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Molecular Genetic Analysis of Citric Acid Accumulation in Citrus Fruit

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Project Title: Molecular Genetic Analysis of Citric Acid Accumulation in Citrus Fruit

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

Citrate, Acidity

Abbreviations commonly used in the report, in alphabetical order:

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Signature
Principal Investigator

Signature
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Refereed (published, in press, accepted)	1	1	3	5
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Invited review papers		1		1
Book chapters				
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Master theses				
Ph.D. theses				
Abstracts		1	4	5
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.

Denqui Fang, SSN: 621-72-7534 _____

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Cooperation Summary (numbers)

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

Description of Cooperation:

1. Exchange of clones for molecular and co-segregation analyses.
2. Combining genetic and molecular biology expertise and approaches for solving problems.
3. Mutual visits to review progress and planning additional experiments.
4. Continuous exchange of knowledge, ideas and results.

Patent Summary (numbers)

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

ABSTRACT

The acid content of the juice sac cells is a major determinant of maturity and fruit quality in citrus. Many citrus varieties accumulate acid in concentrations that exceed market desires, reducing grower income and consumer satisfaction. Pulp acidity is thought to be dependent on two mechanisms: the accumulation of citric acid in the vacuoles of the juice sac cells, and acidification of the vacuole. The major aim of the project was to direct effort toward understanding the mechanism of citric acid accumulation in the fruit. The following objectives were suggested:

1. Measure the activity of enzymes likely to be involved in acid accumulation, and follow their pattern of expression in developing fruit (Sadka, Erner).
2. Identify and clone genes, which are associated with high and low acid phenotypes and with elevated acid level (Roose, Sadka, Erner).
3. Convert RAPD markers that map near a gene that causes low acid phenotype to specific codominant markers (Roose).
4. Use genetic cosegregation to test whether specific gene products are responsible for low acid phenotype (Roose and Sadka).

Objective 1 was fully achieved. Most of the enzymes of organic acid metabolism were cloned from lemon pulp. Their expression was studied during fruit development in low and high acid varieties. The activity and expression of citrate synthase, aconitase and NADP-isocitrate dehydrogenase (IDH) were studied in detail. The role that each enzyme plays in acid accumulation and decline was evaluated. As a result, a better understanding of the metabolic changes that contribute to acid accumulation was achieved. It was found that the activity of the mitochondrial aconitase is greatly reduced early in high-acid fruits, but not in acidless ones, suggesting that this enzyme plays an important role in acid accumulation. In addition, it was demonstrated that increases in the cytosolic forms of aconitase and NADP-IDH towards fruit maturation play probably a major role in acid decline. Our studies also demonstrated that the two mechanisms that contribute to fruit acidity, vacuolar acidification and citric acid accumulation, are independent, although they are tightly co-regulated. Additionally, we demonstrated that sodium arsenite, which reduce fruit acidity, causes a transient inhibition in the activity of citrate synthase, but an induction in the gene expression. This part of the work has resulted in 4 papers. Objective 3 was also fully achieved. Using bulked segregant analysis, three random amplified polymorphic DNA (RAPD) markers were identified as linked to *acitric*, a gene controlling the acidless phenotype of pummelo 2240. One of them, which mapped 1.2 cM from *acitric* was converted into sequence characterized amplified region (SCAR) marker, and into codominant restriction length polymorphism (RFLP) marker. These markers were highly polymorphic among 59 citrus accessions, and therefore, they should be useful for selecting seedling progeny heterozygous for *acitric* in nearly all crosses between pummelo 2240 and other citrus genotypes. This part of the project resulted in one paper. Objective 4 was also fully achieved. Clones isolated by the Israeli group were sent to the American laboratory for cosegregation analysis. However, none them seemed to cosegregate with the low acid phenotype. Both laboratories invested much effort in achieving the goals of objective 2, namely the isolation of genes that are elevated in expression in low and high acid phenotypes, and in tissue cultures treated with arsenite (a treatment which reduces fruit acidity). However, conventional differential display and restriction fragment differential display analyses could not identify any differentially expressed genes. The isolation of such genes was the major aim of a continuation project, which was recently submitted.

ACHIEVEMENTS

Development of markers linked to *acitric* gene (USA)

Fruit juice pH, titratable acidity or citric acid content was measured in 6 populations derived from an acidless pummelo (pummelo 2240) [*Citrus maxima* (Burm.) Merrill]. The acidless trait in pummelo 2240 is controlled by a single gene called *acitric*. Using bulked segregant analysis, three random amplified polymorphic DNA (RAPD) markers were identified as linked to *acitric* (Paper #1). RAPD marker OpZ20₄₁₀, which mapped 1.2 cM from *acitric*, was cloned, sequenced and a sequence characterized amplified region (SCAR) marker (SCZ20) was developed. The SCZ20-410 marker allele that is linked to the *acitric* allele occurs only in pummelo 2240 and other pummelos, and therefore, this SCAR marker should be useful as a dominant or codominant marker for introgressing *acitric* into mandarins and other citrus species. Using the cloned OpZ20₄₁₀ band as a hybridization probe revealed a codominant RFLP marker called RFZ20 that mapped 1.2 cM from *acitric*. Progeny homozygous for the *acitric* allele (genotype *acac*) had citric acid content in juice below 10 μ m, the minimum detection level by HPLC. The citric acid content of fruit juice and marker OpZ20₄₁₀ were studied in 82 progeny of a cross between Chandler pummelo (genotype *Acac*) and *Poncirus trifoliata* (genotype *AcAc*). Progeny predicted to be heterozygous for *acitric* (genotype *Acac*) by the marker had juice with about 30% less citric acid than individuals predicted to be homozygous *AcAc*. The significance of this result is that it indicates that the same gene (or at least chromosome segment) of pummelo 2240 that blocks citric acid accumulation when homozygous will also reduce acidity when heterozygous. Markers OpZ20₄₁₀, SCZ20, and RFZ20 were highly polymorphic among 59 citrus accessions and would allow citrus breeders to select seedling progeny heterozygous for *acitric* in nearly all crosses between hybrids of 2240 pummelo and other citrus genotypes. Thus marker-assisted transfer of *acitric* into other genetic backgrounds should be possible.

The molecular physiology of acid accumulation (Israel)

1. Cloning of the genes for organic acid metabolism from juice sac cells. The cDNAs for a most of the genes for organic acid metabolism (citrate synthase, cytosolic aconitase, NADP and NAD isocitrate dehydrogenase (ICDH), fumarase, malate dehydrogenase, malic enzyme) were cloned from lemon pulp (partially unpublished). This, in addition to other findings, indicates that citrate, among other organic acids, is synthesized in the fruit, and is not translocated from other plant organs.
2. The activity and expression of citrate synthase in acidless lime, acid lemon and in 'Minneola' fruits and tissue cultures treated with arsenite. The mitochondrial citrate synthase was compared between acidless lime and sour lemon (Paper #5). Its mRNA level in sour lemon increased 3-4 fold early in fruit development, in association with acid accumulation. This increase was followed by an increase in the activity of citrate synthase in the mitochondria, as expected from a biosynthetic

enzyme. Similar results were also obtained with sweet lime. Considering these results it seems that the mitochondrial citrate synthase plays a role in citrate accumulation. However, its action cannot explain the difference between low- and high-acid varieties. The activity of citrate synthase of 'Minneola' tangelo fruit was transiently inhibited by sodium arsenite, a treatment that reduced acid content in citrus fruit (paper #3). The recovery in the enzymatic activity could be explained by induction in the transcript level, detected in the fruit and in tissue cultures following arsenite treatment.

3. Aconitase activity and expression in the fruit. It has long been hypothesized that a metabolic block in the mitochondrial aconitase (which catabolizes citrate) plays a major role in citrate accumulation. Indeed, it has been shown that the activity of this enzyme in sour lemon is greatly reduced early in fruit development, while no reduction was detected during the development of acidless fruits (Paper #2). These results provide a reasonable scenario for acid accumulation: the reduction in the mitochondrial aconitase creates a local increase in the citric acid level; the additional acid is removed from the mitochondria to the cytosol, and stored in the vacuole. When the acid level declines toward fruit maturation, citrate is actively removed or leaks from the vacuole. It was expected that aconitase activity would be re-induced at later stages of fruit development. Indeed, it was found that a cytosolic aconitase activity is re-induced in the pulp towards maturation. The cDNA for a cytosolic aconitase was cloned in the lab. The putative amino acid sequence of this clone showed homology to a group of RNA binding proteins, Iron Regulated Proteins (IRP), which also possesses aconitase activity. The involvement of IRP-like protein in citrate catabolism raises an interesting possibility in regards to the involvement of iron homeostasis in citric acid accumulation. It is thought that iron limitation inhibits acid decline. This possibility is currently being investigated in the lab, and preliminary results strongly support it.

4. The activity and expression of NADP-isocitrate dehydrogenase in lemon fruit. Following the catabolism of citric acid to isocitric acid, the later is converted into α -ketoglutarate by isocitrate dehydrogenase (ICDH). The expression and the enzymatic activity of the cytosolic form of this enzyme were examined (Paper #4). Both of them were greatly induced toward fruit maturity. The induction of this enzyme, as well as the cytosolic aconitase, towards fruit maturation suggests that some of the catabolism of citric acid occurs in the cytosol.

5. Fruit total acidity (pH) and citric acid accumulation. Previous work showed that the accumulation of citric acid in the vacuole of the juice sac cells was accompanied by a massive influx of protons generated by a tonoplastic H^+ -ATPase. This proton influx reduces the vacuolar pH to about 2.5, and probably provides a driving force for citrate uptake into the vacuole, where it acts as a buffer. A detailed analysis of citric acid level and total acidity (representing the vacuolar pH) during

'Minneola' fruit development was performed (Paper #3). Differences between the patterns of total acidity and citric acid could be detected, indicating that these two mechanisms are indeed independent, although most likely tightly co-regulated.

Details of cooperation

Genes cloned in California and Israel were exchanged allowing tests for cosegregation in California and characterization of gene expression patterns in Israel. A particularly valuable aspect of the collaboration was combining genetic and molecular biology expertise and approaches to the problem of acid accumulation. Each PI visited the laboratory of the corresponding partner, reviewed progress, and planned additional experiments.

Significance and agricultural or economic impacts

The project showed that the *acitric* gene present in 2240 pummelo is responsible for the reduced acidity seen in progeny of 2240 pummelo. The development of a closely linked genetic marker that can be scored in seedling populations will increase the efficiency of breeders in transferring this gene to other genetic backgrounds (such as mandarin) that are of greater commercial importance. However, this part of the research will not have direct impacts on agriculture until breeders develop new cultivars with altered acidity. The project also resulted in an improved understanding of the molecular physiology of acid accumulation in citrus fruit. The metabolism of citrate in the fruit and factors affecting it are better understood. The possible involvement of iron homeostasis in citrate decline raises interesting possibilities in regards to the development of practical tools to reduce acid content towards fruit maturation. However, the development of molecular tools to control acid accumulation still depends on the identification of controlling genes and factors.

List of publications

1. Fang DQ, Federici, CT and Roose ML (1997) Development of molecular markers linked to a gene controlling fruit acidity in citrus. *Genome* 40, 841-849.
2. Sadka A, Dahan E, Cohen L and Marsh KB (2000) Aconitase activity and expression during the development of lemon fruit. *Physiol. Plant.* 108, 255-262.
3. *Sadka A, Artzi B, Cohen L, Dahan E, Hasdai D, Tagari and Erner Y (2000) Arsenite reduces acid content in citrus fruit, inhibits activity of citrate synthase but induces its gene expression. *J. Amer. Soc. Hort. Sci.* 125, 288-293.
4. Sadka A, Dahan E, Or E and Cohen L (2000) NADP⁺-isocitrate dehydrogenase gene expression and isozyme activity during citrus fruit development. *Plant Sci.* 158, 173-181.
5. Sadka A, Dahan E, Or E, Roose ML and Cohen L (2001) A comparative analysis of mitochondrial citrate synthase gene structure, expression and activity in acidless and acid-containing *Citrus* varieties. *Austral. J. Plant Physiol.* Accepted.
6. Roose, M.L. (2000) Citric acid content in citrus fruit: inheritance and genetic manipulation. Proceedings of The International Society of Citriculture. In preparation

*This publication was chosen by the Journal Scientific Editor to be the cover paper of the May 2000 issue.