proteins that determine fruit quality will provide invaluable tools for the generation of quality control criteria to be used by breeders, growers and regulatory agencies; 2) The identification of genes coding for the sugar, acid and ion transporters in juice fruit cells will serve for the development of quality improvement programs. The discovery of unique citrus genes will provide an economical basis needed by the citrus industry.

Our Proteomics work, combined with the biochemical/molecular characterization of the processes mediating citrate catabolism, revealed a number of interesting observations, among these, evidence showing that citric acid is converted during development to amino acids in the fruits and that fruits contain the enzymes (MHK, gamma-methyl-gamma-hydroxy-alpha-ketoglutaric aldolase and 2-isopropylmalate synthase) capable to synthesize citramalate. Citramalate is a powerful inhibitor of the

cytosolic aconitase, the enzyme that mediates the conversion of citric acid to isocitrate the first required step for the decline of citric acid in the fruits. In order to assess a possible physiological means to arrest the acid decline in fruits, we demonstrated that citramalate can inhibit aconitase both *in vivo* and *in vitro* and that the citramalate-induced inhibition of aconitase resulted in increase citrate contents in citrus calli juice cells and trees sprayed with a solution containing 10 mM citramalate 2.5 months before harvest (these experiments were carried out at the Volcani Center, Israel). The results obtained will serve as a basis for the development of novel field treatments aimed at the improvement of traits associated with fruit quality.

Details of cooperation

The outcome of this project depends on the complementary expertise offered by each lab. Sadka's lab brought to this project their expertise in citrus growth and handling, the molecular characterization of key genes that play an important role in citrate synthesis, and the biochemical characterization of the gene products. Sadka's lab has made already significant progress in the biochemical characterization of the reactions mediated by aconitase and the synthesis of citramalate. Blumwald's lab developed the proteomic tools and the bioinformatics needed for the construction of the citrus functional database. Dr. Ehud Katz and Mr. Mario Fon (Blumwald's lab) were trained in the use of MALDI-TOF-TOF, LC²-MS-MS, GC-MS, HPLC, etc, and much of the methodology employed in the functional complementation of transporters and their role in homeostasis have been pioneered in Blumwald's lab. The synergistic collaboration between the laboratories started four years ago, with the sabbatical leave of Dr. Sadka at the laboratory of Dr. Blumwald. During this time, both researchers developed all of the methodologies needed for the isolation of juice cells, the purification of intact vacuoles, the preparation of citrus membrane fractions and the methods for 2-D-PAGE. During the last three years, Sadka and Blumwald groups have maintained a tight and dynamic collaboration. Dr. Katz (working in Blumwald's lab) has periodically visited Sadka's lab in Israel, and Dr. Sadka have visited Blumwald's lab at UC Davis. The treatment and collection of plant material is done in parallel at the Volcani Center and the University of California, ensuring common methodologies and identical treatments, strengthening the significance of the data obtained.

List of Publications, Papers in refereed journals.

- Shimada, T., Nakano, R., Shulaev, V., Sadka, A. and Blumwald, E. (2006). Vacuolar citrate/H⁺ symporter of citrus juice cells. *Planta* 224:472-480.
- Katz E., Fon M., Nakano, R., Phinney B. S, Sadka A. and Blumwald E. (2007). The Citrus fruit proteome: insights into citrus fruit metabolism. *Planta* 226:989-1005.
- Schlizerman, L. , Marsh, K., Blumwald, E. and Sadka, A. (2007). Iron limitation induces citric acid accumulation and reduces cytosolic aconitase activity in citrus. *Physiol. Plant.* 131: 72-79.

APPENDIX

Papers in preparation

- Nakano, R., Sadka, A., Fon, M., Fu, L. & Blumwald, E. (2007) Post-translational regulation of vacuolar invertase activity in juice sac cells of orange fruit. (to be submitted, J. Exp. Bot).
- Gabriel Bardosh, Alina Sorkina, Luidmila Schlizerman, Samuel Kalderon, Eduardo Blumwald and Avi Sadka (2007). Ovary- and pulp-specific promoter from citrus is active in tomato reproductive organs and fruit

Abstracts of Presentations in Scientific Meetings

- Katz E., Esumi T., Fon M., Nakano, R., Sadka A., Phinney B. S. and Blumwald E. (2007). Fruit Sugar Transport and Sugar Homeostasis: Identification, gene expression and localization analysis of citrus juice sac cell vacuolar sugar transporters and Identification of the Citrus Fruit Proteome. Int'l Plant Membrane Biology Meeting, Valencia, Spain.
- Katz E., Esumi T., Fon M., Nakano, R., Sadka A., Phinney B. S. and Blumwald E. (2007). Fruit Sugar Transport and Sugar Homeostasis: Identification, gene expression and localization analysis of citrus juice sac cell vacuolar sugar transporters American Society of Plant Biologists, Chicago.
- Katz E., Fon M., Nakano, R., Phinney B. S., Sadka A. and Blumwald E. (2007). The citrus fruit proteome. American Society of Plant Biologists, Western Section, Davis, CA.
- Katz, E., Esumi, T., Nakano, R., Sadka, A. and Blumwald, E. (2007). Identification, gene expression and localization analysis of citrus juice sac cell vacuolar sugar transporters. American Society of Plant Biologists, Western Section, Davis, CA.
- Katz E., Fon M., Nakano, R., Phinney B. S., Sadka A. and Blumwald E. (2006). The Citrus fruit proteome. American Society of Plant Biologists, Boston.
- Shimada, T; Nakano R; Sadka A, and E. Blumwald. (2006). Vacuolar citrate transporter of Navel orange juice cells. II American Plant Membrane Transport Meeting. Texas, USA.
- Bardosh G., Schlizerman L, Kalderon S, Sorkin A, Blumwald E and Sadka A (2006) Ovary- and pulp-specific promoter from citrus is active in tomato reproductive organs and fruit ISHS International Congress, Seoul, Korea
- Nakano, R., Sadka, A., Fon, M., Fu, Lianhai & Blumwald, E. (2005) Post-translational regulation of vacuolar invertase activity in juice sac cells of orange fruit. American Society of Plant Biology Annual Meeting, Seattle.
- Shimada, T., Nakano, R., Hui, Z., Sadka, A., & Blumwald, E. (2005). Vacuolar citrate transporter of Navel orange juice sac cells. American Society of Plant Biology Annual Meeting, Seattle.