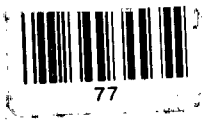

BARD

FINAL REPORT

PROJECT NO. IS-2531-95

Molecular Genetic Analysis of Citric Acid Accumulation in Citrus Fruit

A. Sadka, M.L. Roose, Y. Erner

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Cover Page

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_____

Cooperation Summary (numbers)

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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)				
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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 polymorphyic 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 $OpZ20_{410}$, 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 $OpZ20_{410}$ 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 μm, the minimum detection level by HPLC. The citric acid content of fruit juice and marker $OpZ20_{410}$ 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 $OpZ20_{410}$, 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.

APPENDIXES

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Appendix 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

Appendix 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.

Appendix 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.

Appendix 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.

Appendix 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.

Appendix 6. Unpublished data:

Genetic cosegregation using known genes (USA)

Differential Display (USA)

Development of molecular markers linked to a gene controlling fruit acidity in citrus

D.Q. Fang, C.T. Federici, and M.L. Roose

Abstract: 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 recessive gene called *acitric*. Using bulked segregant analysis, three RAPD markers were identified as linked to *acitric*. RAPD marker $OpZ20_{410}$, which mapped 1.2 cM from *acitric*, was cloned and 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 $OpZ20_{410}$ band as a hybridization probe revealed a codominant RFLP marker called RFZ20 that mapped 1.2 cM from *acitric*. Progeny homozygous (*acac*) for the *acitric* allele had citric acid content below 10 μM, the minimum level detectable by high pressure liquid chromatography. The citric acid content of fruit juice from progeny predicted to be heterozygous (*Acac*) for *acitric* by the above markers was about 30% lower than that of juice from individuals predicted to be homozygous (*AcAc*) for the normal acid allele. Markers $OpZ20_{410}$, SCZ20, and RFZ20 were highly polymorphic among 59 citrus accessions, and using one or more of these markers would allow citrus breeders to select seedling progeny heterozygous for *acitric* in nearly all crosses between pummelo 2240 or its offspring and other citrus genotypes.

Key words: *Citrus*, fruit acidity, citric acid, RAPD, SCAR, RFLP.

Résumé : Le pH du jus de fruit, l'acidité titrable ou le contenu en acide citrique a été mesuré chez six populations dérivées d'un «pummelo» (*Citrus maxima* (Burm.) Merrill) à faible acidité (pummelo 2240). La faible acidité chez le pummelo 2240 est déterminée par un seul gène récessif, *acitric*. Une analyse de ségrégants regroupés a permis d'identifier trois marqueurs RAPD qui sont liés au gène *acitric*. Le marqueur RAPD $OpZ20_{410}$, lequel est situé à 1,2 cM du gène *acitric*, a été cloné et séquencé afin de développer un marqueur SCAR (SCZ20). L'allèle SCZ20-410, qui est lié à l'allèle *acitric*, n'est présent que dans le pummelo 2240 et d'autres pummelos de sorte que ce marqueur devrait s'avérer utile en tant que marqueur dominant ou codominant pour l'introgression du gène *acitric* chez les mandarines et autres agrumes. L'emploi du fragment cloné $OpZ20_{410}$ comme sonde a permis de révéler un marqueur RFLP codominant (RFZ20) qui a été situé à 1,2 cM du gène *acitric*. Des progénitures homozygotes pour l'allèle récessif (*acac*) avaient une teneur en acide critrique inférieure à 10 μM, le seuil minimal détectable par HPLC. La teneur en acide citrique du jus provenant d'individus hétérozygotes (*Acac*), identifiés à l'aide des marqueurs décrits plus haut, était environ 30% inférieur à celle observée chez des individus présumés homozygotes pour l'allèle sauvage (*AcAc*). Les marqueurs $OpZ20_{410}$, SCZ20 et RFZ20 ont montré beaucoup de polymorphisme parmi 59 accessions d'agrumes et l'emploi de l'un ou l'autre de ces marqueurs permettrait aux améliorateurs de sélectionner les individus hétérozygotes pour le gène *acitric* chez presque tous les croisements entre le pummelo 2240 ou ses progénitures et les autres génotypes d'agrumes.

Mots clés : *Citrus*, acidité des fruits, acide citrique, RAPD, SCAR, RFLP.

[Traduit par la Rédaction]

Introduction

One of the most distinctive characteristics of citrus fruit is their accumulation of high levels of organic acids in the vacuoles of juice sac cells. Citric acid is the major acid accumulated in citrus fruit, reaching a level of 1–5% by weight in many cultivars (Vandercook 1977). Acid content makes a major contribution to the flavor and acceptability of citrus fruit, particularly acid content relative to sugars (usually measured as total soluble solids). Many citrus producing regions have legal standard minimums that the sugar:acid ratio of fruit must exceed before it can be sold. Fruit acidity level is a major problem in developing new mandarin and grapefruit cultivars by hybridization and selection, because many crosses produce progeny with higher acid levels than either parent; many hybrid progeny with otherwise desirable attributes are discarded because they are "too tart" (Soost and Cameron 1961).

Knowledge of the genetic control of fruit acidity is quite limited. The trait is usually considered to be quantitatively inherited. However, "acidless" (or very low acid) forms of citrus have been selected in almost all important cultivars.

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including oranges, pummelos, limes, and lemons (Hodgson 1967). The "Siamese sweet" pummelo, known as pummelo 2240, is an acidless cultivar. The titratable acid content of its fruit juice is only about 0.1–0.2% (Cameron and Soost 1977), and its citric acid concentration, as measured by high pressure liquid chromatography (HPLC) analysis, is below the level of detection (about 10 μM). Pummelo 2240 has been crossed with other citrus cultivars that accumulate varying amounts of citric acid to study the inheritance of the acidless trait and to breed new cultivars with acceptable acid levels that retain desirable traits present in the acid parent (Soost and Cameron 1961). Genetic analysis in several populations revealed that the acidless trait of pummelo 2240 is inherited as a single recessive gene (Cameron and Soost 1977) called *acitric* (Canel et al. 1995) (symbol: *ac*). Several other acidless cultivars, such as some acidless oranges, red mandarin-limes, and sweet limes, apparently do not transmit this trait to progeny, perhaps because they are chimeric, or because genetic control is more complex (Cameron and Soost 1977, 1979). Inheritance studies have shown that *acitric* causes very low acidity in homozygous individuals (*acac*) and that hybrids of pummelo 2240 with normal acid cultivars, although variable in acid content, generally have an intermediate level of acidity that is desirable in commercial cultivars (Cameron and Soost 1977, 1979). The intermediate acidity of *acitric* heterozygotes (*Acac*) has been interpreted as indicating that *acitric* is not fully recessive. However, it is also possible that some other gene that is homozygous in pummelo 2240 is responsible for this effect. If one copy of *acitric* were sufficient to reduce citric acid content, then this gene would be an excellent candidate for transfer into other cultivars by crossing or transformation and for breeding new cultivars with moderate acidity levels by selecting *acitric* heterozygotes (*Acac*).

The use of traditional breeding techniques in citrus is hindered by its long generation time, usually from 3 to 8 years. The period prior to evaluation may be even longer when breeding for fruit characters such as fruit acidity, since there is a long period of juvenility during which the typical fruit characteristics of a mature tree are not expressed. Thus, identification of trait-linked markers that allow selection at the seedling stage is highly advantageous to citrus breeders. In citrus, isozymes, restriction fragment length polymorphisms (RFLPs), and random amplified polymorphic DNA (RAPD) have been used to construct genomic maps (Durham et al. 1992; Jarrell et al. 1992; Cai et al. 1994). Of these marker systems, RAPD markers, coupled with bulked segregant analysis (BSA; Michelmore et al. 1991), have already been used to tag genes of horticultural importance, including citrus tristeza virus resistance (Gmitter et al. 1996), nematode resistance (Ling et al. 1994), and dwarfing (Cheng and Roose 1995). However, most RAPD markers are dominant, and thus cannot distinguish between heterozygous and homozygous individuals. Therefore, conversion of RAPD markers to codominant markers, such as sequence characterized amplified region (SCAR) and RFLP markers, facilitates their use in marker-assisted selection. Moreover, codominant markers can allow more precise estimates of the distance between markers and target genes. In this paper, we report the identification of RAPD markers linked to *acitric*, a gene controlling fruit acidity in citrus, conversion of one RAPD marker to a SCAR and to a codominant RFLP marker, and application of these markers to confirm that *acitric* heterozygotes (*Acac*) have reduced citric acid content compared with homozygotes (*AcAc*) for the normal acid allele.

Materials and methods

Plant materials

Six populations segregating for fruit acidity were used (Table 1). The paternal parents of populations 2 and 3 were different progeny trees derived from a cross of pummelo 2240 and Yellow sweet lime. The maternal parent of population 4 was an acidless progeny plant of population 5. The acid content of the parent cultivars ranged from 0.1% to more than 2% (Table 2). Fifty-nine accessions representing 11 cultivar groups belonging to 35 species of 2 genera were sampled from the Citrus Variety Collection at the University of California, Riverside, to study the distribution of markers. Details of the accessions tested and their marker phenotypes are available to readers upon request.

Fruit acidity determination

Mature fruit (3–5 per tree) of populations 1–4 were collected in December and January in the years from 1989 to 1994 and 1996. Most genotypes were sampled in 3 or 4 different years. To determine acid level, each fruit was cut, the juice vesicles macerated with a knife, and a pH probe inserted directly into the macerated fruit tissue. For some samples, juice was extracted with an electric reamer and juice titratable acidity was measured by titration with 0.1 N NaOH. The titratable acidity of fruits in population 5 was measured by Cameron and Soost (1977). To measure the citric acid content in fruit juice, fruits of populations 1–4 and 6 were harvested in November–December 1996. Juice was extracted, filtered through a 0.2-μm filter, and mixed with an equal volume of 1 mM sulfuric acid. Thirty microlitres of juice was injected into a PRP-X300 column (Hamilton Co., Reno, Nev.) for HPLC analysis. The solvent was 1 mM sulfuric acid with a flow rate of 1 mL/min and a measured pressure of 1000–1100 psi (1 psi = 6.895 kPa). Organic acids were detected by absorbance at 210 nm and identified by coelution with standards.

DNA extraction and bulked segregant analysis

Total DNA was extracted from young leaves as described by Fang et al. (1997). To prepare the acid bulk, DNA samples of 10 individuals with juice pH ≤ 3.6 (Fig. 1) from population 1 were pooled at equal ratio and diluted to 10 ng/μL. A bulk sample of DNA from acidless individuals with juice pH ≥ 5.4 was prepared in a similar way. Because of variation in maturity date and the possible effects of other genes that influence acid content, it is not possible to classify acid individuals as *AcAc* or *Acac* based on juice pH or titratable acidity. Consequently, the acid bulk might include both homozygous acid (*AcAc*) and *acitric* heterozygous individuals (*Acac*), but the acidless bulk should only consist of *acitric* homozygous individuals (*acac*). Those primers that revealed polymorphisms between bulks were tested using pummelo 2240 and a second pair of bulks, each composed of DNA samples from 5 individuals of population 2. If a polymorphism was still detected, then all progeny from populations 1 and 2 were amplified using these primers. Primers that amplified a band that showed tight linkage to the *acitric* locus were used to amplify all progeny from populations 3–5. Primer OpZ20 was also used to amplify the DNA of 82 progeny in population 6.

RAPD-PCR amplification

RAPD-PCR amplification was performed on an Ericomp Thermocycler (Ericomp Inc., San Diego, Calif.), using reaction mixtures and temperature cycling profiles described by Cheng and Roose (1995). Random decamer primers were purchased from Operon Technologies, Inc., (Alameda, Calif.). Amplification products were resolved by electrophoresis through 1.8% agarose gel in 1× TBE buffer

Table 1. Populations used for genetic analysis of fruit acidity[a] and segregation ratios for fruit juice pH; progeny were classified as acid if juice pH was less than 5.0.

Population	Parentage	Parental genotypes at the *acitric* locus	No. observed		Expected ratio	χ^2	Total observed
			Acid	Acidless			
1	Chandler × (pummelo 2240 × Red mandarin-lime)	*Acac* × *Acac*	23 (17)	10 (10)	3:1	0.49 ns	33 (27)
2	Chandler × (pummelo 2240 × Yellow sweet lime)	*Acac* × *Acac*	18 (13)	15 (14)	3:1	7.36*	33 (27)
3	Chandler × (pummelo 2240 × Yellow sweet lime)	*Acac* × *Acac*	20 (19)	6 (5)	3:1	0.04 ns	26 (24)
4	((Pummelo 2240 × Kinnow) selfed) × Chandler	*acac* × *Acac*	7 (6)	13 (11)	1:1	1.80 ns	20 (17)
5	(Pummelo 2240 × Kinnow) selfed	*Acac* × *Acac*	28 (2)	12 (11)	3:1	0.20 ns	40 (13)
6[b]	Chandler × Rubidoux trifoliate orange	*Acac* × *AcAc*					82

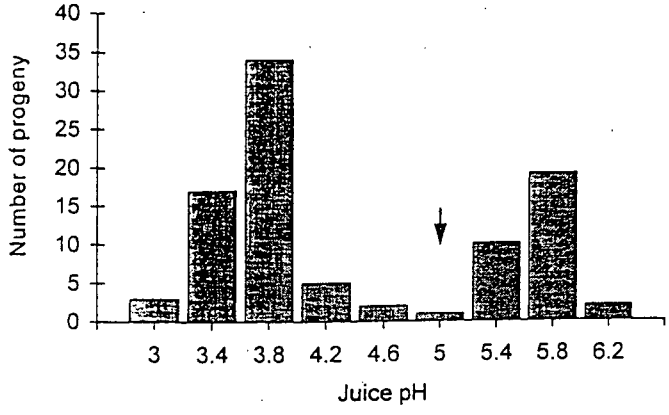
Note: Numbers in parentheses are the numbers of trees available for marker analysis. *$p < 0.05$; ns, not significant.
[a]Some trees died or were discarded before DNA was extracted in 1995.
[b]Population 6 was only used for HPLC analysis.

Table 2. Parent cultivars, genotype at the *acitric* locus, the range of titratable acidity in juice from mature fruit, $OpZ20_{410}$ and SCZ20 phenotypes, and RFZ20 genotypes.

Scientific name	Cultivar	Genotype at the *acitric* locus	Titratable acidity[a] (%)	Phenotype		Genotype
				$OpZ20_{410}$[b]	SCZ20	RFZ20
Citrus maxima (Burm.) Merrill	Pummelo 2241	*AcAc*	1.53–1.59	+	410/450	*gu*
	Pummelo 2240	*acac*	0.10–0.20	–	410	*ww*
	Chandler[c]	*Acac*	1.00–1.20	+	410	*uw*
Citrus limonia Osbeck	Red mandarin-lime[d]	*AcAc*?	0.30–0.40	–	None	*vx*
Citrus limettoides Tanaka	Yellow sweet lime[d]	*AcAc*?	0.20–0.50	–	None	*vx*
Citrus reticulata Blanco	Kinnow mandarin	*AcAc*	1.50-1.75	–	None	*zz*
Poncirus trifoliata (L.) Raf.	Rubidoux trifoliate orange	*AcAc*	2.00–5.00	–	None	*yy*

[a]Data from Soost and Cameron (1961) and Cameron and Soost (1977, 1979).
[b]Marker present (+) or absent (–).
[c]The hybrid pummelo 2240 × pummelo 2241.
[d]This cultivar cannot transmit the acidless trait to the next generation (see Cameron and Soost 1979).

Fig. 1. Frequency distribution of fruit juice pH in populations 1–3 combined. Individuals with pH above 5.0 (arrow) were classified as acidless and those with pH below 5.0 as acid.



(89 mM Tris-borate plus 2 mM EDTA, pH 8.2) and stained with ethidium bromide. In some cases, RAPD products were also separated on 6% nondenaturing polyacrylamide gel containing 3 M urea (300 × 380 × 0.4 mm) in 1× TBE buffer for 12 h at 450 V, and detected by silver staining (Bassam et al. 1991).

Cloning and sequencing the $OpZ20_{410}$ RAPD band

To prepare the 410-bp product amplified from 'Chandler' genomic DNA by primer OpZ20 for cloning, the band was excised from 3% NuSieve (3:1) agarose (FMC, Rockland, Maine) gel and submerged in 10 volumes of water. The gel slice was boiled and cooled to 55°C. Four microlitres was used as template DNA for reamplification using primer OpZ20. The reamplification product was resolved on 1% low-melt agarose gel in 1× TAE buffer (40 mM Tris-acetate plus 1 mM EDTA, pH 8.2). A single 410-bp band was excised from the gel, and the DNA was extracted with AgarAce (Promega, Madison, Wis.) and purified using phenol – chloroform – isoamyl alcohol (25:24:1) extraction and ethanol precipitation. DNA was cloned using the pCR-Script Amp SK(+) cloning vector (Stratagene, La Jolla, Calif.). Recombinant clones were screened by amplification of inserts from intact plasmid DNA using primer OpZ20 with 'Chandler' genomic DNA as control. Further confirmation was conducted by hybridization of the insert as probe to a Southern blot of primer-OpZ20-amplified RAPD products from 25 individuals of population 1, plus pummelo 2240, pummelo 2241, 'Chandler', and Yellow sweet lime. Southern transfer, probe labeling, hybridization, and washing were according to Jarrell et al. (1992). The probe bound only to individuals that had the 410-bp band. The insert DNA was prepared for sequencing using the method of Nicoletti and Condorelli (1993). Sequencing was performed by Li-Cor, Inc. (Lincoln, Nebr.).

Designing SCAR primers and their amplification of genomic regions

Based on the sequence of the cloned $OpZ20_{410}$ RAPD band, a pair of 25-mer primers was designed using the computer program OLIGO 4.0 (Rychlik and Rhoads 1989). Primers were synthesized by Genosys Biotechnologies Inc. (The Woodlands, Tex.). PCR amplification with SCAR primers was carried out in a 25 ul reaction mixture containing 26.5 ng of template DNA, 2.5 μL 10× buffer, 2.0 mM $MgCl_2$, 128 μM of each dNTP, 0.40 μM of each primer, and 1.25 U of Taq polymerase (Promega, Madison, Wisc.). The amplification was performed on an Ericomp Thermocycler with the following program: 94°C for 4 min; followed by 40 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; with 7 min at 72°C for final extension. PCR products with or without restriction endonuclease digestion were electrophoresed on 3% NuSieve (3:1) agarose gel in 1× TBE buffer and stained with ethidium bromide, and also electrophoresed on 6% nondenaturing polyacrylamide gel with silver staining, as described above.

Development of the RFLP marker

The probe used in this experiment was the cloned $OpZ20_{410}$ band amplified from 'Chandler' genomic DNA described above. DNA samples from pummelo 2241, pummelo 2240, 'Chandler', Red mandarin-lime, Yellow sweet lime, 'Kinnow' mandarin, one acid progeny plant, and one acidless tree of population 1, were digested with 27 restriction endonucleases according to manufacturers' recommendations and with the addition of 3 mM spermidine. Each reaction had about 4 μg of DNA and 30 U of restriction endonuclease. Digestion was conducted at 37°C for 14 h. DNA electrophoresis, Southern transfer, probe labeling, hybridization, and washing were as described above. Membranes were exposed to Phosphor Image Screens for 12 h. Autoradiographic images were obtained by scanning the screens with a Phosphor Imager (Molecular Dynamics Ltd., Sunnyvale, Calif.). After the completion of restriction endonuclease screening, the most promising endonuclease, *Eco*RV (Gibco–BRL, Life Technologies, Gaithersburg, Md.), was employed to digest genomic DNA samples from all 108 progeny of populations 1–5 and 59 citrus accessions. Subsequent electrophoresis, Southern transfer, and hybridization were carried out as described above.

Linkage analysis

Segregation data for fruit acidity and RAPD and RFLP markers were mapped to the region of *acitric* by analyzing populations 1–5 with the program JOINMAP 2.0 (Stam and Van Ooijen 1995). Linkage was considered significant if the LOD score was equal to or greater than 4.0.

Results and discussion

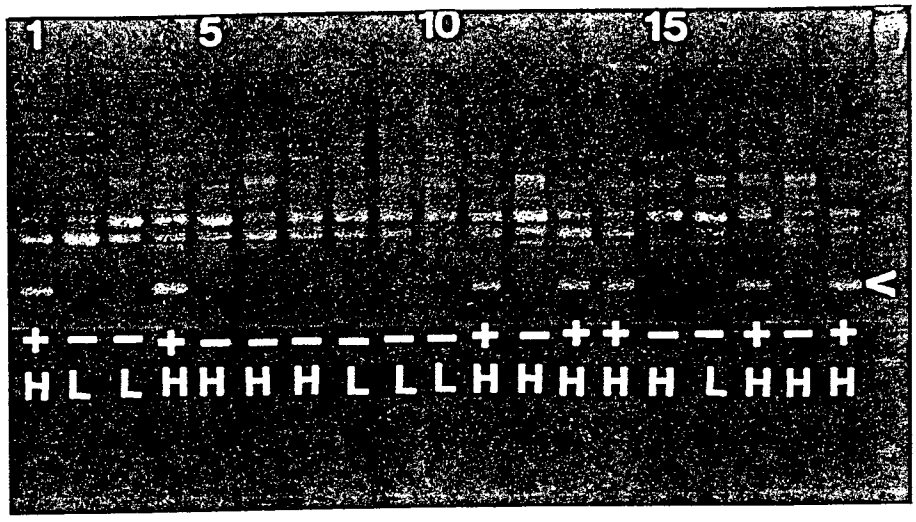
Inheritance of fruit acidity

We evaluated populations 1–4 for fruit acidity over 4 years by measuring the pH of fruit juice. The frequency distribution of juice pH in populations 1–3 is clearly bimodal (Fig. 1) and nonoverlapping, with acid fruit averaging pH 3.6 and acidless fruit averaging about pH 5.6. Individuals can easily be classified as acid or acidless. A trimodal distribution might be expected if the *ac* allele is not fully recessive. However, a bimodal distribution was observed because the range of acidity within the *Acac* class has considerable overlap with that of the *AcAc* class and, as shown below, few *AcAc* individuals occur in populations 1–3. As shown in Table 1, only population 2 deviated significantly from the 3:1 ratio expected for a single recessive gene for the acidless trait. A combined test over populations 1–3 gave a ratio of 61 acid : 31 acidless progeny ($\chi^2 = 3.71$, ns). Population 4 segregated 1:1, as expected. Population 5, which was analyzed for total titratable acidity rather than juice pH, segregated 3:1, as expected. These results confirmed the inheritance patterns observed by Cameron and Soost (1977, 1979) based on segregation in different populations derived from pummelo 2240. In summary, the inheritance of fruit acidity in progeny derived from pummelo 2240 is controlled by a single recessive gene called *acitric*.

Identification of RAPD markers linked to *acitric*

A total of 360 decamer primers was screened against the acid and acidless bulks. Most primers amplified from 1 to 13 bands ranging from about 200 to 2500 bp. OpG10 only generated one intense band and 10 primers produced no product. Of the primers screened, 77 revealed polymorphisms between bulks.

Fig. 2. RAPD products of genomic DNAs from progeny of population 1 using primer OpZ20. Lane 20 is a 123-bp ladder. Arrow indicates the RAPD marker $OpZ20_{410}$ linked to the *acitric* locus. "+" indicates the presence of a marker, while "–" indicates the absence of a marker. "H" means that the individual was classified as an acid progeny, while "L" means that the individual was an acidless progeny. Please see text for explanation of six acid progeny trees (lanes 5, 6, 7, 12, 15, and 18) that do not have this marker band.



The acid bulk had a band that was not amplified from the acidless bulk with 53 of these primers. When these 53 primers were tested on the second pair of bulks and pummelo 2240, only 12 revealed polymorphisms. After testing in populations 1 and 2, only three primers, OpB12, OpC10, and OpZ20 (Fig. 2), generated 3 markers ($OpB12_{800}$, $OpC10_{440}$, and $OpZ20_{410}$) that were linked to *acitric*. Since the presence of these RAPD markers was associated with the *Ac* allele, and the absence of the markers was associated with the *ac* allele, these 3 RAPD markers were linked to *acitric* in coupling phase. These markers also showed linkage to *acitric* in populations 3 and 4, but they did not segregate in population 5. $OpZ20_{410}$ was linked to *acitric* with a LOD value of 22.0 and a map distance of 1.2 centimorgans (cM). $OpB12_{800}$ and $OpC10_{440}$ mapped to the opposite side of *acitric* from $OpZ20_{410}$ at distances of 29.5 and 17.9 cM, respectively. Because these three markers did not appear in pummelo 2240, Red mandarin-lime, Yellow sweet lime, 'Kinnow' mandarin, or 'Rubidoux' trifoliate orange, but did appear in 'Chandler' and pummelo 2241, it is evident that the amplified sequences must originate from pummelo 2241. Consequently, these RAPD markers are only informative in certain crosses involving pummelo 2241 or its hybrids such as 'Chandler'. Indeed, as shown in Fig. 2, 6 acid progeny trees (lanes 5, 6, 7, 12, 15, and 18) have no $OpZ20_{410}$ RAPD marker band. These progeny might have received the acid allele from Red mandarin-lime. The faint band seen at the approximate position of the $OpZ20_{410}$ band in lanes 6, 7, 8, 12, and 18 in Fig. 2 was clearly smaller than the marker band when the same RAPD products were separated on a polyacrylamide gel.

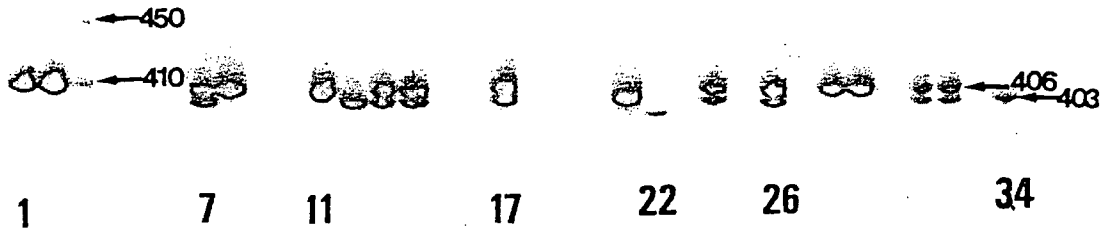
The BSA method developed by Michelmore et al. (1991) has been widely used to rapidly identify markers linked to the gene of interest. BSA is particularly useful for cases for which no near-isogenic lines exist, as for most traits in fruit crops. In their paper, Michelmore et al. (1991) calculated that the probability of an unlinked locus being polymorphic between bulks of 10 individuals is 2×10^{-6}. However, a high frequency of false positives was observed in the present experiment; 21.4% of primers gave polymorphic patterns between the first bulk pair, but only two thirds of these primers generated polymorphic bands present only in the acid bulk (coupling phase). This percentage decreased to 3.33% after testing the the second bulk pair. Finally, only 3 primers (0.83% of primers screened) were found to generate 3 markers linked to *acitric*. In a separate experiment identifying markers linked to the citrus tristeza virus resistance gene, we also found a high rate of false positives (D.Q. Fang and M.L. Roose, unpublished data). A high rate of false positives in BSA has been observed by other researchers (Haley et al. 1993; Young and Kelly 1996). A low repeatability of RAPD might be one cause of false positives. Very small (less than 200 bp), very large (more than 2000 bp), or faint bands are not reliable. They may vary with different DNA polymerases, Mg^{2+} concentrations, and many other factors. To reduce false positives and amplification work, it is helpful to construct two different bulk pairs with one of them being used as retest bulks, and to include one parent contributing the gene of interest as a control.

The populations studied here were developed by Cameron and Soost (1977, 1979) to study the inheritance of acidity, and were not designed for efficient marker identification. The use of 'Chandler' pummelo as one parent means that only markers differing between its parents, pummelo 2240 and pummelo 2241, will be detected by using the BSA. A more efficient strategy would be to use a hybrid between pummelo 2240 and a more divergent genotype (e.g., mandarin) as a parent, so that a larger proportion of markers would differ between the acid and acidless chromosomes. We did not develop and use such populations because of the long time (about 10 years) required for fruiting. Successful identification of a marker closely linked to *acitric*, even when using populations not ideal for marker identification, illustrates the power of the BSA method.

Development of the SCAR marker

Based on the sequence of the cloned $OpZ20_{410}$ band, one pair of primers was synthesized: 5′-TTTGGCGGCCCCAT-

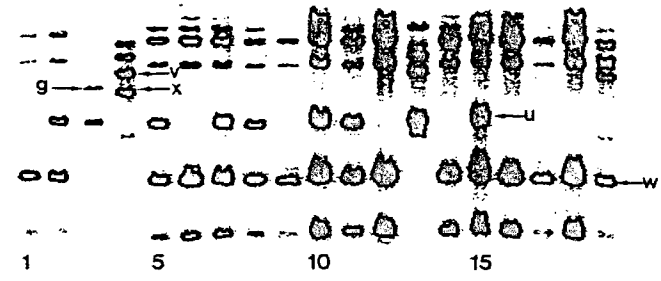
Fig. 3. Amplification products of genomic DNAs of citrus accessions using a pair of SCAR primers derived from RAPD marker $OpZ20_{410}$. Products were separated on 6% polyacrylamide gel containing 3 M urea and detected by silver staining. Lanes, from the left: 'Chandler', pummelo 2240, pummelo 2241, 'Eureka' lemon, Key lime, 27 mandarin accessions, and *Citrus maderaspatana* (Kitchli) (lane 34). Arrows and numbers indicate band size. Lane 22 is a 100-bp ladder and the band is 400 bp.



AAATCCCTAAT-3′ and 5′-GGCGGGAAATCATGACCC-TAAACTA-3′ (the sequence underlined is derived from the primer OpZ20). These primers amplified a single 410-bp band from pummelo 2241, pummelo 2240, and 'Chandler', and no product from Red mandarin-lime, Yellow sweet lime, or 'Kinnow' mandarin, when the products were resolved on a 3% NuSieve (3:1) agarose gel. When this primer pair was tested on genomic DNA from progeny of the 6 populations, all progeny had a single 410-bp band. The polymorphism that was detected in RAPD analysis by primer OpZ20 could not be detected with these SCAR primers. In an attempt to recover polymorphisms, 20 restriction endonucleases (11 6-cutters and 9 4-cutters) were used to digest the SCAR-primer-amplified products of pummelo 2240 and 'Chandler'. However, no polymorphism between pummelo 2240 and 'Chandler' was detected. In summary, the SCAR marker named SCZ20 reported here is not informative in most of the populations used in the present study. It may segregate in population 5, since it does not amplify from 'Kinnow' mandarin, but the two acid progeny available to us both have the 410-bp band, as would be expected if they were *acitric* heterozygotes. Since no amplification product was detected in Red mandarin-lime, Yellow sweet lime, 'Kinnow' mandarin, or 'Rubidoux' trifoliate orange, the marker SCZ20 would probably segregate as a dominant marker in certain F_2s or backcrosses derived from them and pummelo 2240.

Paran and Michelmore (1993) developed the technology to convert dominant RAPD markers to codominant and reliable SCAR markers. In converting RAPD markers to SCAR markers in lettuce, they found that 6 of the 9 polymorphic RAPD markers linked to downy mildew resistance genes were caused by mismatches in one or a few nucleotides in the priming sites, and that the other 3 were caused by sequence divergence or by rearrangements. In the present study, a pair of primers, synthesized on the basis of the sequence of the RAPD band, amplified one single 410-bp product from pummelo 2240, pummelo 2241, and their hybrid 'Chandler'. Though several 4-cutter restriction enzymes, such as *Rsa*I, *Mbo*I, and *Alu*I, digested the SCAR products, no length polymorphism between 'Chandler' and pummelo 2240 was found after digestion. These observations indicate that the polymorphisms revealed by the RAPD marker might be caused by mismatches in the priming sites. The sequence of the $OpZ20_{410}$ RAPD band from 'Chandler' differed from that of the 410-bp band amplified from pummelo 2240 by the SCAR primers by a 1-base deletion in the priming site of the latter fragment. Since SCAR primers are longer than the RAPD primer, SCAR primers could tolerate this mismatch and generate SCAR products. When we resolved the SCAR products on 6% polyacrylamide gel, a band of about 450 bp was observed in pummelo 2241 in addition to the 410-bp band (Fig. 3). We could not detect two bands on any of several kinds of agarose at different concentrations, although 410- and 450-bp DNA fragments should be readily separated on an agarose gel. We cannot explain the origin of the 450-bp band.

Fig. 4. RFZ20 patterns of parent cultivars and progeny of population 1. DNA was digested with restriction endonuclease *Eco*RV and hybridized with the cloned $OpZ20_{410}$ band. Lanes: 1, pummelo 2240; 2, 'Chandler'; 3, pummelo 2241; 4, Red mandarin-lime; and 5–19, 15 progeny from population 1. Arrows and letters indicate allele designation.



Development of a codominant RFLP marker

DNA samples of the 6 parent cultivars and 2 progeny were digested with 27 restriction endonucleases and hybridized with the cloned $OpZ20_{410}$ band. Informative polymorphisms were observed with 10 enzymes, *Bcl*I, *Bfa*I, *Bgl*II, *Bsto*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Mbo*I, *Msp*I, and *Sac*I, with *Eco*RV, *Mbo*I, and *Sac*I giving the clearest polymorphisms. When DNA samples of 108 progeny from populations 1–5 were digested with *Eco*RV, a codominant RFLP marker named RFZ20 that segregated in all populations was detected (Fig. 4; Tables 2 and 3). This marker was tightly linked to *acitric* with a LOD score of 22.6 and a genetic distance estimate of 1.2 cM. Only 1 progeny plant (in population 4) was recombinant. All individuals having the $OpZ20_{410}$ RAPD band had RFZ20 allele "*u*," which was inherited from pummelo 2241.

Table 3. Segregation analysis of marker RFZ20 and fruit acidity in 5 populations.

Population	RFZ20 genotype of parents[a]	No. of progeny: RFZ20 genotypes *uv* or *ux*		RFZ20 genotypes *uw*, *vw*, *wx*, or *wz*		RFZ20 genotype *ww*	
		Acid	Acidless	Acid	Acidless	Acid	Acidless
1	*uw* × *vw*	2	0	15	0	0	10
2	*uw* × *wx*	3	0	10	0	0	14
3	*uw* × *wx*	5	0	14	0	0	5
4	*ww* × *uw*			5	0	1	11
5	*wz* × *wz*			2	0	0	11

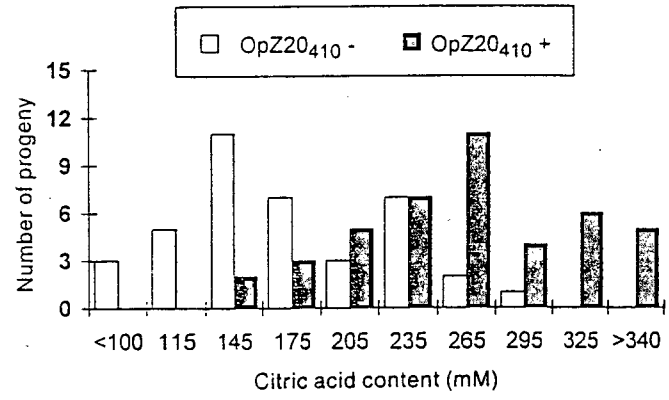
[a]The "RFZ20-*w*" allele is in *cis* with *acitric*. The "RFZ20-*u*", "-*v*", "-*x*," and "-*z*" alleles are in *trans* with *acitric*.

Citric acid levels in *acitric* heterozygotes (*Acac*)

Because 'Chandler' and other hybrids derived from pummelo 2240 had reduced acidity compared with hybrids derived from other pummelos (Soost and Cameron 1961; Cameron and Soost 1977, 1979), we speculated that *acitric* is not fully recessive. However, it was possible that reduced acidity in hybrids of pummelo 2240 was caused by some other gene inherited from pummelo 2240. The development of a codominant genetic marker closely linked to *acitric* allows us to test the hypothesis that *acitric* is not fully recessive by using the marker to infer which "normal acid" progeny are homozygous normal (*AcAc*) and which are heterozygous (*Acac*) for *acitric*. Because RFZ20 marker genotypes suggest that only 10 progeny from populations 1–3 are homozygous for the acid allele, it may be hard to draw reliable conclusions from these populations. However, our pH and titratable acidity data showed that, in populations 1–3, homozygous acid progeny (RFZ20 genotype *uv* or *ux*) had a slightly lower average pH value and higher titratable acidity content than *acitric* heterozygous individuals (RFZ20 genotype *uw*, *vw*, or *wx*). The HPLC analysis showed that the average citric acid content in juice of the homozygous acid (*AcAc*), *acitric* heterozygous acid (*Acac*), and *acitric* homozygous acidless (*acac*) progeny were 84.626, 58.747, and 0 mM, respectively. These differences were statistically significant ($p < 0.01$).

We further tested the segregation of the $OpZ20_{410}$ RAPD marker and measured the citric acid content in fruit juice of 82 progeny in population 6. Forty-three progeny had marker $OpZ20_{410}$, while the other 39 did not. Since this RAPD marker is linked to *acitric* in coupling phase and is not present in 'Rubidoux' trifoliate orange, nearly all progeny with the marker are expected to be homozygous acid progeny (*AcAc*). Nearly all progeny without the marker are expected to be heterozygous (*Acac*). Within each marker genotype class, the frequency distribution of citric acid content did not differ significantly from normal (Fig. 5). Although the two distributions overlapped, the mean citric acid content was 262 ± 59 (SD) mM for progeny having the $OpZ20_{410}$ RAPD marker, and 173 ± 57 mM for progeny without this marker. The estimate of dominance effect (d) is 42 mM and is significantly different from 0 ($t = 4.25$, $p < 0.001$). As shown in Fig. 5, selection for an $OpZ20_{410}$-derived marker linked to *acitric* would substantially reduce the acid content of the population.

Fig. 5. Frequency distributions of citric acid content in progeny from population 6 that had alternative alleles of RAPD marker $OpZ20_{410}$. The $OpZ20_{410}$ + allele is linked to the acid allele from pummelo 2241 and the $OpZ20_{410}$ – allele is linked to the acidless allele *acitric* from pummelo 2240.



Thus, in populations 1–3 and in a cross of 'Chandler' pummelo with 'Rubidoux' trifoliate orange, a distantly related, very high acid parent, the citric acid content in progeny inferred to be heterozygous for *acitric* is one third lower than that in progeny inferred to be homozygous acid. These results suggest that a single copy of *acitric* is sufficient to reduce citric acid content in fruit of all citrus genotypes.

Distribution of markers $OpZ20_{410}$, SCZ20, and RFZ20 among citrus accessions

Genomic DNA samples extracted from 59 citrus accessions representing 11 cultivar groups belonging to 35 species of 3 genera (Table 4) were amplified with the primer OpZ20. Only 4 pummelo cultivars, i.e., pummelo 2241, 'Chandler', 'Kao Phuang', and 'New Guinea', had the $OpZ20_{410}$ RAPD band. However, Southern blots of primer-OpZ20-amplified products that were hybridized with the cloned $OpZ20_{410}$ band showed that, in addition to these 4 pummelo cultivars, some mandarin and sour orange accessions had hybridization signals. When SCAR primers were used to amplify the genomic DNAs of these 59 accessions, polymorphisms among them were detected. Six types of SCAR banding patterns, i.e., "none,"

Table 4. Citrus accessions and their SCZ20 phenotypes.

Cultivar groups	No. of accessions tested	SCZ20 phenotypes[a]
Pummelos	5	410 or 410/450
Grapefruits	2	None
Sweet oranges	3	None
Citron	1	None
Lemon	1	None
Limes	3	None
Sweet lemons	3	None
Sour oranges	4	None or 403
Mandarins	33	None, 403, 403/406, or 406
Tangelos	3	None
Trifoliate orange	1	None

[a]Number indicates the SCAR product length as shown in Fig. 3.

"SCZ20-403," "SCZ20-403"/"SCZ20-406," "SCZ20-406," "SCZ20-410," and "SCZ20-410"/"SCZ20-450," were observed. (Fig. 3; Table 4). Bands SCZ20-410 and SCZ20-450 were only observed in pummelos. Pummelo 2241 had pattern SCZ20-410/SCZ20-450. The other 4 pummelo cultivars, i.e., 'Chandler', 'Kao Phuang', 'New Guinea', and pummelo 2240, had only a single band, SCZ20-410. Grapefruits, sweet oranges, citron, limes, lemon, and sweet lemons had no SCAR product (the phenotype none). The mandarin and sour orange accessions that had the SCZ20-403 band also had hybridization signals on Southern blots of OpZ20-amplified products. Four mandarin accessions, 'Tien Chieh', 'Tim Kat', 'Kinokuni', and 'Willowleaf', which gave no hybridization signal, had only one intense band, SCZ20-406.

Genomic DNA of these 59 accessions was digested with *Eco*RV and hybridized with the cloned $OpZ20_{410}$ band. Many different phenotypes were observed in various cultivar groups. The band present in pummelo 2240 and scored as allele RFZ20-*w* in segregating populations was not observed in any other accessions. The phenotypes suggest that the cloned $OpZ20_{410}$ RAPD marker hybridized to one or perhaps two loci in all accessions. The phenotypes were consistent with a single locus having at least 7 alleles, but additional genetic analyses would be needed to confirm this. Because the *acitric* allele is linked to a unique RFLP allele, RFZ20-*w*, it should be possible to use this RFZ20 for marker-assisted selection of the pummelo 2240 derived *acitric* allele in almost any cross.

One of our long-term breeding objectives is to breed new mandarin and grapefruit cultivars with moderate acidity levels. Since the marker SCZ20 is polymorphic among mandarins, grapefruits, and pummelos, it will be helpful in selecting *acitric* heterozygotes in backcrosses and later generations derived from pummelo 2240. In crosses in which the marker SCZ20 is not informative, the codominant marker RFZ20 or the dominant RAPD marker $OpZ20_{410}$ can be used.

In summary, the *acitric* gene of pummelo 2240 prevents citric acid accumulation in citrus fruit when homozygous and reduces citric acid content by about 30% when heterozygous. We have identified RAPD, SCAR, and RFLP markers that are highly polymorphic and closely linked to *acitric* and which should be able to assist citrus breeders in selecting *acitric* heterozygotes at the seedling stage.

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APPENDIX 2

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Aconitase activity and expression during the development of lemon fruit

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Citrus fruits are characterized by the accumulation of high levels of citric acid in the juice sac cells and a decline in acid level toward maturation. It has been suggested that changes in mitochondrial aconitase (EC 4.2.1.3) activity affect fruit acidity. Recently, a cytosolic aconitase (cyt-Aco) homologous to mammalian iron-regulated proteins was identified in plants, leading us to re-evaluate the role of aconitase in acid accumulation. Aconitase activity was studied in 2 contrasting citrus varieties, sweet lime (*Citrus limettioides* Tan., low acid) and sour lemon (*Citrus limon* var. Eureka, high acid). Two aconitase isozymes were detected. One declined early in sour lemon fruit development, but was constant throughout sweet lime fruit development. Its reduction in sour lemon was associated with a decrease in aconitase activity in the mitochondrial fraction. Another isozyme was detected in sour lemon toward maturation, and was associated with an increase in aconitase activity in the soluble fraction, suggesting a cytosolic localization. The cyt-Aco was cloned from lemon juice sac cells, but in contrast to the changes in isozyme activity, its expression was constant during fruit development. We present a model, which suggests that reduction of the mitochondrial aconitase activity plays a role in acid accumulation, while an increase in the cyt-Aco activity reduces acid level toward fruit maturation.

Introduction

Citrus fruits are characterized by the accumulation of high levels of citric acid, which account for ~90% of the total organic acids (Sinclair 1984). The acid begins to accumulate early in fruit development, reaches a peak, and then, in most varieties, declines toward maturation (Erickson 1968). Usually, the citric acid concentration in the juice peaks at 0.1–0.2 *M*, but in sour lemon it reaches 0.3 *M* (Sinclair 1984).

Citric acid is synthesized in the juice sac cells and not translocated from the leaves to the fruit (Ramakrishnan 1971). The synthesis occurs via the condensation of acetyl-CoA with oxaloacetate to yield the 6-carbon citrate, a reaction catalyzed by citrate synthase (CS). The citric acid is then reversibly isomerized to isocitrate via *cis*-aconitate by aconitase (EC 4.2.1.3). Citrate synthesis in the cells probably takes place in the mitochondria (through the tricarboxylic acid [TCA] cycle) and the acid accumulates in the vacuoles of the juice sac cells, where it reaches high concentrations (Echeverria and Valich 1988, Chamarro et al. 1991). Thus, citrate accumulation also requires movement across the tonoplast. Tonoplast vesicles from citrus juice cells have the capacity for ATP-driven citrate uptake, in addition to a H^+-ATPase activity (Canel et al. 1995, Müller et al. 1996, 1997, Brune et al. 1998). Bogin and Wallace (1966) compared organic acid metabolism in sour and sweet lemon varieties and hypothesized that an inhibition of mitochondrial aconitase activity creates a metabolic block in the TCA cycle, leading to citrate accumulation in sour lemon fruits.

Aconitase belongs to a family of more than 30 proteins that participate in isomerization, catalysis, and RNA binding (Gruer et al. 1997). The enzyme contains a single, labile [Fe-S] cluster, which is composed of [3Fe-4S] in the inactive state. Activation of the enzyme involves binding of a fourth labile Fe (Rouault and Klausner 1996). Two forms of the enzyme have been described in plants, a mitochondrial and a cytosolic enzyme (Verniquet et al. 1991, De Bellis et al. 1993, 1994, 1995, Hayashi et al. 1995, Peyret et al. 1995). So far, only the cytosolic enzyme has been cloned from higher plants, and direct evidence has been provided for its cytosolic location (Hayashi et al. 1995, Peyret et al. 1995). While the mitochondrial enzyme most likely participates in the TCA cycle, the cytosolic enzyme might play a role in different metabolic pathways. For instance, its participation was suggested in the glyoxylate cycle in pumpkin cotyledons

Abbreviations – CS, citrate synthase; cyt-Aco, cytosolic aconitase; IDH, isocitrate dehydrogenase; IRP, iron-regulated protein; PEPC, PEP carboxylase.

(Hayashi et al. 1995). The cytosolic enzyme shows a remarkably high similarity with proteins of one subfamily of the aconitase, the iron-regulated proteins (IRPs), which possess RNA binding activity and regulate the translation of genes playing a role in iron homeostasis in animal cells (reviewed in Klausner et al. 1993, Hentze 1996, Rouault and Klausner 1996). One group of the IRPs, the IRP1s, consists of bifunctional proteins which act as cytoplasmic aconitases when iron is not limited, and as RNA binding proteins under iron deficiency.

The aim of the present study was to re-evaluate the role of the mitochondrial and cytosolic aconitase (cyt-Aco) in citric acid accumulation in the juice sac cells of citrus fruits. We show that one isozyme, which is correlated with the mitochondrial form of aconitase, is greatly reduced early in fruit development, while a different one, correlated with a soluble form, is induced toward fruit maturation. The results are fitted into a model, which summarizes current knowledge of acid accumulation and decline in the fruit.

Materials and methods

Plant material and fruit acidity measurements

Fruits of sour lemon (*Citrus limon* var. Eureka) and Palestine sweet lime (*C. limettioides* Tan.) were collected from orchards in the central-coastal region of Israel. The total acidity of the fruit was determined by the titration of 2 ml of juice extract with 0.1 *M* NaOH, with phenolphthalein as an indicator. Citric acid is the major organic acid in the juice, and the variations in acidity during fruit development are due chiefly to changes in its concentration (Sinclair 1984). Therefore, the total acidity, calculated as if citric acid were the only acid in the extract, is given as a percentage of juice volume (w/v). In acid lemon, 0.3 *M* of citric acid in the juice forms ~ 6% (w/v) total acidity.

Juice sac cells total extraction and fractionation

Depending on the sizes of the fruits and the juice contents of the vesicles, 1–4 g of juice vesicles from 4 or 5 fruits, at similar developmental stages, were divided into two halves and kept on an ice bath. In order to prevent enzyme inhibition by the low pH of the extracts, we routinely made a trial extraction, using half of the juice sacs. The contents of one tube were homogenized in 100–500 µl of extraction buffer containing 100 m*M* Tris-HCl, pH 7.5, 10 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* polyvinylpyrrolidone (40 kDa), and 14.3 m*M* β-mercaptoethanol (Soltis et al. 1983). The homogenate was adjusted to pH 7.5 by the addition of 5 *M* KOH. The same volume of KOH was then added to the extraction buffer prior to its addition to the content of the second half. If necessary, the pH was then corrected to 7.5. Following quick extraction in an ice bath, cell debris was removed by centrifugation at 1000 *g* for 30 min at 4°C and the supernatant, considered as the total extract, was assayed.

Soluble and mitochondrial preparations were obtained essentially as described by Bogin and Erickson (1965), with modifications. Juice sacs were collected and extracted as described above, with a buffer containing 0.6 *M* sucrose, 250 m*M* Tris-Cl, pH 7.8. Cell debris was removed by centrifugation at 2000 *g* for 10 min at 4°C and the supernatant was re-centrifuged at 20000 *g* for 30 min. The resulting supernatant was regarded as the soluble fraction and was mixed with 1 volume of extraction buffer prior to its assay. The pellet was washed with a solution containing 0.6 *M* sucrose, 25 m*M* Tris-HCl, pH 7.8, and was pelleted by centrifugation at 20000 *g* for 30 min at 4°C. It was then resuspended with 100–200 µl of extraction buffer to give a fraction enriched with mitochondria.

The protein contents of the total and the fractionated extracts were determined with Bradford reagent (Bio-Rad, Hercules, CA, USA).

Aconitase assay

The aconitase assay was performed in gel, as described previously (Soltis et al. 1983, Wendel and Weeden 1989), with modifications. We used a non-denaturing polyacrylamide mini-gel system containing an upper gel of 4% (w/v) acrylamide-bisacrylamide (30:1, v/v) in 125 m*M* Tris-HCl, pH 6.8, and a separating gel containing 7% (w/v) acrylamide-bisacrylamide (30:1, v/v) in 375 m*M* Tris-HCl, pH 8.0. The electrode buffer contained 25 m*M* Tris-HCl and 192 m*M* glycine. Before sample loading, the gel was pre-run for 30 min with 10 m*M* glutathione in order to block unpolymerized acrylamide. Total protein extract (20–40 µg protein in 20 µl) was mixed with 10 µl of gel-loading buffer containing 50% glycerol and bromophenol blue. Separation was performed under 60–120 mV at 4°C. When the bromophenol blue had reached the bottom of the gel, an additional aliquot of loading buffer was loaded, and the separation was stopped when the second dye had reached half way. Staining was performed at 37°C in a solution containing 100 m*M* Tris, pH 8.5, 4 m*M* *cis*-aconitic acid, 10 m*M* $MgCl_2$, 0.130 m*M* $NADP^+$, 1.2 m*M* (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 3.26 m*M* phenazine methosulfate (PMS) and 7 units of isocitrate dehydrogenase (IDH; EC 1.1.1.42, Sigma I2002), until at least one band was visible. Replacing the staining solution with 7% (v/v) acetic acid stopped the reaction.

Aconitase assay was also performed in a reaction volume of 1 ml using protein extract equivalent to 0.1 g fresh weight with *cis*-aconitate as a substrate, essentially as described by Hirai and Ueno (1977).

RNA extraction and analysis

RNA from juice vesicles, leaves, and flowers was extracted by means of the phenol-chloroform method of Ausubel et al. (1988), with modifications. Approximately 5 g of tissue was ground in liquid nitrogen and added to a tube containing 17 ml of extraction buffer and 8 ml of phenol saturated with 0.2 *M* Tris-HCl, pH 8.2, 0.1 *M* LiCl, and 5 m*M* EDTA. The mixture was blended in a Polytron (Kinematica, Bazel, Switzerland), and 8 ml of chloroform was added to it, followed by a 30-min incubation at 50°C with occasional shaking. The mixture was centrifuged at 10000 *g* for 20 min and the upper phase was re-extracted with 1 volume of phenol-chloroform (1:1, v/v), and recentrifuged. LiCl was

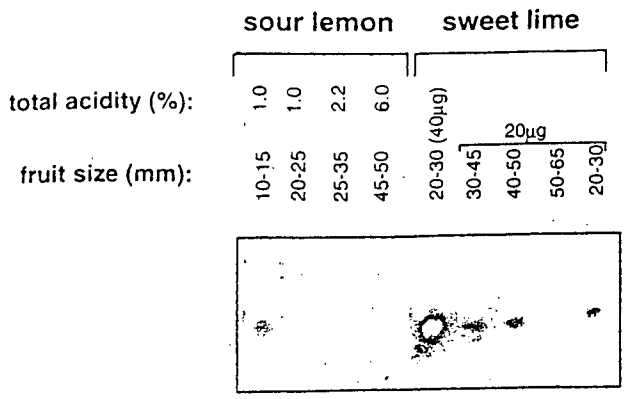

Fig. 1. A developmental analysis of aconitase activity in acidless (sweet lime) and high acid (sour lemon) varieties. Protein extracts of fruits of increasing developmental stages, as indicated by their size and total titratable acid, were separated on non-denaturing PAGE. Aconitase isozymes were detected using *cis*-aconitic acid as a substrate, and visualized by staining with tetrazolium salt. The two leftmost lanes represent extracts of lemon vesicles from fruits of differing sizes, but with 1% total acidity. All fruits were analyzed with 20 µg of protein extract, but the smaller fruits of sweet lime were analyzed also with 40 µg of protein extract.

then added to the upper phase to a final concentration of 2 *M* (from 8 *M* stock solution), followed by an overnight incubation at 4°C and centrifugation at 10000 *g* for 20 min at 4°C. The pellet was dissolved in 0.5 ml of H_2O and briefly centrifuged to remove undissolved material. We did not observe any differences in the RNA yield and quality in fruits at different developmental stages, except that in mature yellow fruits the yield was low, and the RNA was usually quite degraded. Northern blot analyses were performed with [α^{32}P]dCTP-radiolabeled probes (Sambrook et al. 1989). The membranes were exposed to X-ray films; they were also scanned with a Fujifilm BAS-1500 Phosphoimager (Fuji Photo Film Co., Tokyo, Japan), and the resulting images were quantified with TINA2 software (Fuji Photo Film Co.). The ethidium bromide (EtBr)-stained gel in Fig. 4A was scanned and quantified with the same software.

Results

The activity of the mitochondrial aconitase is reduced early in sour lemon fruit development

The activity of aconitase was examined by non-denaturing PAGE, with *cis*-aconitic acid as a substrate. This method has the potential to detect changes in different isozymes in a total extract simultaneously, and to follow a change in one isozyme against a background of others, which might not be altered. A single isozyme was detected early in sour lemon fruit development (Fig. 1, 10–15-mm fruits). This isozyme was reduced later in development, when the acid level increased. To allow for the possibility that inadequate neutralization, increased salt, or increased acid concentration could affect the assay, more developed fruits (as indicated by their size, 20–25 mm), with similar acid levels (1%) were also analyzed (Fig. 1). These results showed that the isozyme prevalent in 10- to 15-mm fruit was reduced in 20- to 25-mm fruits of similar acidity. By contrast, a similar isozyme identified in sweet lime remained approximately

Table 1. Aconitase activity during the early development of sour lemon. The values represent means of results from triplicate experiments ± SE.

Fruit size (mm)	Total acidity (%)	Mitochondria		Soluble		Total extract	
		Total activity (nmol g^{-1} FW min^{-1})	Specific activity (nmol mg^{-1} protein min^{-1})	Total activity (nmol g^{-1} FW min^{-1})	Specific activity (nmol mg^{-1} protein min^{-1})	Total activity (nmol g^{-1} FW min^{-1})	Specific activity (nmol mg^{-1} protein min^{-1})
15–20	0.52 ± 0.05	7.53 ± 1.05	88.5 ± 10.7	0.52 ± 0.31	3.01 ± 0.32	10.59 ± 1.24	30.25 ± 2.44
20–30	1.34 ± 0.12	1.22 ± 0.63	21.3 ± 1.1	0.68 ± 0.27	2.42 ± 0.15	2.03 ± 0.76	5.31 ± 1.02

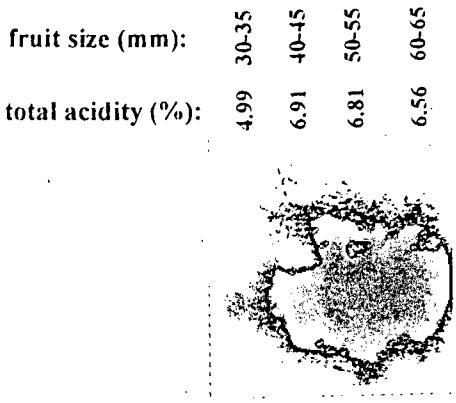

Fig. 2. Analysis of aconitase activity in sour lemon near fruit maturation. Fruits of increasing developmental stages, as indicated by their size and total titratable acid, were extracted, and 20 μg of protein extract were analyzed for aconitase isozymes as described in Fig. 1.

constant throughout most stages of fruit development, and was reduced only in nearly mature and mature fruit of 50–65 mm (Fig. 1). The reduction in the aconitase activity in young sour lemon fruits was also confirmed by in tube assay. Pulps of 15–20- and 20–30-mm fruits were fractionated into soluble and mitochondrial fractions and aconitase was assayed (Table 1). In both samples, the specific activities of the mitochondrial fractions were 10- to 20-fold higher than those of the soluble fractions. This suggested that the mitochondrial enzyme was the dominant one in young fruits, and contributed to most of the activity detected in the total extracts. In the mitochondrial fraction of the larger fruits, 20–30 mm in diameter, a ~4-fold decrease in the specific activity was detected in comparison with the smaller fruits (15–20 mm). The reduction in the activity of the mitochondrial fraction correlated well with the decrease in the isozyme of young sour lemon fruit (Fig. 1), supporting the view that this isozyme was the mitochondrial form of aconitase. The reduction in its activity in sour lemon (high acid), but not in sweet lime (low acid), provided support for the suggestion that a reduction in the mitochondrial aconitase activity contributed to citrate accumulation (Bogin and Wallace 1966). The reduction in the total and specific activities might result either from a reduced amount of enzyme, binding of an inhibitory substance, which is not released during gel separation, or some other modification of the enzyme.

A soluble aconitase is induced in sour lemon fruits toward maturation when acid peaks

In most citrus fruits, citric acid starts to decline toward fruit maturation, but this decrease can be as little as 30–40 m*M* (~0.5–0.7%, w/v, in total acidity) in acid lemon. We examined aconitase activity late in fruit development, when acid content reaches a peak. A remarkable increase in one isozyme was observed toward maturation in sour lemon fruit 50–65 mm in diameter (Fig. 2). This suggests that re-induction of aconitase activity precedes citric acid decline, normally detected in yellow mature fruits (not included in this experiment). A comparison of two groups of young fruit (10–15 and 15–20 mm) and two groups of mature fruit (40–55 and 55–65 mm) shows that the two isozymes are different (Fig. 3A). The early isozyme is slow migrating, and corresponds to the one shown in Fig. 1, while the later one is fast migrating and corresponds to the one shown in Fig. 2. In addition, in some cases (fruit of moderate size, 20–30 mm and 1.88% acidity) both isozymes were detected in the same extract (Fig. 3B). We next examined the activity of aconitase in soluble and mitochondrial preparations of fruits toward maturation. Pulps of large fruits, 30–60 mm, were fractionated as described above, and the results are shown in Table 2. The specific activity in the mitochondrial preparations remained low in all fruits chosen for the experiment. In contrast, a > 10-fold induction was detected in the soluble fraction of 50–60 mm-fruits, as compared with 30–35-mm fruits. This strongly suggests that the isozyme, which is induced toward fruit maturation, is associated with the soluble fraction, and is most likely the cytosolic form of aconitase.

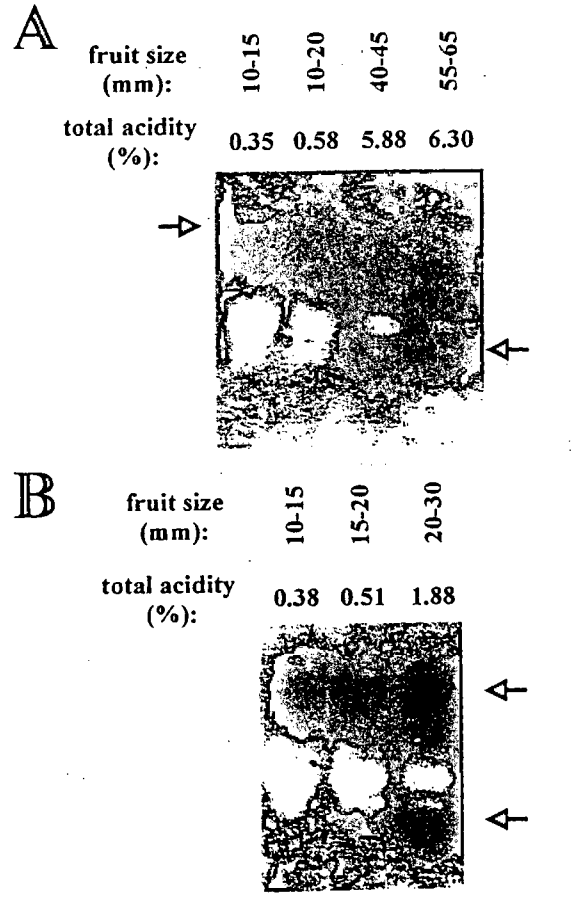

Fig. 3. Two isozymes are developmentally regulated during sour lemon fruit development. A. Extracts of two groups of young fruits and two groups of mature fruits, as indicated by their size and total titratable acidity were analyzed for aconitase isozymes, as described in Fig. 1. Arrows mark the two isozymes. The upper one corresponds to the one shown in Fig. 1, and the lower one to the one shown in Fig. 2. B. Extracts of fruits of moderate size and acidity were analyzed for aconitase isozyme as described above. The arrows mark the position of the two isozymes. In all cases, 20 μg of protein extracts were analyzed. The white bands (see also Fig. 2), contain probably pigments, which due to their hydrophobic nature are not stained by the background staining.

Table 2. Aconitase activity in mid-sized and nearly mature fruits. The values represent means of results from triplicate experiments ± SE.

Fruit size (mm)	Total acidity (%)	Mitochondria		Soluble		Total extract	
		Total activity (nmol g^{-1} FW min^{-1})	Specific activity (nmol mg^{-1} protein min^{-1})	Total activity (nmol g^{-1} FW min^{-1})	Specific activity (nmol mg^{-1} protein min^{-1})	Total activity (nmol g^{-1} FW min^{-1})	Specific activity (nmol mg^{-1} protein min^{-1})
30–35	4.97 ± 0.23	2.03 ± 0.44	19.31 ± 2.10	1.04 ± 0.56	3.11 ± 0.23	2.25 ± 0.42	5.31 ± 0.12
40–45	5.67 ± 0.14	0.83 ± 0.56	6.12 ± 0.37	4.54 ± 1.01	24.42 ± 2.01	7.58 ± 0.93	35.26 ± 5.02
50–60	5.98 ± 0.32	0.42 ± 0.32	7.04 ± 2.12	10.07 ± 1.27	53.20 ± 0.10	17.93 ± 1.25	81.34 ± 4.32

Cytosolic aconitase is constitutively expressed in the fruit pulp

We moved next to study whether the changes in the aconitase isozyme and enzyme activity in the soluble fraction would also be reflected at the gene expression level. An *Arabidopsis* probe for cyt-Aco gene, homologous to the mammalian IRP, was used as a probe to screen a λ-Zap-Express cDNA library (Stratagene, La Jolla, CA) of lemon juice vesicles. A full-length cDNA clone of ~3.4 kbp was cloned and sequenced (GenBank Accession no. AF073507). It contains a 5'-untranslated region (UTR) of 455 bases, a deduced coding region of 898 amino acids, and a 3'-UTR of 216 bases. The deduced amino acid sequence shows 80–90% identity with pumpkin, *Arabidopsis*, potato, and watermelon cyt-Aco (not shown). The plant clones, including the lemon one, were compared with the human IRP1, which possesses aconitase activity in addition to RNA binding activity, and with IRP2, which lacks aconitase activity and acts only as RNA binding protein (Phillips et al. 1996). A higher identity and similarity were found with IRP1 than with IRP2. For example, the lemon clone showed 62% identity and 70% similarity with IRP1 and 54% identity and 64% similarity with IRP2. Moreover, like IRP1, the plant clones lack a domain of 73 amino acids, which is hypothesized to prevent IRP2 potential aconitase activity.

The clone was used as a probe in northern blot analyses with RNA extracted from juice vesicles of sour lemon and sweet lime (Fig. 4). Fruits at various developmental stages, as indicated by their size and total acidity, were analyzed. Lemon aconitase was highly and continuously expressed throughout

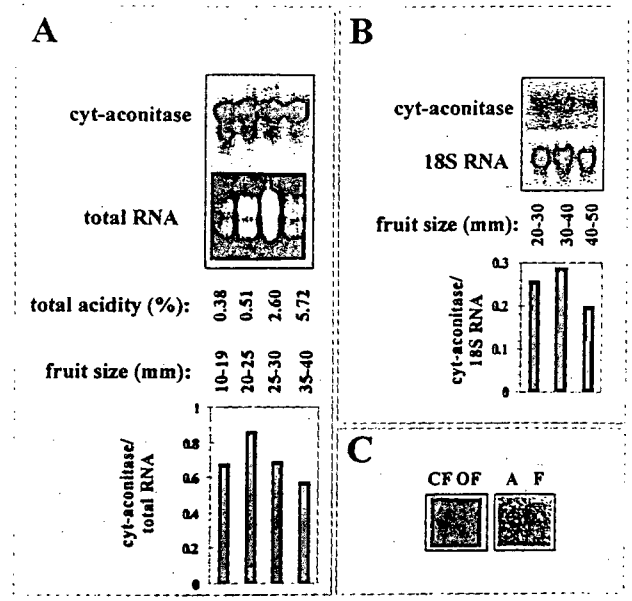

Fig. 4. The expression of lemon cyt-Aco. RNA was extracted from sour lemon and sweet lime fruits of several developmental stages, as indicated by the size and total acidity of the fruits. A. RNA from sour lemon juice vesicles. The upper panel shows hybridization results with cyt-Aco clone; the middle panel, EtBr-stained gel; and the bottom panel, standardization of the cyt-Aco signal with the total RNA, as described in Materials and methods. B. The same as A, but for RNA of sweet lime using the same probe (upper panel), or a probe for 18S rRNA as a standard (middle panel). C. RNA from closed flowers (CF) and open flowers (OF) of lemon, albedo (A) and flavedo (F) of 35-mm sour lemon fruits.

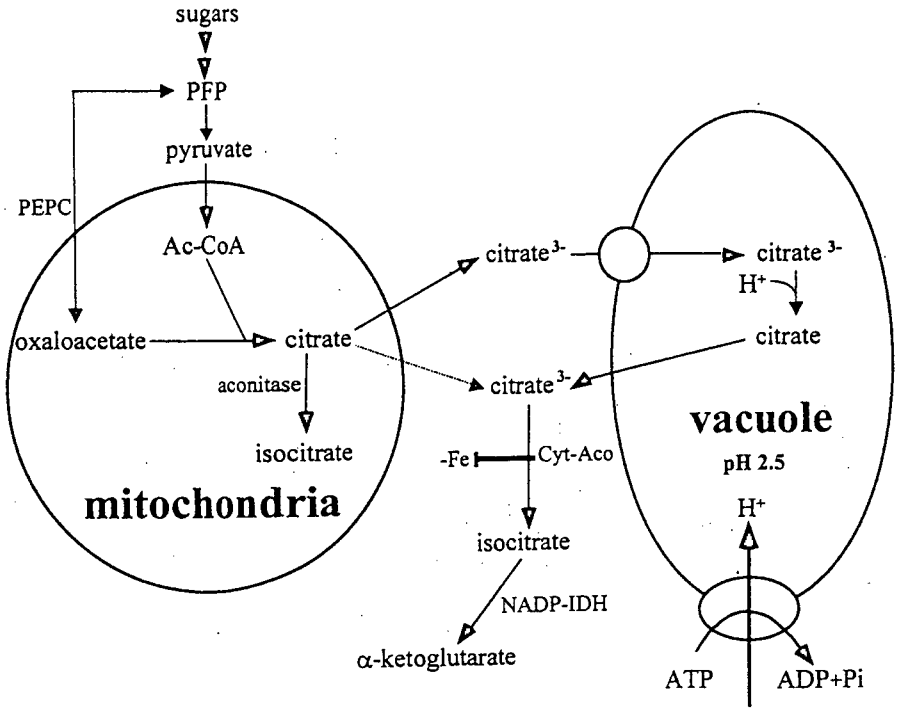

Fig. 5. A model showing the reactions involved in the accumulation and decline of citrate in citrus fruits. Citric acid synthesis results from the condensation of acetyl Co-A with oxaloacetate by CS. PEPC can directly supply oxaloacetate for the synthesis of citrate. Reduction in mitochondrial aconitase activity in sour lemon, but not in sweet lime, leads to a local increase in citrate that is translocated to the vacuole via a specific carrier in an ATP-dependent manner. In the vacuole, citric acid acts as a buffer and absorbs protons, which are pumped in via a tonoplastic H^+-ATPase. When acid reaches a steady-state level in lemon fruit, or declines in other citrus fruits, it is removed to the cytosol. The cyt-Aco activity is responsible for the conversion of citric acid into isocitric acid. Iron availability might play a role in this process. Next, isocitrate is converted into α-ketoglutarate by NADP-IDH. Further steps might occur in the cytosol or in the mitochondria. It is likely that some of the citrate is converted into isocitrate, utilized for amino acid synthesis, during acid accumulation stage (dashed arrow).

sour lemon fruit development (Fig. 4A); the slight increase in expression early in fruit development (20–25 mm-fruit) did not seem to be physiologically relevant. Aconitase expression in sweet lime was somewhat lower than that in sour lemon, but it was also constant throughout fruit development (Fig. 4B). Some expression of aconitase was also observed in closed and open flowers, before fruit set; very low expression was observed in the outer peel, the flavedo, and none in the inner peel, the albedo (Fig. 4C).

Discussion

The compartmentation of citric acid in the vacuole seems to play a major role in its accumulation in the fruit. However, other mechanisms at the metabolic level, might also play a role in citrate accumulation. Dark CO_2 fixation in the juice sac cells, mainly by PEP carboxylase (PEPC), could serve as a controlling point for acid accumulation (Bean and Todd 1960, Clark and Wallace 1963, Young and Biale 1968, Yen and Koch 1990). However, no correlation was found between PEPC and changes in acid content during fruit development, or between low- and high-acid varieties (Sinclair 1984). Citric acid is synthesized in the mitochondria as part of the TCA cycle by CS. Several studies (Bruemmer et al. 1977, Canel et al. 1996, A. Sadka, E. Dahan and L. Cohen, unpublished results) show that changes in CS activity and expression do not correlate with changes in citric acid level in low- and high-acid varieties. A model proposing a block in mitochondrial aconitase activity explains the accumulation of acid in sour lemon compared with sweet lime (Fig. 5).

Early in fruit development, mitochondrial aconitase activity is reduced (slow migrating isozyme, Figs. 1 and 3B, Table 1), which 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. No increase in the level of citrate is expected to occur in sweet lime, since its mitochondrial aconitase activity is not reduced (Fig. 1). This part of the model is consistent with the aconitase-block hypothesis that was proposed by Bogin and Wallace (1966). Their model was based on the finding that the level of citramalate, a competitive inhibitor of aconitase, is higher in sour lemon than in sweet lime. Reduction in aconitase activity creates a complete or, most likely, a partial block in the TCA cycle. Such a block is not unique for citrus fruits; in photosynthetically active leaves, the TCA cycle is not fully operative, and supplies citrate for amino acid synthesis in the cytosol (Hanning and Heldt 1993). In citrus, a partial block would not abolish organic acid metabolism, but would be sufficient to create a local increase in citric acid, which is immediately translocated from the mitochondria. Oxaloacetate, required for citric acid accumulation, could be supplied by the activity of PEPC (Fig. 5).

It has been proposed that citrate (Fig. 5) is translocated into the vacuole via a specific carrier (Canel et al. 1995, Brune et al. 1998) and that the tonoplastic H^+-ATPase pumps protons into the vacuole (Müller et al. 1996, 1997). This reduces the pH to ~2.5 and probably provides a driving force for citrate uptake into the vacuole, where it acts as a buffer. Although most of the citrate is stored in the vacuole, it is likely that small portion of it is converted into isocitrate in the cytosol, even during the acid accumulation stage. Indeed, aconitase activity in the soluble faction is maintained at some level even at early stages of fruit development (Table 1). Moreover, the soluble NADP-IDH, which probably plays a role in citrate catabolism, is also active at these stages of fruit development (A. Sadka, E. Dahan, E. Or and L. Cohen, unpublished results).

The model (Fig. 5) also assumes that when the acid reaches a steady state level in lemon, and declines in other citrus fruits, it is exported from the vacuole. Aconitase activity then increases, catabolizing citric acid into isocitric acid. Re-induction of a second aconitase isozyme toward fruit maturation is clearly demonstrated in Fig. 2. Based on our results presented in Table 2, we suggest that this isozyme corresponds to the cyt-Aco. Hirai and Ueno (1977) similarly showed a second phase of aconitase activity occurring at late stages of Satsuma mandarin fruit development, and a developmental change in different isoforms of the enzyme was also described in other plants (De Bellis et al. 1995). The induction of the cyt-Aco precedes citrate decline, suggesting that other regulatory points for acid catabolism might exist, possibly at the vacuolar export stage. In contrast to isozyme pattern, RNA analysis (Fig. 4) shows that gene expression of the cyt-Aco does not change significantly during fruit growth. This may indicate that the level of regulation is post-transcriptional, although we can not exclude the possibility that the clone does not encode for the isozyme shown in Fig. 2. Animal IRP1, which possesses aconitase activity, is regulated at the post-translation level, and this might also be the case in lemon fruit.

The model also implies that the iron level in the cell might affect acid content. Iron responsive elements (IRE) were identified in the 5'-end of the mRNA of the mammalian mitochondrial aconitase and of the iron-sulfur protein subunit of succinate dehydrogenase of *Drosophila* (Gray et al. 1996, Kim et al. 1996). These RNAs have been shown to bind IRP, so they are not translated. These findings show that a regulatory link between iron homeostasis and organic acid metabolism exists in animal cells. IREs have not yet been identified in plant genes involved in organic acid metabolism, nor in any other plant genes. However, leaves of iron-deficient plants may show a remarkable increase in organic acid levels (DeKock and Morrison 1958, Clark 1968, Wallace 1971), and in one case a reduced aconitase activity (Bacon et al. 1961). We hypothesize that under Fe-limiting conditions, aconitase activity in the cytosol would decline, causing citric acid to accumulate and this is currently being studied in our laboratory using juice vesicles in callus and cell cultures.

In summary, we present data supporting the view that changes in aconitase activity can explain the pattern of acid accumulation in sour lemon. Other factors, however, are likely to be needed to account for differences between low- and moderate-acid varieties, and for physiological responses within the same variety. The scheme presented in Fig. 5 provides a useful framework for discussing these differences in acidity.

Acknowledgements – This work was supported by The USA-Israel Binational Agricultural Research and Development Fund (BARD grant no. IS-2531-95), and by The Israeli Citrus Board. Contribution No. 214/99 from Agricultural Research Organization, Institute of Horticulture, Bet Dagan, Israel.

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APPENDIX 3

JOURNAL OF THE AMERICAN SOCIETY FOR HORTICULTURAL SCIENCE

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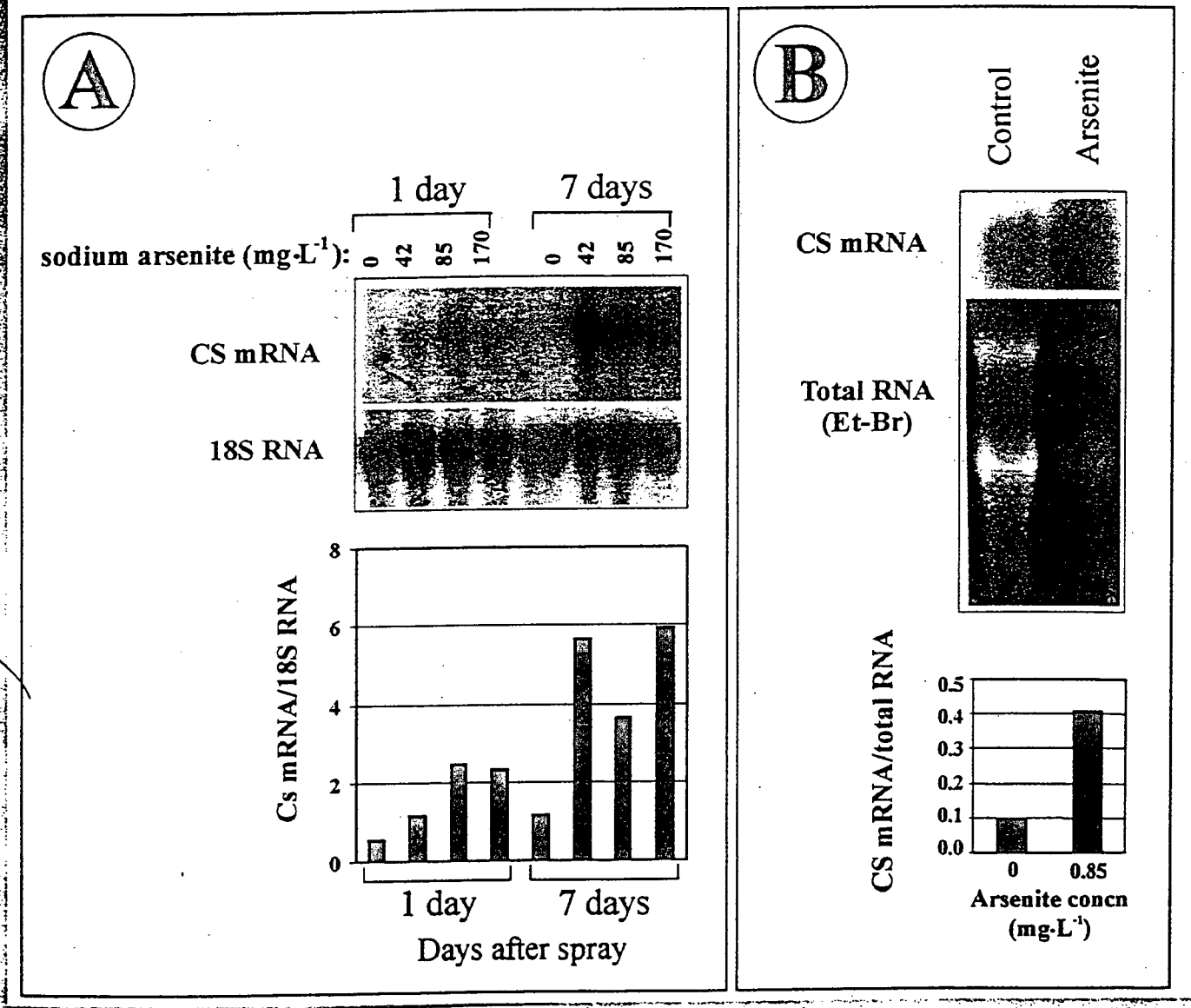

On the Cover

Effects of sodium arsenite treatment on expression of citrate synthase gene in (A) 'Minneola' tangelo fruit and (B) 'Eureka' lemon callus. RNA was extracted from treated 'Minneola' tangelo fruit and collected 1 or 7 d following application, or from 'Eureka' lemon calli grown for 6 weeks in the presence of 0.85 mg·L^{-1} sodium arsenite (see p. 291).

Photo courtesy of Avi Sadka et al.

RESEARCH SPOTLIGHT

We will call your attention to the following papers appearing in this issue of the journal. They caught my interest while I was editing these manuscripts and a similar response is anticipated from many of our readers.

Frank Blazich

Reproduction and Horticultural Performance of Transgenic Ethylene-insensitive Petunias

The ability to develop ethylene-insensitive plants with delayed flower senescence has great commercial potential in the floriculture industry. Since plants have evolved elaborate hormone perception systems, it is logical to hypothesize that constitutive suppression of the perception of any hormone might be detrimental to some stage(s) of whole plant flower development. A series of experiments by **Gubrium et al. (p. 277)** characterized a range of effects of the *etr1-1* transgene on the gross morphology of ethylene-insensitive transformed petunia (*Petunia ×hybrida*) plants. This characterization involved an examination of the growth and development (horticultural performance) of ethylene-insensitive transgenic plants. Horticultural performance of these plants was evaluated in various areas that are closely related to crop production and pertinent to the commercial viability of the plants. The authors reported that transgenic *etr1-1* plants had longer lasting flowers, but fruit development was delayed, seed germination was inhibited, and adventitious rooting of stem cuttings was significantly decreased. In using the *etr1-1*, transgene driven by the constitutively expressed CaMV35S promoter, it was clearly demonstrated that developmental processes, other than flower senescence were altered which have important ramifications and must be considered.

Arsenite Reduces Acid Content in Citrus Fruit, Inhibits Activity of Citrate Synthase, but Induces Its Gene Expression

Pulp acidity of citrus fruit (*Citrus* sp.) is correlated with the citric acid concentration, and is a major factor in determining fruit maturity and quality. Citrate begins to accumulate during the second phase of fruit development, when the fruit and its juice vesicle cells enlarge rapidly. The accumulation continues for a few weeks, reaching a peak when the fruit volume is ≈50% of its final volume, and then, in most citrus species, declines gradually as the fruit matures. Citrate concentration in the juice usually reaches a peak of 0.1 to 0.2 M (3% to 4% titratable acidity) and it decreases to 20 to 50 mM (≈1% total titratable acidity in mature fruit. In many citrus species, high acid content in mature fruit reduces quality or delays harvest. Unfortunately, management tools that influence acidity are limited. For instance, a few rootstocks can partially reduce acid content at harvest time but, in many cases, they also have negative characteristics. Several arsenic compounds, including lead arsenate, calcium arsenate, sodium arsenite, and para-aminobenzenarsonic acid, in the soil or as a foliar spray, have been found to reduce the acid content in citrus fruit. However, arsenic compounds, including sodium arsenite, are prohibited for commercial use. **Sadka et al. (p. 288)** initiated a study to elucidate the mechanism of arsenite action, to develop alternative tools to improve fruit acidity. They present the first detailed study of changes in the kinetics of citric acid accumulation and total titratable acidity, which follow sodium arsenite treatment. They demonstrated that arsenite effects become detectable immediately following a lag in acid accumulation early in fruit development. They also show that an initial and temporal inhibition of citrate synthase activity, which lasts for a few days following arsenite application, is accompanied by induction of its gene expression. The authors report that arsenite treatment was very effective in reducing total fruit acid concentration to a desired level of 1% ≈2 weeks sooner than in the controls, thus allowing early harvest. Although use of this chemical is prohibited, a better understanding of its mode of action might lead to development of a nonhazardous alternative means to reduce fruit acidity. This research does not answer the question of the mechanism of arsenite effect but it raises interesting possibilities for future research.

Surface Energy Balance Affects Gas Exchange and Growth of Two Irrigated Landscape Tree Species in an Arid Climate

There is lack of information on how water loss of isolated trees in arid climates is influenced by nonvegetative urban surfaces. Past research on water loss of isolated trees has been conducted primarily in regions of high relative humidity. In such regions leaf-to-air vapor pressure difference (LVPD) is generally lower than LVPD in arid regions and urban environments. There is also little information on how isolated landscape trees respond to urban environments found in arid regions. Therefore, **Montague et al. (p. 299)** conducted the following research to investigate the

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Arsenite Reduces Acid Content in *Citrus* Fruit, Inhibits Activity of Citrate Synthase but Induces Its Gene Expression

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Additional index words. citric acid, 'Eurieka' lemon, 'Minneola' tangelo

Abstract. **Arsenic compounds generate diverse effects in all living organisms. In citrus (*Citrus* L. sp.), they reduce acidity and improve fruit quality by unknown mechanisms. The major organic acid in citrus fruit is citric acid, which begins accumulating early in fruit development, reaches a peak in middle-sized fruit and then, in most species, declines as the fruit matures. In an attempt to understand the basis of the effect of arsenite, it was applied to 'Minneola' tangelo (*Citrus paradisi* Macf. x *C. reticulata* Blanco) ≈6 weeks postanthesis, and a detailed analysis of total titratable acidity and citric acid concentration was performed throughout fruit growth. Within 35 days after arsenite application, total acid content and citrate concentration were slightly lower compared with the controls, and this difference persisted throughout fruit development. The concentrations of other organic acids were not reduced by the treatment. Sodium arsenite reduced the citrate concentration in 'Eurieka' lemon callus [*Citrus limon* (L.) Burm.] also, without affecting tissue growth. Extractable activity of citrate synthase in treated fruit was inhibited within 1 day following arsenite spray, but recovered to a normal level a few days later. In contrast, gene expression was remarkably induced 1 day following treatment, which might explain the recovery in enzyme activity. Data suggest that reduction in acid accumulation may not be related to the initial inhibition of citrate synthase activity.**

Pulp acidity of citrus fruit (*Citrus* sp.) is correlated with the citric acid concentration, and is a major factor in determining fruit maturity and quality (Sinclair, 1984). Citrate begins to accumulate during the second phase of fruit development, when the fruit and its juice vesicle cells enlarge rapidly (Erickson, 1968). The accumulation continues for a few weeks, reaching a peak when the fruit volume is ≈50% of its final value, and then, in most citrus species, declines gradually as the fruit matures. Citrate concentration in the juice usually reaches a peak of 0.1 to 0.2 M (3% to 4% total titratable acidity), and it decreases to 20 to 50 mM (about 1% total titratable acidity) in mature fruit (Sinclair, 1984).

The biosynthesis of citric acid in the mitochondria is catalyzed by citrate synthase, which condenses the four-carbon acid, oxaloacetate, with the two-carbon molecule, acetyl-CoA. Next, citrate is isomerized into isocitrate by aconitase. It has long been hypothesized that a metabolic block in the mitochondrial aconitase plays a major role in citrate accumulation (Bogin and Wallace, 1966). Indeed, we have shown recently that activity of this enzyme in 'Eurieka' lemon (*Citrus limon*) is greatly reduced early in fruit development (Sadka et al., 2000). Following synthesis, citrate is transported and stored in the vacuole (Brune et al., 1998; Canel et al., 1995; Echeverria and Valich, 1988).

In many citrus species, high acid content in mature fruit reduces quality or delays harvest. Management tools that influence acidity are limited. For instance, a few rootstocks can partially reduce acid content at harvest time but, in many cases, they also have negative characteristics (Davies and Albrigo, 1994). Several arsenic compounds, including lead arsenate, calcium arsenate, sodium arsenite, and para-aminobenzenarsonic acid, in the soil or as a foliar spray, have been found to reduce the acid content in citrus fruit (Erner et al., unpublished; Miller et al., 1933; Wilson and Obreza, 1988; Yamaki, 1990a). In an attempt to understand the mechanism of the arsenate effect, Vines and Oberbacher (1965) treated mitochondria from 'Shamouti' orange [*Citrus sinensis* (L.) Osbeck] fruit pulp with lead arsenate, and showed that treatment uncoupled phosphorylation while having little effect on oxidation. It has also been shown that lead arsenate reduces citrate synthase activity and acetyl-CoA concentration by 20% to 30% during the 5 weeks following the spraying of 'Satsuma' mandarin [*Citrus unshiu* (Mak.) Marc.] (Yamaki, 1990a, 1990b). The effect of arsenite, which also reduces fruit acidity, has not been studied previously in citrus. Similar to arsenate, it is probably taken up by the phosphate transport system (Lenartowicz, 1990; Meharg and Macnair, 1992; Yompakdee et al., 1996). However, unlike arsenate, which acts as a phosphate analogue, inhibits phosphorylation-dependent processes and competes with phosphate as an electron acceptor, arsenite binds to vicinal thiol groups and inhibits enzymes which require SH groups for their catalytic activity (Lenartowicz, 1990; Lopez et al., 1990; Narayan and Nair, 1989). Therefore, arsenite is considered to be more toxic than arsenate, and it may affect fruit acidity through a different mechanism from that of arsenate.

Arsenic compounds, including sodium arsenite, are prohibited for commercial use. Therefore, we initiated a study to elucidate the mechanism of the arsenite action, in order to develop alternative tools to improve fruit acidity. We present herein the first detailed study of changes in the kinetics of citric acid accumulation and total titratable acidity, which follow sodium arsenite treatment. It is demonstrated that arsenite effects become detectable immediately following a lag in acid accumulation early in fruit development. We also show that an initial and a temporal inhibition of citrate synthase activity, which lasts for a few days following arsenite application, is accompanied by induction of its gene expression.

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Materials and Methods

PLANT MATERIAL AND TISSUE CULTURES. Fruit of 'Minneola' tangelo trees (*Citrus paradisi* x *C. reticulata*) grafted on sour orange rootstocks (*Citrus aurantium* L.) grown in the central-coastal region of Israel were used in this study. Tissue cultures originating from 'Eurieka' lemon (*C. limon*) fruit juice sacs were also used. The lemons were surface sterilized with 70% ethanol and cut into halves, and the juice sacs, including the stalks, were placed in 50-mL glass tubes containing 10 mL of a medium described by Erner and Reuveni (1981), except for omission of orange juice. The explants were incubated in the dark at 28 °C, and calli were formed within 4 to 6 weeks. Pieces weighing about 100 mg were cut from the callus under aseptic conditions, transferred to fresh medium and allowed to grow for an additional 4 weeks. At least two cycles of growth were performed before calli were used in experiments.

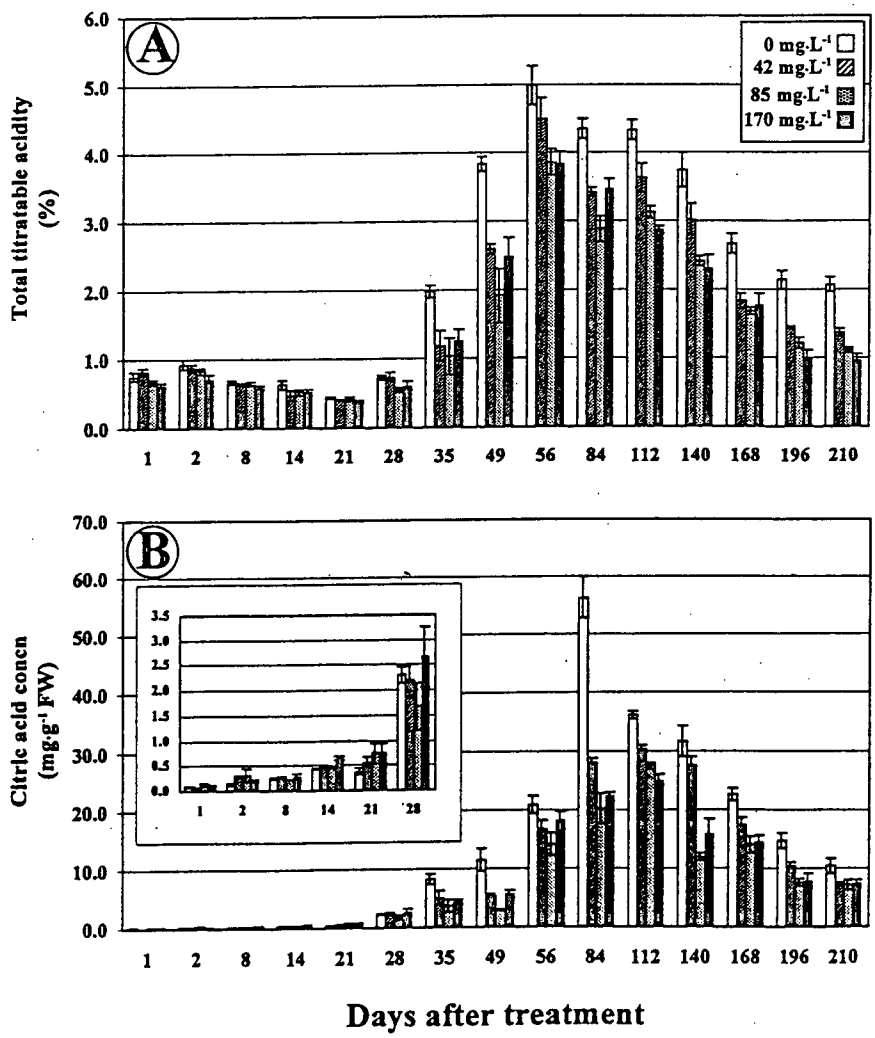
SODIUM ARSENITE TREATMENTS. Whole tree arsenite sprays were applied during 1997 and 1998. In 1997, arsenite was applied 14 July, ≈12 weeks postanthesis when fruit diameters were 25 to 30 mm. In 1998, the treatment was applied 3 June, about 6 weeks postanthesis, when fruit diameters were 10 to 15 mm. Aqueous solutions containing 0, 42, 85 or 170 mg·L^{-1} of sodium arsenite were adjusted to pH 3.5 with HNO_3, and sprayed in the presence of 0.025% Extravon surfactant (Novatis AG, Bazel, Switzerland). In both years, the experiments were performed on four replicates in randomized blocks, each containing four trees, with a border row between the blocks. Ten west-sided fruit from each block, located about 1.5 m above ground level, were collected at various times following the spray treatments, as indicated in Fig. 1. Fruit diameters in a few sampling dates were: 20 to 25 mm 28 d after treatment (7 July), 40 to 45 mm 84 d after treatment (8 Aug.), and 80 to 90 mm 210 d after treatment (30 Dec.). Following harvest, fruit were placed on ice for further analysis in the laboratory. Pieces of 'Eurieka' lemon calli weighing about 100 mg were placed on fresh media containing 0, 0.085, 0.425 or 0.850 mg·L^{-1} sodium arsenite and allowed to grow as described above for 4 to 6 weeks before analysis.

TOTAL TITRATABLE ACID AND ORGANIC ACID ANALYSES. Total titratable acid content of the fruit pulp was determined by titration (Sinclair, 1984). Depending on the juice content of the fruit, either 200 to 500 mg of juice sacs (young fruit), or 0.5 mL of juice sac extracts was analyzed for organic acids with a 330 gas chromatograph (Varian, Walnut Creek, Calif.) according to Erner and Reuveni (1981). We used a Megabore DB17 column (J & W Scientific, Folsom, Calif.) with the following conditions: N_2 flow-through, 10 mL·min^{-1}; N_2 makeup, 20 mL·min^{-1}; H_2, 30 mL·min^{-1}; air, 400 mL·min^{-1}. Detection was performed with a 4290 Integrator (Varian). When calli were used for analyses, they were extracted similarly to young fruit.

CITRATE SYNTHASE ACTIVITY. Depending on the size of the fruit and the juice content of the sacs, 0.6 to 2.0 g of juice sacs from 10 or 30 fruit, at similar developmental stages, were divided evenly between two tubes, and kept in an ice bath. In order to prevent enzyme inhibition by the low pH of the vesicles, we routinely made a trial extraction from half of the juice sacs. The contents of one tube were homogenized in a Polytron (Kinematica AG Littau, Luzern, Switzerland), in 0.3 to 1.0 mL of extraction buffer containing 50 mM HEPES-NaOH, pH 8.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% Tween-20, 0.5 mM phenyl-methylsulfonylfluoride, and 10% glycerol (Landschutze et al., 1995). The homogenate was adjusted to pH 7 by addition of a measured volume of 5 M KOH, and the same volume was then added to the extraction buffer prior to its addition to the second tube. Following quick extraction in an ice bath, cell debris was removed by centrifugation at 5,000 g_n for 10 min at 4 °C, and the supernatant was assayed for enzyme activity. The enzymatic assay was performed by the 5'5'-dithiobis-(-2-nitrobenzoate) method at 412 nm, as described by Srere (1969).

RNA EXTRACTION AND ANALYSIS. RNA was extracted from juice vesicles by means of the phenol-chloroform method of Ausubel et al. (1988), with modifications. About 5 g of tissue was ground with liquid nitrogen, and added to a tube containing 17 mL of extraction buffer and 8 mL of phenol saturated with 0.2 M Tris-HCl (pH 8.2),

Fig. 1. Effect of sodium arsenite sprays on (A) total titratable acidity and (B) citric acid concentration of 'Minneola' tangelo fruit. Trees of 'Minneola' tangelo were sprayed with various concentrations of sodium arsenite on 3 June 1998, ≈6 weeks postanthesis (10 to 15 mm fruit). Fruit were collected at different times, as indicated, and analyzed for titratable acidity and citric acid concentration. Mean values of four independent replicates ± SE. Legend in A applies to both figures. Inset (in B), citric acid concentration on an expanded scale.



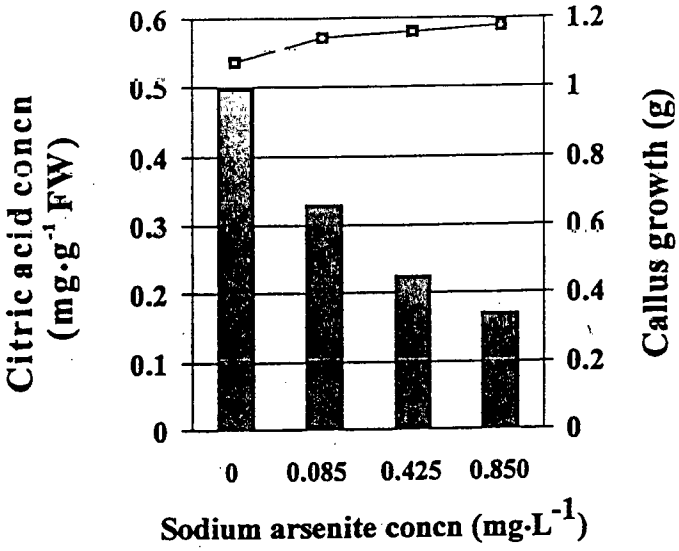


Fig. 2. Effect of sodium arsenite concentration on citric acid concentration (columns) and growth (■) of 'Eurieka' lemon callus. Small pieces of callus (50 to 100 mg) originating from lemon juice vesicles were placed on solid media containing various sodium arsenite concentrations, as indicated. After 6 weeks, the calli were weighed and analyzed for citric acid concentration. Results are means of three independent experiments. The linear component of the treatment effect on citric acid concentrations was highly significant ($P = 0.0046$), whereas the deviation from linearity was nonsignificant ($P = 0.611$). Effect of arsenite treatments on callus growth was not significant ($P = 0.877$).

0.1 M LiCl and, 5 mM EDTA. The mixture was blended by means of a Polytron (Kinematica AG Littau), and 8 mL of chloroform was added to it, followed by a 30-min incubation at 50 °C with occasional shaking. The mixture was centrifuged at 10,000 g_n for 20 min, and the upper phase was reextracted with one volume of phenol-chloroform (1:1, v/v), and recentrifuged. Lithium chloride was then added to the upper phase to a final concentration of 2 M, followed by overnight incubation at 4 °C and centrifugation at 10,000 g_n for 20 min at 4 °C. The pellet was dissolved in 0.5 mL H_2O, and centrifuged briefly to remove nonprecipitated material. Northern blot analyses (Sambrook et al., 1989) were performed with [a^{32}P]dCTP-radiolabeled probe for pummelo citrate synthase cDNA (Canel et al., 1996), provided by Mikeal Roose, Riverside, Calif. The membranes were autoradiographed with X-ray film, and were also scanned with a Fujifilm BAS-1500 Phosphoimager (Fugi Photo Film Co., Tokyo, Japan). The images were quantified with the aid of TINA2 software (Fugi Photo Film Co., Tokyo, Japan) and the ethidium bromide (EtBr)-stained RNA was scanned and quantified with the same software.

Statistical analysis. Two-way analysis of variance for arsenite concentrations and experiments were carried out on citric acid and calli growth (Fig. 2) followed by partitioning the concentration sum of squares into linear and deviation from linear components. One-way analysis of variance for arsenite concentrations and citrate synthase activity (Fig. 3) was followed by Tukey's Studentized range test.

Results

Results from 1997 and 1998 were very similar, therefore, only 1998 results are presented. Total titratable acidity (Fig. 1A) decreased slightly during the first 21 d after arsenite application. Between 21 and 56 d after treatment, total acidity of control fruit increased 10 fold, before decreasing to about three times the initial level toward fruit maturation (210 d after treatment). The pattern of acid accumulation in the arsenite treated fruit was similar to that in the controls, but the acid concentration was significantly lower on each sampling date, starting from 35 d after treatment. On a few sampling dates, 56, 112, 140, 196, and 210 d after treatment, a dose response of arsenite concentration was usually found: increasing the arsenite concentration resulted in a larger reduction in the total titratable acidity. However, on the other sampling dates there was no difference in total acidity among the arsenite concentrations. By 196 d after treatment, the acid content of treated fruit was 1% to 1.2%, allowing commercial

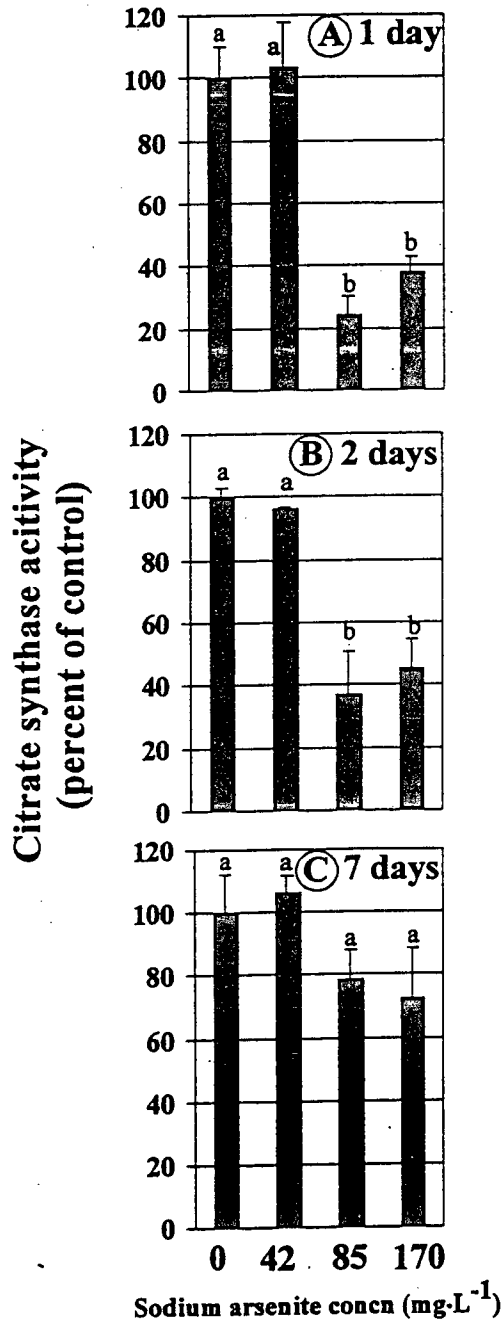


Fig. 3. Effects of sodium arsenite concentration on citrate synthase activity of 'Minneola' tangelo fruit harvested at various intervals following application. Citrate synthase activity was measured in treated fruit as described in Fig. 1, and collected (A) 1, (B) 2, and (C) 7 d following application. Lower case letters denote mean separation by Tukey's Studentized range test ($P < 0.001$).

Table 1. Effect of sodium arsenite concentration on the concentrations of organic acids in 'Minneola' tangelo fruit 35 d following sodium arsenite sprays (n = 4, means ± SE).

Arsenite concn ($mg \cdot L^{-1}$)	Organic acids content ($mg \cdot g^{-1}$ fresh wt)			
	Malonic	Fumaric	Succinic	Citric
0	0.0303 ± 0.0053	0.0193 ± 0.003	0.0193 ± 0.003	8.156 ± 0.881
42	0.0388 ± 0.0038	0.0266 ± 0.006	0.0242 ± 0.003	4.962 ± 1.318
85	0.0280 ± 0.0018	0.0196 ± 0.002	0.0177 ± 0.004	3.640 ± 1.107
170	0.0280 ± 0.0018	0.0196 ± 0.002	0.0177 ± 0.004	4.382 ± 0.408

harvest, while the acid content of control fruit was 1% higher and therefore too high for harvesting.

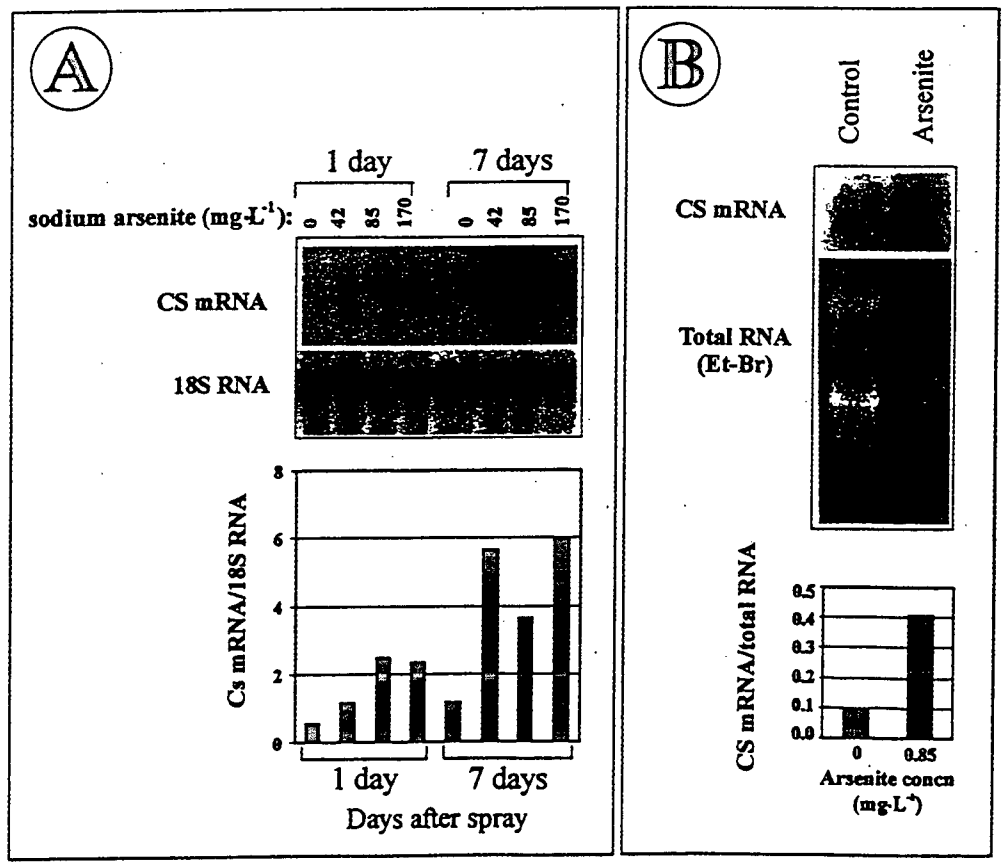
The pattern of citrate accumulation in the control fruit was similar to that of total titratable acidity. There was little accumulation the first 21 d after treatment, more than a 100-fold increase between 21 and 84 d, and a decline toward fruit maturation. The decrease in citrate concentration continued for 14 d longer than that in total acidity. The effect of arsenite treatment on citric acid concentration was also very similar to its effect on total titratable acidity. The inhibition became evident only when the major increase in citric acid concentration was detected after 35 d. Although there was no difference between control and treated fruit in the time of maximum titratable acid accumulation (Fig. 1A), the citric acid concentration peaked 28 d later in the treated fruit than in the control fruit (Fig. 1B). Only at 112 d after treatment was the reduction in citrate level correlated with the increase in arsenite concentration. On the other dates, 84, 140, 168, and 196 d after treatment, the two highest arsenite concentrations gave similar values of citrate concentrations. On the other sampling dates, the difference between the arsenite concentrations was not significant.

The effect of arsenite on total titratable acidity appeared to be correlated primarily with its effect on citric acid, since it had no effect on the concentrations of other organic acids found in the fruit (Table 1). Growth of 'Eurieka' lemon callus was unaffected by arsenite, up to 0.85 $mg \cdot L^{-1}$, in the culture medium (Fig. 2). However, there was a gradual reduction in the citric acid concentration in callus as the sodium arsenite concentration increased. The highest concentration (0.85 $mg \cdot L^{-1}$) resulted in a 2.5-fold decrease in acid level, as compared with the controls. Higher concentrations of sodium arsenite in the medium greatly inhibited callus growth (data not presented).

A 3- to 4-fold decrease in citrate synthase activity was observed in fruit treated with the two higher sodium arsenite concentrations, within 1 d after application (Fig. 3A). After an additional day (Fig. 3B), the activities started to recover approaching that of the controls, and after 7 d, no difference was detected (Fig. 3C). No differences in citrate synthase activity between treated and control fruit were detected during the remainder of the fruit development period.

Citrate synthase mRNA was undetectable in young control fruit (Fig. 4A). However, in the arsenite-treated fruit, expression was induced within 1 d, with the greatest increase evident at the two higher arsenite concentrations. A further increase was detected after 7 d. Although 42 $mg \cdot L^{-1}$ sodium arsenite did not inhibit activity of citrate synthase (Fig. 3), it induced citrate synthase mRNA to levels similar to that of the other treatments after 7 d. Gene expression was also induced in callus grown in the presence of 0.85 $mg \cdot L^{-1}$ sodium arsenite (Fig. 4B).

Fig. 4. Effects of sodium arsenite treatment on expression of citrate synthase gene in (A) 'Minneola' tangelo fruit and (B) 'Eurieka' lemon callus. RNA was extracted from treated 'Minneola' tangelo fruit as described in Fig. 1, and collected 1 or 7 d following application, or from 'Eurieka' lemon calli grown for 6 weeks in the presence of 0.85 $mg \cdot L^{-1}$ sodium arsenite. Standardization of the results was performed with (A) 18S RNA, which was used as a probe, or with (B) ethidium bromide (Et-Br)-stained total RNA.



Discussion

In spite of the broad-spectrum effects of arsenic compounds in all living organisms, the concentrations of sodium arsenite used in the present study did not alter fruit growth and development (data not presented). Even tissue cultures, exposed continuously to sodium arsenite, up to 0.85 mg·L^{-1}, grew normally. In addition, sugar accumulation was unaffected in the fruit following arsenite spray (data not presented), suggesting that metabolic pathways, other than acid accumulation, were not altered. We describe herein two effects of arsenite treatment. First, a reduction in citric acid accumulation that was detected 35 d following treatment and which proceeded throughout fruit development; and second, a transient decrease in citrate synthase activity that occurred 1 to 2 d following treatment and was accompanied by induction of gene expression.

Pulp acidity of citrus fruit is thought to be dependent on two mechanisms: the accumulation of citric acid in the vacuoles of the juice sac cells (Brune et al., 1998; Canel et al., 1995; Sadka et al., 2000), and acidification of the vacuole by a tonoplastic H^+-ATPase, which creates a proton influx and reduces vacuolar pH to about 2.5 (Müller et al., 1996, 1997). Although the patterns of citric acid concentration and total titratable acidity (representing the vacuolar pH) were similar, slight differences between them might indicate that these two mechanisms are independent. For instance, total titratable acidity decreased slightly during the first 21 d following treatment, while there was a minor increase in citric acid concentration. This might indicate that following its transport into the vacuole, citrate is protonated (Müller et al., 1996) and slightly reduces the total titratable acidity. Similarly, the increase in citric acid concentration between 56 and 84 d might have caused the reduction in total titratable acidity during that time. The two mechanisms are most likely coregulated and inhibiting one of them may inhibit the other. Indeed, in the present study the inhibitory effects of sodium arsenite on total titratable acidity and citrate concentration became evident at the same time, 35 d after treatment, and affected both parameters almost simultaneously throughout the season. On most sampling dates during the acid decline stage, the two highest arsenite concentrations usually elicited the largest reductions in total titratable acidity and citrate concentration. On the other dates, especially during the acid accumulation stage, the sample size was probably not large enough to show differences among the various arsenite treatments, in citrate level or total titratable acidity.

Citrate synthase activity was reduced transiently following application of arsenite at the two higher concentrations. It is possible that the lowest arsenite concentration also reduced the activity, but that the recovery time was shorter than 24 h; by the following sampling dates, activity of citrate synthase in the sodium arsenite-treated fruit was similar to that in the controls. The long delay between enzymatic inhibition, which lasted 7 d following arsenite application, and the onset of the effect on acid accumulation, which was detected only after 35 d, lessen the possibility that the two phenomena are directly related. The detection method should, in principal, be able to detect differences even with the low acid concentrations found during the first 21 d after treatment, as small differences were detectable in the tissue cultures. In addition, the lowest arsenite concentration (42 mg·L^{-1}) reduced acid accumulation without a detectable affect on the enzyme activity, unless the latter recovered sooner than 24 h. It is therefore concluded that sodium arsenite does not reduce fruit acidity through a direct effect on the activity of citrate synthase. This is in contrast to the effect of lead arsenate, which caused a reduction in the activity of citrate synthase during the 5 weeks following treatment, in parallel to the reduction in acid level, and also inhibited enzymatic activity when added to the assay medium (Yamaki, 1990a).

It is well established that arsenite inhibits activity of pyruvate dehydrogenase (PDH), which requires an SH group for its activity (Hu et al., 1998; McKay et al., 1988). PDH catalyses synthesis of acetyl-CoA, used for citrate synthesis, and this might be the reason for the reduction in acid level in citrus fruit. A reasonable scenario, which might explain our results, distinguishes between the short- and long-term effects of sodium arsenite. Immediately following application, activity of citrate synthase is inhibited, probably by indirect effects of arsenite, and it returns to the normal level quite rapidly, because of gene induction (see below). The activity of PDH is also inhibited following treatment, but it remains relatively low, resulting in a reduced supply of acetyl-CoA. Citrate synthase is operated, in vivo, under lower rates without a detectable effect on its extractable activity, and so causing the long-term effect on citrate accumulation. Interestingly, it has been reported that lead arsenate also reduces the concentration of acetyl-CoA, in parallel to the reduction in citrate synthase activity (Yamaki, 1990b). However, it should be considered that lead arsenate was used at a concentration of 3000 mg·L^{-1}, and if it contained even a minor amount of arsenite, this might have caused the effect. It should also be considered that prokaryotes and eukaryotes can metabolize one arsenic form into another, pump it out of the cell, or detoxify it by methylation (Kuroda et al., 1998; Mass and Wang, 1997; Silver and Phung 1996). This raises the possibility that even when a particular material is used on citrus, conversion or detoxification mechanisms may be operating to change it to a different form.

Reduction in citrate synthase activity was followed by a major increase in its mRNA accumulation, which could already be detected 1 d after treatment. Arsenite induced the expression of a few genes belonging to the heat-shock family in *Arabidopsis thaliana* (L.) Heynh., but this probably reflected a general stress response (Milioni and Hatzopoulos, 1997). In citrus, the increase in citrate synthase gene expression most likely played a role in the fast recovery of enzyme activity. The mechanism of arsenite action on gene induction might be either indirect, through the accumulation of citrate synthase substrate (Avsian-Kretchmer et al., 1999), or direct, as in the case of methotrexate, which inhibits the activity of dihydrofolate reductase (DHFR), but induces its mRNA level through gene amplification (Schimke, 1984). Interestingly, arsenite resistance in *Leishmania tarentolae* cell line TarIIWT has also been associated with amplification of a few genes (Grondin et al., 1997).

In summary, arsenite treatment was very effective in reducing total fruit acid concentration to a desired level of 1% ≈2 weeks sooner than in the controls, thus allowing early harvest. Since use of this chemical is prohibited, a better understanding of its mode of action might lead to development of a nonhazardous alternative means to reduce fruit acidity. Although the work described herein does not answer the question of the mechanism of arsenite effect, it raises interesting possibilities for future research.

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NADP$^+$-isocitrate dehydrogenase gene expression and isozyme activity during citrus fruit development

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Abstract

The accumulation of citric acid and its decline toward fruit maturation is typical of citrus fruit. We studied NADP$^+$-isocitrate dehydrogenase (NADP-IDH), an enzyme involved in citrate metabolism. A cDNA encoding the enzyme was cloned from lemon (*Citrus limon*) juice sac cells, and is the first-reported NADP IDH from fruits. Sequence comparisons and phylogenetic analysis indicate that it most probably belongs to a monophyletic clade of plant cytosolic enzymes. The mRNA level in the juice sac cells was induced during lemon fruit growth, and increased by about 15-fold to a peak as the fruit neared maturation. Spectrophotometric assay of the NADP-IDH activity in the pulp during fruit development showed that in young fruit, most of the activity was associated with the mitochondrial preparation and that, as the fruit grew, the activity shifted to the soluble fraction. The two activities could also be distinguished by isozyme gel electrophoresis: while one isozyme was detected in the mitochondrial preparation of young fruit and declined later, the other was induced in the soluble fraction of older fruit and increased as the fruit grew. The increasing activity of NADP-IDH in the soluble fraction throughout fruit development correlated well with the increase in gene expression, which suggests that the soluble activity is regulated by the expression of the cytosolic NADP IDH gene. The possible role of this form of the enzyme in citric acid catabolism in the pulp is discussed.

Keywords: Citrus fruit; *Citrus limon*; Citric acid; NADP$^+$-isocitrate dehydrogenase

1. Introduction

Many fruits accumulate considerable amounts of organic acids during various stages of their development [1]. Most citrus fruit are characterized by a massive accumulation of a single organic acid, citric acid, which is synthesized in the juice sac cells and accumulates in their vacuoles [2–5]. Acid content reaches a peak when the fruit reaches about 50% of its final volume and, in most varieties, it gradually declines during later stages of fruit maturation [6]. Usually, acid concentration in the juice peaks at 0.1–0.2 M, but in sour lemon it reaches 0.3 M, and declines only slightly thereafter.

Research in our laboratory aims to understand the metabolic and molecular mechanisms controlling acid accumulation in the fruit. One approach involves the analysis of citric acid-metabolizing genes and their products throughout fruit development. The six-carbon citrate molecule results from the condensation of acetyl-CoA with oxaloacetate, a reaction catalyzed by citrate synthase (EC 4.1.3.7). The first two steps of citric acid catabolism are catalyzed by aconitase (EC 4.2.1.3) and isocitrate dehydrogenase (IDH) in the following reversible reactions:

$$\text{citrate} \leftrightarrow [\textit{cis}\text{-aconitate}] \leftrightarrow \text{isocitrate}$$

$$\text{isocitrate} + \text{NAD}^+ \text{ (or NADP}^+\text{)} \leftrightarrow 2\text{-oxoglutarate} + \text{CO}_2 + \text{H}^+ + \text{NADH (or NADPH)}$$

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In a previous paper we studied the role of aconitase in citric acid accumulation [7]; in the present paper we describe the activity and expression of IDH. Two forms of IDH, widely distributed in higher plants, can catalyze the conversion of isocitrate to 2-oxoglutarate (2OG): an NAD-dependent form (EC 1.1.1.41) and an NADP-dependent form (EC 1.1.1.42). The NAD-IDH is localized exclusively in the mitochondria, where it acts in the TCA cycle. By contrast, the NADP-IDH is found in several cell compartments: cytosol, chloroplasts, peroxisomes and mitochondria (reviewed in Refs. [8,9]). Most of the NADP-IDH cDNA clones described previously are hypothesized to encode for cytosolic isozymes [8–13]. They share about 90% identity in their deduced amino acid sequences, and direct evidence was provided for the cytosolic localization of the tobacco clone's product [13]. A cDNA clone for a mitochondrial NADP-IDH has been isolated from tobacco [14] and an homologous clone has also been isolated from potato [15].

2OG provides an important precursor of glutamate, and it is hypothesized that the major site of its production is in the cytosol [16]. Therefore, it is thought that the cytosolic form of NADP-IDH plays a role in the metabolic flow between carbonaceous and nitrogenous compounds in the cell: amino acid synthesis from 2OG, on the one hand, and ammonia re-assimilation resulting from protein degradation, on the other hand [8,15]. In a few cases, a good correlation between the activity of the cytosolic NADP-IDH and glutamate synthesis was established (reviewed in [9]). However, in Scots pine cotyledons and hypocotyls, NADP-IDH activity was not correlated with those of GS, GOGAT and Rubisco, suggesting it might had another biological role [9,17]. In addition, antisense inhibition of NADP-IDH did not result in any phenotypic effects on plant growth and development, or in a reduction in 2OG level [15]. The physiological role of the mitochondrial NADP-IDH has not yet been described, but it has been hypothesized that it acts in the production of NADPH, which serves to reduce glutathione [14,18].

Only a few workers have studied the activity of NADP-IDH in citrus fruits [19–21]. In the present paper we report the isolation of a putative cytosolic NADP-IDH cDNA clone from lemon juice sac cells; it is, to the best of our knowledge, the first such cloning from a fruit. The expression of the gene(s) during fruit development is compared with the activities of various isozymes by means of fractionation studies, and the role of NADP-IDH in citric acid metabolism in citrus fruit is discussed.

2. Materials and methods

2.1. Plant material and fruit acidity measurements

Fruits of sour lemon (*Citrus limon* var. Eurieka) were collected from orchards in the central-coastal region of Israel. The total acidity of the fruit, an accepted indicator of citric acid level, was determined by the titration method [7].

2.2. NADP-IDH cloning from lemon juice sac cells

Poly-A mRNA was isolated from total RNA extracted from pulps of 15–35-mm lemon fruits, by means of the PolyATackt mRNA isolation system III (Promega, Madison, WI) according to the manufacturer's instructions. About 5 μg of poly-A mRNA were used to construct a Lambda ZAP-Express cDNA library (Stratagene, La Jolla, CA), according to manufacture's instructions. The library was screened with a full-length potato *icdh1* clone [12] kindly provided by B. Müller-Röber, Germany. Hybridization was performed in a solution containing 5 × SSC, 5 × Denhardt, 25% formamide and 100 μg/ml salmon sperm DNA, for 17 h at 42°C [22]. Three 30-min washes were performed at 45°C with a solution containing 1 × SSC and 0.1% SDS.

2.3. Phylogenic tree construction

Sequence alignment was performed using CluterW software [23]. Brach lengths and phylogenetic tree were obtained with the neighbor-joining method [24] using the ClusterW program correcting for multiple replacements.

2.4. RNA extraction and analysis

RNA was extracted from juice vesicles flavedo and albedo by means of the phenol–chloroform method as described by Sadka et al. [25]. Northern

blot analyses were performed with [α^{32}P]dCTP-radiolabelled probes [22]. The hybridizations were carried out at 65°C in a solution containing 5 × SSC, 5 × Denhardt, 1% SDS and 100 μg/ml salmon sperm DNA using the full-length citrus cDNA clone. Following hybridization, the membranes were washed three times (20 min each time) at 50°C in a solution containing 0.1 × SSC and 0.1% SDS. The membranes were exposed to X-ray films; they were also scanned with a Fujifilm BAS-1500 phosphoimager (Fujifilm, Tokyo, Japan), and the resulting images were quantified with TINA2 software. The ethidium bromide (EtBr)-stained gel in Fig. 2A was scanned and quantified with the same software.

2.5. Juice sac cells extraction and fractionation

The procedure for extraction of juice sac cells was essentially as described by Sadka et al. [7], except that the extraction buffer contained 50 mM Hepes, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 1 mM polyvinylpyrrolidone (40 kDa), 0.002% Tween 20 and 14.3 mM β-mercaptoethanol. Soluble and mitochondrial preparations were obtained essentially as described by Sadka et al. [7], expect that the extraction buffer contained 0.6 M sucrose, 200 mM Hepes, pH 7.8, and the washing buffer contained 0.6 M sucrose, 20 mM Hepes, pH 7.8.

The protein contents of the total and the fractionated extracts were determined with Bradford reagent (BioRad, Hercules, CA).

2.6. NADP-IDH assay

NADP-IDH was measured spectrophotometrically by monitoring the production of NADPH at 340 nm, essentially as descried by Gallardo et al. [26]. Activity assays were also performed in gel, as described previously [27,28], with modifications. We used a non-denaturing acrylamide mini-gel system containing an upper gel of 4% (w/v) acrylamide–bisacrylamide (30:1, v/v) in 125 mM Tris–HCl, pH 6.8, and a separating gel containing 7% (w/v) acrylamide–bisacrylamide (30:1, v/v) in 375 mM Tris–HCl, pH 8. The electrode buffer contained 25 mM Tris–HCl and 192 mM glycine. Before sample loading, the gel was pre-run for 30 min with 10 mM glutathione in order to block unpolymerized acrylamide. Total extract (20–40 μg in 20 μl) was mixed with 10 μl gel-loading buffer containing 50% (v/v) glycerol and bromophenol blue. Separation was performed using 60–120 mV at 4°C. When the bromophenol blue had reached the bottom of the gel, an additional aliquot of loading buffer was loaded, and the separation was stopped when the second dye had reached half way. Staining was performed at 37°C in a solution containing 100 mM Tris pH 8.0, 3.87 mM isocitric acid, 50 mM $MgCl_2$, 0.13 mM $NADP^+$, 1.2 mM (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and 3.26 mM phenazine methosulphate (PMS), until the bands were visible. Replacing the staining solution with 7% (v/v) acetic acid stopped the reaction.

3. Results

The *icdh1* clone from potato, for which indirect evidence was given for the cytosolic localization of its product [12], was used as a probe to screen a cDNA library from lemon juice sac cells. Numerous positive clones were isolated, and the larger one, 1708 bp in length, was sequenced (GenBank Accession Number AF176669). It contained a 5′-untranslated region (UTR) of 155 bp, a deduced coding region of 414 amino acids and a 3′-UTR of 274 bp. The deduced amino acid sequence showed approximately 90% identity with the potato clone. It was compared with plant and animal NADP–IDH, and a phylogenetic tree (Fig. 1) was constructed (with the help of D. Graur and T. Pupko, Tel-Aviv University, Israel). As expected, the animal clones formed two monophyletic clades of mitochondrial and cytosolic enzymes [29]. Similarly, the two plant mitochondrial clones were grouped separately of the other plant clones (cytosolic-like). Most of the cytosolic-like clones formed a monophyletic clade, but three of them, *Arabidopsis*, soybean I and carrot I, were outgroups (see below, Section 4). The lemon clone, which was grouped among the cytosolic enzymes, showed approximately 90% identity with the putative plant cytosolic clones, while about 75% identity was found with the tobacco mitochondrial clone and with its potato homologue. It is, therefore, concluded that the lemon clone most likely encodes for a cytosolic NADP-IDH and not for a mitochondrial one.

The lemon clone served as a probe for Northern analysis using RNA from lemon juice sac cells that

had been extracted from fruits at various developmental stages (Fig. 2A). One major transcript was detected in young fruits (10–19 mm, 0.38% total titratable acidity); its abundance increased throughout fruit development, reaching a peak level of about 15 times the abundance in young fruits (10–19 mm), in 50–60-mm fruits (nearly mature). By its relative migration, which was slightly faster than the 18S RNA (not shown), it was concluded that the transcript is at the expected size of about 1700 bp. An additional, longer transcript of a lower intensity was also detected in the fruit pulp; it was slightly induced in fruits of 10–19 mm to 20-25 mm, but remained constant during the remaining stages of fruit development. A single major transcript was detected in the fruit rind also, in both the flavedo (outer layer) and the albedo (inner white layer), in neither of which was there any difference in its abundance between young and mature fruits (Fig. 2B). The flavedo generally had a slightly higher expression of IDH than the albedo. Unlike the case of the fruit pulp, no additional transcript was detected in the fruit rind.

The enzymatic activity of NADP-IDH was analyzed in total extracts, fraction-enriched with mitochondria, and in the soluble fraction of lemon pulps, during fruit development. The results, presented in Table 1, showed a gradual increase in the specific activity of NADP-IDH in the total extracts, during fruit development. However, the distribution of the activity changed remarkably as the fruit grew. In young fruits (10–15 mm), about 80% of the total activity was found in the mitochondrial fraction and only around 9% in the soluble fraction. In larger fruits (30–40 mm), the specific activity in the mitochondria was reduced, while that in the soluble fraction was greatly enhanced. Further increase in the specific activity in the soluble fraction was detected in the largest

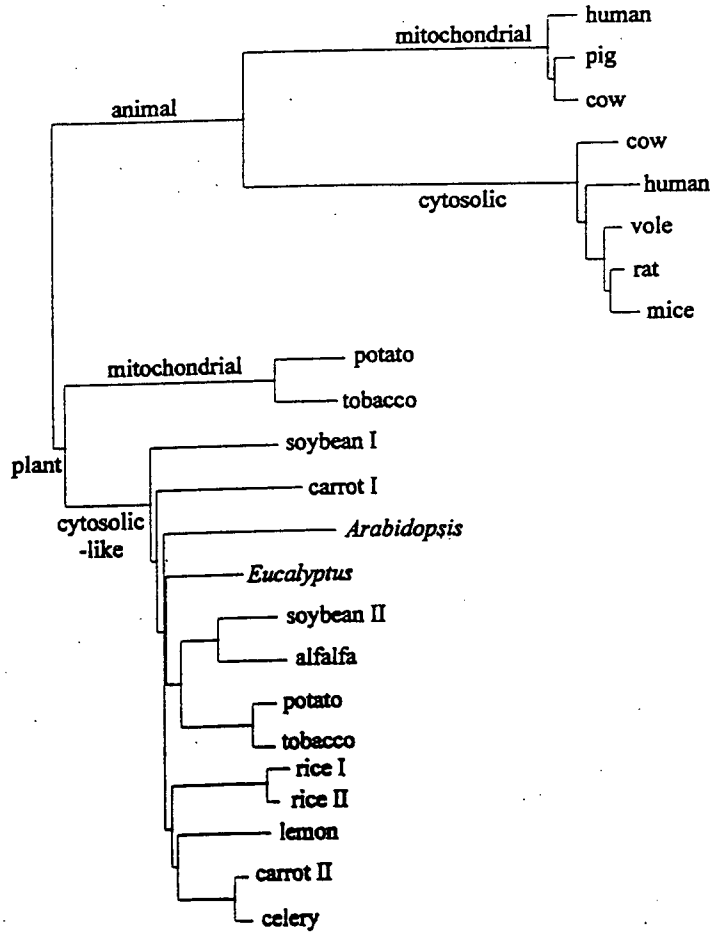

Fig. 1. Phylogenetic tree of animal and plant NADP-IDH. The clone accession numbers from the top are as follows: p48735, p33198, q04467, af136009, af020038, af048831, l35317, af020039, x92486, x96728, af095445, ab019328, aad25614, u80912, q06197, q40345, p50217, p50218, af155333, af176669, ab019327, and y12540.

Table 1
NADP-IDH activity in total, soluble and mitochondrial preparations of fruits at successive stages of development, as indicated by their sizes[a]

Fruit size (mm)	Activity in fractionated extracts				Activity in total extracts	
	Mitochondria		Soluble		Total activity (nmol min^{-1}) per g FW	Specific activity (nmol mg^{-1} protein min^{-1})
	Total activity (nmol min^{-1}) per g FW	Specific activity (nmol mg^{-1} protein min^{-1})	Total activity (nmol min^{-1}) per g FW	Specific activity (nmol mg^{-1} protein min^{-1})		
10–15	23 ± 7	227 ± 53	2 ± 1	13 ± 3	28 ± 5	567 ± 12
30–40	2 ± 1	31 ± 5	85 ± 9	466 ± 125	75 ± 6	198 ± 22
50–60	4 ± 2	54 ± 18	179 ± 22	816 ± 272	189 ± 10	653 ± 113

[a] Mean values of three independent replicates ± S.E.

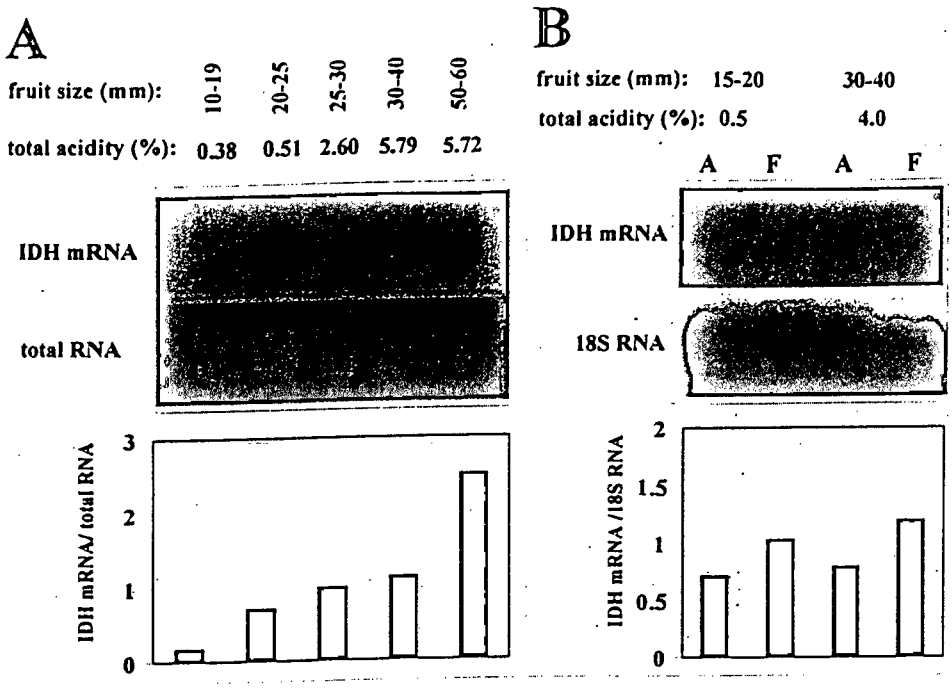

Fig. 2. Northern analysis of lemon fruit RNA with the NADP-IDH clone. RNA was extracted from juice sac cells at different developmental stages as indicated by fruit size and total acidity (A), and from flavedo and albedo of fruits of two sizes (B). Standardization of the results was performed with ethidium bromide-stained total RNA (A), or with 18S RNA (B) which was used as the probe following the stripping of the IDH probe by washing the membrane twice in a solution containing 0.1 × SSC and 0.1% SDS at 98°C.

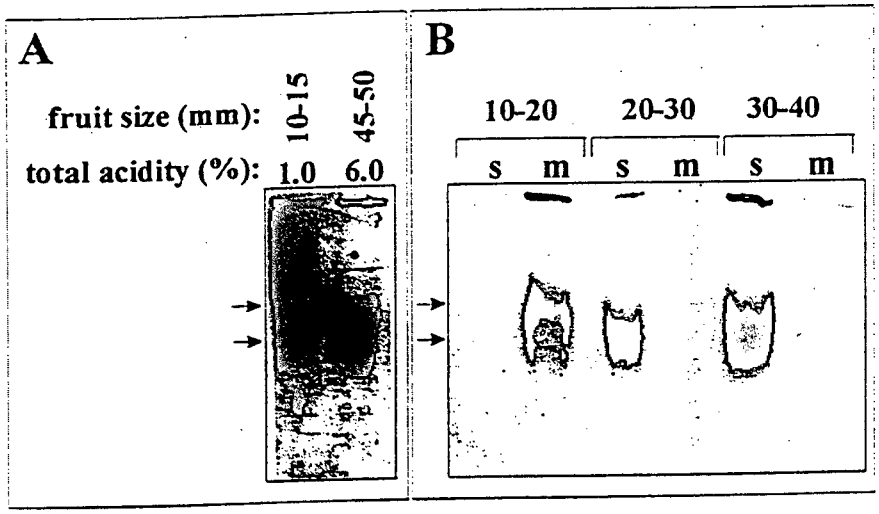

Fig. 3. NADP-IDH isozymes detected in the pulp during lemon fruit development. (A) Total extracts of young (10–15 mm) and nearly mature (45–50 mm) fruit pulps were subjected to NADP-IDH isozyme PAGE. The arrows indicate the positions of the isozymes. (B) Pulps of fruits at successive stages of development, as indicated by fruit size, were fractionated into soluble (s) and mitochondrial (m) preparations and subjected to isozyme PAGE. The arrows indicate the positions of the isozymes.

fruits (50–60 mm), while that in the mitochondrial preparation remained low. Overall, the increase in the activity of the total extracts could be attributed directly to that of the soluble fraction. In parallel, we used isozyme gel electrophoresis to examine the enzyme activity of NADP-IDH. Total extracts of young (10–15 mm, 1% total acidity) and almost mature (45–50 mm, 6% total acidity) fruits were analyzed (Fig. 3A). One isozyme was detected in the small fruit, and a different one, with a slightly higher electro-mobility in the large fruit. When pulps of three fruits of successively

larger sizes were fractionated into soluble and mitochondrial preparations, the distributions of the activity throughout fruit growth were similar to those detected in the spectrophotometric assays (Fig. 3B): in the youngest fruits (10–20 mm), the activity was detected in the mitochondrial fraction (Fig. 3B, m), but as the fruits grew (20–30 mm) it was detected in the soluble fraction (Fig. 3B, s), in which it was further enhanced in the largest fruits (30–40 mm). Although the differences among the relative electrophoretic mobilities were somewhat less remarkable in the fractionated extracts than in the total extracts (Fig. 3A), it seemed that the isozyme with the slower electrophoretic mobility represented the mitochondrial NADP-IDH, and the one with the faster electro-mobility the soluble enzyme. Both the spectrophotometric and the isozyme gel electrophoresis results strongly suggest that the mitochondrial enzyme is active in young fruits, and that its activity diminishes as the fruit grows. In contrast, the soluble enzyme, which most likely represents the cytosolic form, becomes predominant in mid-sized and nearly mature fruits. The increase in the activity of the enzyme in the soluble fraction, detected during fruit growth, was consistent with the increase in the expression of the putative cytosolic gene toward fruit maturation (Fig. 2A). This provides further support for the hypothesis that the isozyme is indeed the cytosolic NADP-IDH, and suggests that the enzyme is regulated at the gene expression level.

4. Discussion

It has been suggested that cytosolic and mitochondrial isoforms in animals and fungi arose from gene duplication, which occurred independently in the two kingdoms [29]. The phylogenic tree (Fig. 1) suggests that this may also be the case in plants, as previously suggested [9]. An alternative explanation may be that the paralogous genes underwent gene conversion events among the animal, plant and fungal kingdoms. From the tree in Fig. 1, it is also evident that the rate of evolution of the animal genes is more than twice as fast as their homologs in plants. It should also be considered that the group containing plant cytosolic-like clones (Fig. 1) might also include clones of organellar enzymes. For instance, it is suggested that the alfalfa clone contains an extra N-terminal peptide with some sequence similarity to thylakoid transit peptide [10]. In addition, the soybean I and carrot I clones contain a C-terminal SKL sequence, which is a typical peroxisomal signal motif. As mentioned above, Fig. 1 shows that these two clones, along with the *Arabidopsis* clone (which contains a C-terminally SRL sequence), are outgroups of the other plant clones, which form a monophyletic clade.

Although the clones encoding mitochondrial isoforms are easily distinguishable from the other plant clones, the level of similarity is quite high. For instance, the tobacco and potato cytosolic clones share about 72% identity with their mitochondrial counterparts at the DNA level, supporting the view that they originated from a common gene. The results of the Northern analysis (Fig. 2A) clearly show an additional transcript, with a somewhat different pattern of expression. The relative migration of the two ribosomal RNAs (the 28S, about 5 kb, and the 18S, about 2 kb), shown in the lower panel of Fig. 2B, suggests that the two transcripts may also vary considerably in length. The major one migrates somewhat faster than the 18S RNA (not shown), as expected from the clone size (1.7 kbp). The upper band migrates between the two ribosomal bands (not shown), and is expected to be much longer than the major band. This eliminates the possibility that the probe cross hybridizes with the mitochondrial form, as the two are unlikely to differ greatly in length. It is difficult to predict at this stage what might be the nature of the additional transcript.

In the leaves of higher plants, most of the cellular NADP-ICDH activity is localized in the cytosol (Ref. [30] and the references therein). It is also predominant in potato tubers, and is the only detectable form in pine cotyledons [9]. In tomato fruits, the cytosolic NADP-IDH contributes about 99% of the total activity found in mature green and red fruits [26]. This is obviously not the case in lemon fruits smaller than 30 mm, where the mitochondrial form is the dominant one. Only as the fruit grows does the cytosolic form become the dominant one, as in other plant systems.

The reduction in the activity of the mitochondrial form of NADP-IDH was detected early in fruit development, before the content of citrate in the pulp had dramatically increased. It was previously suggested that the increase in citrate level in lemon fruits may be partially due to an inhibition

of the activity of the mitochondrial aconitase, leading to slower operation of the TCA cycle [7,19]. This partial arrest would create a local increase in the level of citric acid, which would immediately be translocated to the vacuole, where it is stored. The reduction in the mitochondrial form of NADP-IDH was detected approximately when the aconitase activity was reduced [7], but it is questionable whether this reduction plays a direct role in citric acid accumulation. However, if indeed the TCA cycle were operating more slowly, it might be expected that the levels of isocitrate, and of other organic acids, in the mitochondria would be reduced. Therefore, the reduced activity of NADP-IDH might only reflect a general reduction in organic acid metabolism in the mitochondria, a hypothesis that should be investigated.

As mentioned above, it is thought that the cytosolic NADP-IDH is a key enzyme in the metabolism of amino acids and proteins, and that 2OG is the metabolic signal that regulates the coordination of carbon:nitrogen metabolism (reviewed in Ref. [9]). The action of the enzyme has been interpreted in various ways: ammonia re-assimilation during senescence, long-distance transport of amino acids, glutamate accumulation in ripe tomato fruits, etc. [8,15,26]. Isolated mitochondria of spinach leaves showed a reduced rate of the TCA cycle under photosynthesis conditions [31]. It was therefore suggested that a lower rate of the TCA cycle supplies citrate for the cytosolic synthesis of glutamine and glutamate, in agreement with the original hypothesis that the cytosol is the major site for 2OG production [16]. In citrus fruits, the block in the mitochondrial aconitase activity results in the accumulation of citric acid in the vacuole. We recently suggested that a small proportion of it may be converted to 2OG in the cytosol, and not stored in the vacuole [7]. It is possible that the cytosolic NADP-IDH plays a role in protein and amino acid metabolism in citrus fruits, also. Indeed, the contents of protein and a few free amino acids in the pulp increase during fruit growth [3,32], and they are also synthesized (although they do not accumulate) in the fruit rind [3]. The conversion of citric acid to glutamate via isocitrate and 2OG might occur during the acid accumulation phase, or after the acid level peaks. Our results show that there is a gradual increase in the expression of the gene (Fig. 2A) accompanied by a gradual increase in the activity of the enzyme in the soluble fraction (Fig. 3B, and Table 1). These observations favour the possibility that the conversion of citrate into 2OG is a continuous process, which takes place in the cytosol during fruit growth, even before the acid peaks. However, since the expression of the gene and the enzymatic activity were strongly enhanced at later stages of fruit development, it is considered that the major portion of the catabolism may take place after the acid accumulation stage. It should also be considered that the activity of the cytosolic form of NADP-IDH must, in principle, be coordinated with the activity of the cytosolic aconitase. Indeed, we have recently shown that the main increase in the latter occurs after the acid level has reached its peak [7], which also favours the possibility that most of the catabolism takes place at that stage. This interpretation also has a metabolic rationale. The acid accumulation rate in lemon must be maximal to allow its concentration to increase in spite of the dilution, caused by fruit growth. It is, therefore, reasonable to assume that active catabolism occurs mostly after the acid content has peaked at a level sufficient to compensate for the dilution effect, and when the accumulation rate has fallen somewhat. According to this rationale, it is expected that in other citrus varieties, which, unlike lemon, show a remarkable decrease in citrate level, the level of expression and the activity of NADP-IDH would be considerably higher, at least at the acid diminution stage.

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Comparative analysis of mitochondrial citrate synthase gene structure, transcript level and enzymatic activity in acidless and acid-containing *Citrus* varieties

Running head: Citrate synthase in *Citrus* fruit

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Abstract. Most citrus (*Citrus* L. spp.) fruits accumulate a considerable amount of citric acid in the vacuoles of the juice sac cells. As part of a research aimed to understand the mechanism of acid accumulation, we compared the gene structures and transcript levels of citrate synthase in sour lemon (high acid, *C. limon* (L.) Burm.), "Shamouti" orange (moderate acid, *C. sinensis* (L.) Osbeck) and sweet lime (acidless, *C. limettioides* Tan.). Southern analyses suggested that a single gene for citrate synthase was present in the genomes of all three *Citrus* varieties. The gene structures seemed to be very similar, with minor differences in "Shamouti" orange. Overall, the transcript levels of citrate

synthase were similar in sweet lime and sour lemon, and about two-fold lower in "Shamouti" orange. The enzymatic activity of citrate synthase was compared between sour lemon and sweet lime. In sour lemon, the specific activity of the enzyme was induced early in fruit development, in parallel with the increase in acid content, reaches a maximal level, and did not diminish significantly towards fruit maturation; the pattern and level of activity detected during sweet lime fruit development were similar. These results suggest that the difference in acid accumulation between acidless and acid-containing fruits may not be attributed to changes in the activity of citrate synthase.

Keywords: fruit acidity, fruit development.

Introduction

Many commercial fruits accumulate considerable amounts of organic acid, which make a major contribution to their flavour. The two most abundant acids are malate and citrate, which are synthesized in the fruit from photosynthetic assimilates (Tucker 1993). Usually, greater amounts of acids accumulate than are required for energy generation, and they decline toward fruit maturation and ripening. Citrate is the major acid of citrus fruit, contributing more than 90% of the total organic acids (Sinclair 1984). Its accumulation starts about 5-6 weeks post anthesis, reaches a peak when the fruit reaches about 50% of its final volume and then, in most varieties, declines toward fruit maturation (Erickson 1968).

Citric acid is formed by citrate synthase, which condenses the four-carbon molecule oxaloacetate with the two-carbon molecule acetyl-CoA to form the six-carbon citrate. In citrus fruits the synthesis probably takes place in the mitochondria of the juice sac cells, as part of the TCA cycle. It has long been hypothesized that a metabolic block in the mitochondrial aconitase, which catabolizes citrate into isocitrate, plays a major role in citrate accumulation (Bogin and Wallace 1966*b*). Indeed, a comparison between the aconitase activities of acidless sweet lime and high-acid sour lemon showed a remarkable reduction in aconitase activity early in sour lemon development (Sadka *et al.* 2000*b*). It is assumed that the arrest in the mitochondrial aconitase activity in those varieties which accumulate citrate, is sufficient to create a local increase in citrate level. The acid is then transported and stored in the vacuoles, a process that is accompanied by a massive influx of protons, which acidify the vacuole to a pH of about 2.5 (Canel *et al.* 1995; Müller *et al.* 1996; 1997; Brune *et al.* 1998).

Although partial block in the mitochondrial aconitase activity may provide a sufficient metabolic scenario for citrate accumulation, the question remains open, of whether the rates of acid synthesis by citrate synthase also differ between low- and high-acid fruits. Several forms of citrate synthase have been described in higher plants: mitochondrial, glyoxisomal and peroxisomal (Kato *et al.* 1995; La Cognata *et al.* 1996; Papke and Gerhardt 1996). In yeast, mitochondrial and peroxisomal genes were described (Lewin *et al.* 1990; Jia *et al.* 1997). The mitochondrial enzyme catalyses the first reaction of the TCA cycle, and therefore plays a central role in energy and metabolites production. Increased mitochondrial citrate synthase activity, which results in enhanced citrate extrusion from roots and cells, plays a major role in aluminium resistance (del la Fuente *et al.* 1997; Koyama *et al.* 1999). The glyoxisomal enzyme participates in the utilization of acetyl-CoA, generated during fatty acid β-oxidation, for sucrose and energy production. The physiological role of the peroxisomal enzyme is not known yet (Papke and Gerhardt 1996). The cDNAs for mitochondrial enzymes were cloned from *Arabidopsis*, potato, pummelo, tobacco, poplar tree, sugar beet and carrot (Unger *et al.* 1989; Landschutze *et al.* 1995*a*; Canel *et al.* 1996; La Cognata *et al.* 1996; Takita *et al.* 1999). The glyoxisomal enzyme has so far been cloned only from pumpkin, and it shows a greater homology with the prokaryotic citrate synthases than with the plant mitochondrial enzymes (Kato *et al.* 1995; Stevens *et al.* 1997). In *Citrus*, the activity of citrate synthase has been detected in leaves, fruit pulp and fruit rind of lemon and orange, and the enzyme has been partially purified from mitochondria of young lemon juice sac cells (Srere and Senkin 1966; Bogin and Wallace 1969). Similarly to citrate synthase from other sources, the activity of the citrus enzyme was inhibited by ATP, which reduced its affinity to acetyl CoA (Bogin and Wallace 1966*a*). The activity of the enzyme was remarkably increased during the first half of fruit

development in 'Satsuma" mandarin (*C. unshiu* (Mak.) Marc.), in parallel with the increase in citric acid level (Hirai and Ueno 1977). However, a further induction of the specific activity was also detected as 'Satsuma and "Hamlin" (*C. sinensis* (L.)) fruits approached maturity (Hirai and Ueno 1977; Roe *et al.* 1984). Arsenite, which reduces fruit citric acid content, transiently reduced the extractable activity of citrate synthase, but induced its transcript level (Sadka *et al.* 2000*a*).

In a previous publication, the transcript level of citrate synthase was compared between acidless and normal-acid pummelo fruits (Canel *et al.* 1996). Both varieties showed similar levels of mRNA, which were constant throughout fruit development. In addition, the gene did not cosegregate with the acidless phenotype. Yet, these findings do not solve the question of whether the acid synthesis rate may differ between acidless and acid-containing fruits. In the present work, we addressed this question by comparing the activities of citrate synthase in sour lemon (high-acid) and sweet lime (acidless) throughout fruit development. We also carried out a molecular comparison of citrate synthase gene structure and expression. As a reference case to those found in sour lemon and sweet lime, the molecular analyses also include "Shamouti" orange, which accumulates moderate levels of citric acid.

Materials and methods

Plant material and fruit acidity measurements

Fruits of 'Eurieka' sour lemon (*Citrus limon* (L.) Brum.), Palestine sweet lime *(C. limettioides* Tan.*)* and "Shamouti" orange (*C. sinensis* (L.) Osbeck) were collected from orchards in the central-coastal region of Israel. The relationships between fruits sizes

and their developmental stages are summarized in Table 1. The total acidity was determined by the titration of 2 ml of juice extracted from five-seven fruits with 0.1 *M* NaOH, with phenolphthalein as an indicator. Citric acid is the major organic acid in the juice, and the variations in acidity during fruit development are due chiefly to changes in its concentration (Sinclair 1984). Therefore, the total acidity, calculated as if citric acid were the only acid in the extract, is given as a percentage of juice volume (w/v). In acid lemon, 0.3 *M* of citric acid in the juice forms about 6% (w/v) total acidity.

Isolation of citrate synthase probe from lemon juice-sac cells

A cDNA fragment of about 1000 bp was cloned from a cDNA library constructed in Lambda ZAP-Express (Stratagene, La Jolla, CA, USA) by PCR, with primers generated from the pummelo clone (Canel et al. 1996). The forward primer was complementary to primer A5, and the reverse primer was A1. The PCR reaction involved 5 µl of the amplified cDNA library, 100 pmol of each primer, 10 mM Tris-HCl pH 9, 2 µM dNTPs, 50 mM KCl, 0.1 % Triton X-100, 2 mM $MgCl_2$ and 1 unit of *Taq* DNA Polymerase (Promega). Thirty cycles of 1 min at 94^0C, 2 min at 45^0C and 3 min at 72^0C, followed by 5 min at 72^0C were performed. A PCR product of about 1000 bp, the expected size, was separated on 1% agarose gel, purified with the Geneclean II Kit (Bio101 Inc., La Jolla, CA), and partially sequenced to confirm its identity.

DNA extraction and analysis

DNA was extracted from leaves essentially as described by Webb and Knapp (1990), with modifications. Leaves were ground in liquid nitrogen, and 2 g were mixed for 30 s with 15 ml of extraction buffer containing 0.5% CTAB, 50 mM tris-HCl, pH 8, 0.7 M NaCl and 10 mM EDTA, in a Polytron (Kinematika, Bazel, Switzerland) at a

speed setting of 3-4. The mixture was incubated at 60^{0}C for 1 h with occasional shaking, followed by extraction with 15 ml chloroform, and centrifugation for 10 min at 5,000 *g*. The upper phase (15 ml) was collected and 19 ml of precipitation buffer containing 0.5% CTAB, 50 mM tris-HCl, pH 8 and 10 mM EDTA was added to it, followed by overnight incubation at room temperature, and 10 min centrifugation at 5,000 *g* at room temperature. The pellet was dissolved in 400 µl of 1 M NaCl, and RNAse A (Sigma) was added to it to a final concentration of 50 µg/ml. Following 20 min incubation at 37^{0}C, 400 µl of phenol were added, and the mixture was extracted for 5 min by inversions. The upper phase, separated by 10 min centrifugation at 13,000 *g* at room temperature, was re-extracted with 400 µl of chloroform, followed by 5 min centrifugation at 13,000 *g* at room temperature. One volume of cold isopropanol was added, and the mixture was incubated at –20^{0}C for 1 h. The DNA was collected by 10 min centrifugation at 13,000*g* at 4^{0}C, washed with cold 70% ethanol, dried and dissolved with 100-200 µl of TE. Digestion of 20 µg DNA with restriction enzymes (MBI Fermentas) was carried out according to the manufacturer's instructions, followed by Southern blot analysis with the lemon probe for citrate synthase.

RNA extraction and analysis

Juice sacs were collected from 5-7 fruits, and about 5 g of tissue were used for RNA extraction essentially as described by Sadka *et al.* (2000b). Northern blot analysis was carried out, using a [α^{32}P]dCTP-radiolabelled pummelo probe for the mitochondrial citrate synthase (Canel *et al.* 1996). The membranes were exposed to X-ray films and were also scanned with a Fujifilm BAS-1500 Phosphoimager (Fuji Photo Film Co., Tokyo, Japan), and the resulting images were quantified with TINA2 software (Fuji Photo Film Co., Tokyo, Japan). The probe was then stripped by a wash with a solution

containing 0.1X SSC and 0.1% SDS for 15 min at 95^{0}C, and the membrane was rehybridized with a [α^{32}P]dCTP-radiolabelled probe for the 18S RNA, followed by quantification, as described above. The radioactive counts, resulted from citrate synthase probe hybridization, were normalized with that of the 18S RNA probe.

Differential display

RNA was extracted from 5 g juice sac cells of five-seven fruits (18-25-mm) of sweet lime and sour lemon, and 100-µg samples were treated with RQ1 RNAse-free DNAse (Promega) in a reaction volume of 100 µl containing 40 mM Tris-HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$ and 6 units of enzyme for 1 h at 37^{0}C. The reaction mixture was then extracted with one volume of phenol-chloroform (1:1). First-strand synthesis was performed with 10-mer poly-dT primer containing an additional A, C or G base at the 3'-end. The reaction mixture consisted of 0.2 µg RNA, 20 µM dNTPs, 0.2 µM primers, 25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 1.5 mM $MgCl_2$, and 25 mM DTT in a total volume of 20 µl; it was incubated for 10 min at 37^{0}C, 5 units of SuperScript II RNAseH$^-$-Reverse transcriptase (BRL) were added, and the reaction was allowed to proceed for a further 50 min. Two microlitres of the first-strand reaction mixture were taken for a polymerase chain reaction in a mixture containing 25 µM random 10-mer primers, 25 µM oligo-dT primers, used for the first-strand synthesis, 2 µM dNTPs, 0.2 µl of [$\alpha-^{32}$P]dCTP, 50 mM KCl, 10 mM Tris-HCl pH 9, 0.1 % Triton X-100, 2 mM $MgCl_2$ and 1 unit of *Taq* DNA Polymerase (Promega) in a total volume of 20 µl. Thirty cycles of 30 s at 94^{0}C, 2 min at 40^{0}C and 30 s at 72^{0}C, followed by 5 min at 72^{0}C were performed. Samples were separated on a 6% acrylamide-bisacrylamide (20:1) gel containing 50% urea, 45 mM Tris-borate and 1 mM EDTA.

Citrate synthase activity

Crude mitochondrial preparation was isolated from juice sacs of four to seven fruits, as described by Sadka *et al.* (2000b). The final pellet, enriched with mitochondria, was suspended in 50-100 µl of extraction buffer containing 50 mM HEPES-NaOH, pH 8.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% Tween-20, 0.5 mM phenylmethylsulphonylfluoride and 10% glycerol (Landschutze *et al.* 1995a). The protein contents of the fractionated extracts were determined with Bradford reagent (BioRad, Hercules, CA, USA), according to the manufacture's instructions. The activity of citrate synthase was performed by means of the 5'5'-dithiobis-(-2-nitrobenzoate) (DTNB) method at 412 nm, as described (Srere 1969). Ten to twenty five microliters of extracts were pre-incubated with 0.1 mM DTNB and 0.3 mM of acetyl-CoA in a total volume of 950 µl for 3 min at room temperature, prior to the addition of 50 µl of 10 mM oxaloacetate. The reaction was allowed to proceed for additional 6-8 min at room temperature.

Results

A comparison of mRNA populations from sour lemon and sweet lime

In the present study, we compared the expression and gene structure of citrate synthase in three citrus varieties: sour lemon, sweet lime and "Shamouti" orange. In addition, the activity of citrate synthase was compared between sour lemon and sweet lime. Wile sour lemon accumulates citrate to a high level (0.3M, 6-7% total titratable acidity, w/v), sweet lime is characterized by a very low acid level (0.1-0.2% total titratable acidity, w/v), and 'Shamouti" orange accumulates acid to a moderate level (3-

4% total titratable acidity, w/v). Sour lemon and sweet lime, although taxonomically related, are considered as being two different species (Vardi and Spiegel-Roy 1978). We therefore attempted, first, to establish the relevance of the physiological comparison between them, by comparing their mRNA populations by means of differential display (Liang *et al.* 1993), at a stage when fruits were rapidly growing and accumulating acid (the sour lemon). For all 18 pairs of primers used, very similar patterns were obtained with acid lemon and sweet lime, suggesting that they express similar arrays of genes (Fig. 1, example for eight pairs of primers).

The mitochondrial citrate synthase gene copy number and structure

Based on a partial sequence analysis, the lemon cDNA clone showed about 90% identity at the DNA level with the pummelo mitochondrial citrate synthase (Canel *et al.* 1996). The lemon clone was used as a probe to analyze gene copy number and gene structure and compare them among those of sour lemon, sweet lime and "Shamouti" orange. While digestion of the "Shamouti" orange DNA with *EcoRI* resulted in two hybridizing bands, both sour lemon and sweet lime presented three major bands hybridizing with the probe (Fig. 2). Since digestion of the lemon PCR product, used as a probe, also revealed three bands (not shown), it is concluded that the three *EcoRI* fragments probably resulted from a single gene. Digestions with *BamHI*, HindIII, *SalI* and *XhoI* resulted in a single, large band (only the results of *BamHI* digestion are shown in Fig. 2). This also support the conclusion that a single copy of the citrate synthase gene is present the genomes of the three varieties. The identical sizes of the fragments from sweet lime and sour lemon suggest that the gene structures in these two varieties are very similar. One *EcoRI* restriction site seemed to be missing from the "Shamouti"

orange DNA, and the upper band resulting from this digestion was somewhat shorter then in sour lemon or sweet lime. However, in spite of these changes, the overall structure of the "Shamouti" orange gene seems to be quite similar to that of sour lemon and sweet lime.

The expression of the mitochondrial citrate synthase throughout citrus fruit development

The expression of the gene for mitochondrial citrate synthase was examined during fruit development using two independent RNA preparations, which gave very similar results. The results of one analysis are shown in Fig. 3. A single transcript, whose abundance increased fourfold as the fruits grew from 10-20 mm to 20-25 mm in diameter, was detected early in lemon fruit development (Fig. 3*A*). The induction in the mRNA level preceded the major increase in acid content from 1.45 to 5.41%. The transcript level reached a peak in 20-25-mm fruit, and then gradually declined as the fruits grew, although the acid level continued to increase. A similar transcript was detected in sweet lime, but its level seemed to be constant throughout fruit development. The expression of the gene was also analyzed in closed and open lemon flowers (Fig. 3*B*) where, interestingly, it was almost 10 times higher than in the fruits.

We also compared the expression of citrate synthase in high-acid-accumulating lemon with that in "Shamouti" orange. The analysis was performed twice using two independent RNA preparations with very similar results. The results of one analysis are shown in Fig. 4. The level of citrate synthase transcript in "Shamouti" orange increased about 1.5- to twofold, as compared with three- to fourfold in sour lemon fruits. Overall,

it seems that the mRNA level in "Shamouti" orange is about half of that in sour lemon. In contrast to its behaviour in sour lemon, in which a sharp decline in the transcript level was detected while the acid was still increasing, in "Shamouti" orange the mRNA level remained more or less constant even during the acid decline stage.

The activity of citrate synthase during citrus fruit development

Juice vesicles of sour lemon and sweet lime were fractionated into a soluble and a crude fraction enriched with mitochondria. Activity was detected only in the fraction enriched with mitochondria (Fig. 5), whereas no activity was detected in the soluble fraction (data not shown). In sour lemon, the specific activity was induced early in fruit development, and gradually increased until it peaked in 30-40-mm fruits (Fig. 5*A*). The induction and the peak in the specific activity followed that of gene expression, and accompanied the increase in acid level. The pattern of enzymatic activity and the specific activities during the development of sweet lime fruit were very similar to that in sour lemon (Fig. 5*B*).

Discussion

The relationships between sour lemon and sweet lime

In the present study, the gene structure, transcript level and enzymatic activity of citrate synthase were compared between sweet lime (*C. limettioides*) and sour lemon (*C. lemon*) fruits, which differ remarkably in their acid contents. Based on morphological and other parameters, these two varieties have been defined as separate species

(Swingle and Reece 1967), although affinity studies suggested that both of them are trihybrids originated from the same parent species (Vardi and Spiegel-Roy 1978). Recent analyses using DNA markers grouped them in the same monophyletic clade, although with differing distances (Federici *et al.* 1998; Nicolosi *et al.* 2000). Further support for their close relationship was provided by the analysis of cytoplasmic markers generated from chloroplastic DNA (Nicolosi *et al.* 2000). Considering the taxonomy of *Citrus* and the movement of the diverse varieties around the world (Vardi 1979), it may be that these two "species" are more appropriately considered as two cultivars rather than two different species. The regeneration of sour lemon protoplasts yielded two trees with the morphology of sweet lime (A. Vardi, personal communications), which may provide support for the above view. Regardless of the taxonomic relationships between C. *limon* and C. *limetioides*, the differential display analysis presented in the present study shows that fruits of similar size express an almost identical array of genes, which makes the physiological comparison between them relevant. Additional support for the close relationship of the two varieties comes from the Southern analysis: the results suggested that the gene structures of citrate synthase are very similar in sweet lime and sour lemon.

A single gene for citrate synthase is present in Citrus

The digestions of the lemon DNA with *EcoRI* resulted in three hybridizing bands, all originated from sites inside the gene. Several other enzymes gave a single hybridizing band. These results strongly suggest that only one copy of the mitochondrial citrate synthase is present. There is a theoretical possibility that under less stringent hybridization conditions, more than one copy of the gene might be

detected. In *Arabidopsis* and in yeast, for example, there are two copies of the mitochondrial citrate synthase. However, southern analysis of pummelo, naval orange and trifoliate orange DNAs also showed only one hybridizing band with various enzymes (Canel *et al.*, 1996). It is suggested, therefore, that similarly to carrot (Takita *et al.* 1999), only one copy of the mitochondrial citrate synthase gene is present in *Citrus*.

Citrate synthase and the control of acid accumulation

The comparison between sweet lime and sour lemon clearly shows that the two varieties display almost identical levels of citrate synthase mRNA. Similarly, a comparison between the low-acid pummelo genotype, 2240 and the normal-acid pummelo, showed that the mRNA levels of citrate synthase were the same (Canel *et al.* 1996). In addition, genetic analysis showed that the mitochondrial citrate synthase gene did not co-segregate with *acitric*, the gene causing the acidless phenotype of 2240 (Canel *et al.* 1996). It was shown in the present study that sweet lime and sour lemon also display similar values of extractable citrate synthase specific activities throughout fruit development. The increase in the specific activity of citrate synthase coincides with the increase in acid level in the first half of sour lemon fruit development. This demonstrates the importance of citrate synthesis step for its accumulation. In a previous study, we also suggested that early in fruit development the first step of citrate catabolism, catalyzed by aconitase, is blocked in sour lemon but not in sweet lime (Sadka *et al.* 2000b). This blockage probably reduces the rate of the TCA cycle, and leads to citrate accumulation. The combined data presented here and in the previous paper (Sadka *et al.* 2000b) suggest that in sweet lime, citrate is synthesized at a normal

rate, but that, since its catabolism is not blocked, no accumulation occurs. The nature of the factor that controls the metabolic block is under investigation.

The regulation of citrate synthase

The mRNA of citrate synthase showed a remarkable induction early in lemon fruit development, as the fruits grew from 20 to 30 mm in diameter. The increase in the enzymatic activity somewhat lagged behind that of the mRNA, and was accompanied by a massive increase in total acidity. These are typical responses of a biosynthetic enzyme. The results also suggest that early in lemon fruit development, the enzyme is regulated, at least partially, at the mRNA level. A similar phenomenon, induction in citrate synthase activity and expression, has been reported in young leaves of a few plant species (Landschutze et al. 1995a; La Cognata et al. 1996). However, in other citrus fruits, and even in lemon fruit, as they approach maturity, the situation might be quite different. Pummelo fruit shows a constant level of citrate synthase mRNA during its development (Canel et al. 1996), and the present results show that this might also be the case in sweet lime. "Shamouti" orange seems to represent an intermediate case between lemon and pummelo: a roughly twofold increase in the mRNA level was detected early in fruit development, suggesting that if there is regulation at the mRNA level, it is less important than in sour lemon. Differences in the expression of the gene were also detected at later stages of fruit development. In sour lemon, the mRNA level started to decrease while the enzyme specific activity was still high, and the acid level continued to increase. On the other hand, no decrease in the mRNA level was detected in "Shamouti" orange when the acid level reached its peak, or during its decline. Overall, these differences could be explained in terms of different regulatory

mechanisms controlling the enzyme during different stages of fruit development and in different citrus varieties. Whereas, the enzyme might be regulated at the transcriptional or mRNA stability level early in sour lemon fruit development, at a later stage (40-mm fruits), increased translatability and protein stability might be responsible for maintaining a high activity level. In "Shamouti" orange and pummelo different regulatory mechanisms might control the enzyme.

Citrate synthase and cellular metabolism

Besides the association of citrate synthase expression and activity with citric acid accumulation, there were two observations that suggested that the enzyme might play an additional physiological role. First, the mRNA level was about 10 times higher in flowers (which do not accumulate acid) than in the juice-sac cells. High levels of expression in buds or flowers have been reported in potato and in other plant species (Landschutze *et al.* 1995*a*; La Cognata et al. 1996), and it has been suggested that, along with other TCA cycles enzymes, citrate synthase plays an important role in the transition from vegetative to reproductive meristem and in the early stages of flower development (Landschutze *et al.* 1995*b*). This might be the case also in *Citrus*. The second observation concerns the high level of citrate synthase activity in sour lemon and sweet lime fruits as they approach maturity. This response is probably typical of citrus fruit, as it has been reported in other citrus varieties also (Hirai and Ueno 1977; Roe *et al.* 1984). These observations might indicate that at later stages of fruit development the rate of citrate synthesis is similar to or even higher than in the early stages, regardless of the total decline in its content. This might represent an acceleration of the

TCA cycle, for respiration or other metabolic processes, such as amino acid and protein synthesis (Sadka *et al.* 2000*c*).

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Table 1. The relationships between fruit size and its developmental stage among the *Citrus* varieties used in this study.

	Sour lemon			**Sweet lime**			**"Shamouti" orange**			
Fruit size (mm)	10-25	25-40	40-50	20-30	30-40	40-50	20-25	25-30	35-65	65-70
Weeks after full bloom	4-9	11-24	28-29	8-11	17-22	26-28	8-9	11-12	14-33	36-37
Fruit stage (Bain 1958)	I	II	II-III	I-II	II	III	I	I-II	II	III
Duration of fruit development	33-36 weeks			33-36 weeks			42-45 weeks			

Figure legends

Fig. 1. A comparative analysis of mRNA populations of 18-25-mm sour lemon and sweet lime fruits. RNA from sweet lime and sour lemon fruits was analyzed by differential display. Eight pairs of primers, marked 1 to 8 are shown. The left-hand lane of each primer pair represents RNA from sour lemon and the right hand lane that of sweet lime. The experiment was repeated once using independent RNA preparations with similar results.

Fig. 2. Southern blot analysis of citrate synthase gene in sour lemon, sweet lime and 'Shamouti' orange. Twenty micrograms of DNA were digested with *EcoRI* (E), *BamHI* (B) or both enzymes, separated on agarose gel, blotted into a nitrocellulose membrane and hybridized with a PCR fragment containing about 1000 bp of the sour lemon citrate synthase cDNA. Following hybridization, the membrane was exposed to X-ray film.

Fig. 3. Citrate synthase mRNA level during sour lemon and sweet lime fruit development (*A*), and in closed (Cf) and open (Of) lemon flowers (*B*). RNA was extracted from fruit pulps at various developmental stages, as indicated by fruit size and total acidity, and from flowers, and hybridized with a fragment of about 1100 bp containing the pummelo citrate synthase cDNA (CS mRNA). The same membrane was re-hybridized with a probe for the 18S rRNA (18S RNA). Bottom panel, normalization of the CS mRNA signal with the 18S RNA.

Fig. 4. Citrate synthase mRNA level during the development of sour lemon and 'Shamouti' orange fruits. RNA was extracted from fruits at various developmental stages, as indicated by their size and total acidity (lower panel), hybridized with a fragment of about 1100 bp containing the pummelo citrate synthase cDNA (CS

mRNA), followed by re-hybridization with a probe for 18S rRNA (18S RNA). Middle panel, normalization of the citrate synthase signal with that of 18S RNA.

Fig. 5. Mitochondrial citrate synthase specific activities during the development of sour lemon and sweet lime fruits. Crude mitochondrial preparations were extracted from fruits at various developmental stages, as indicated by fruit size and total acidity, and the activity of citrate synthase was measured. The results are mean value of three independent experiments; bars represent *SE*.

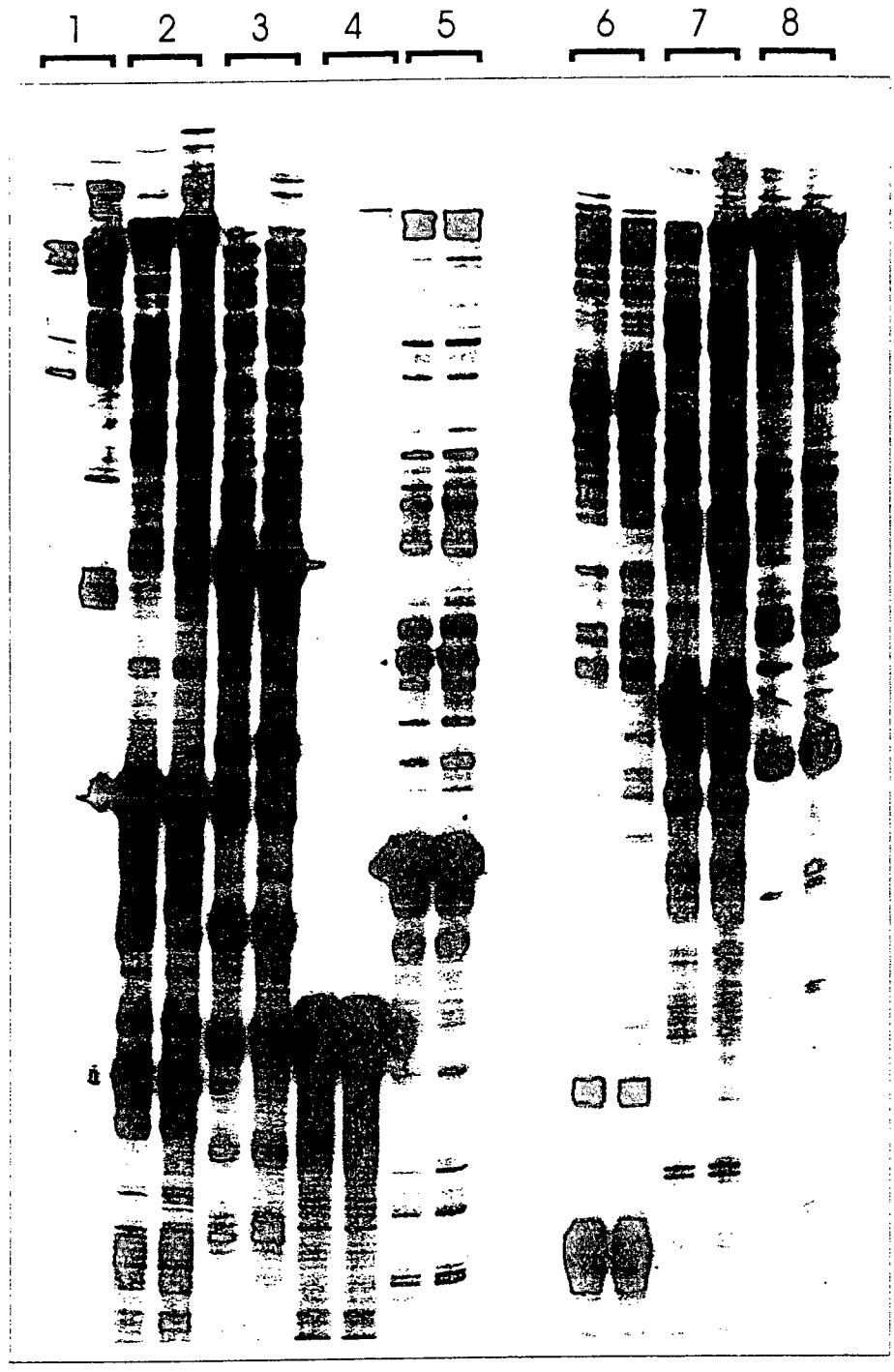

Fig.1

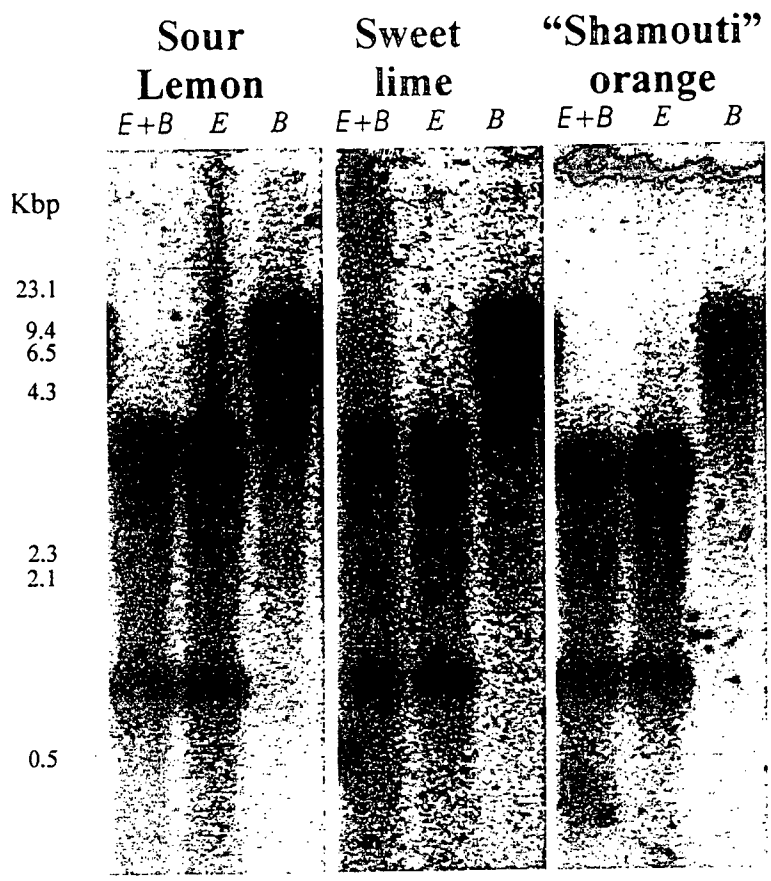
Sour Lemon
Sweet lime
"Shamouti" orange
E+B E B
E+B E B
E+B E B
Kbp
23.1
9.4
6.5
4.3
2.3
2.1
0.5

Fig. 2

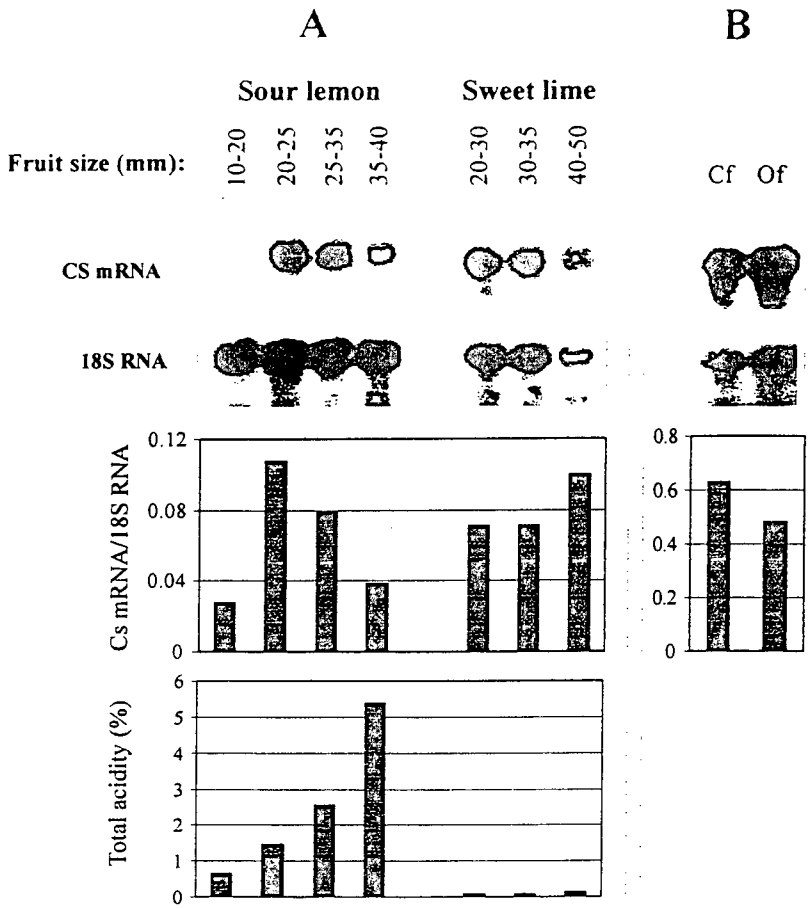
A
B
Sour lemon
Sweet lime
Fruit size (mm):
10-20
20-25
25-35
35-40
20-30
30-35
40-50
Cf
Of
CS mRNA
18S RNA
Cs mRNA/18S RNA
0.12
0.08
0.04
0
0.8
0.6
0.4
0.2
0
Total acidity (%)
6
5
4
3
2
1
0

Fig. 3

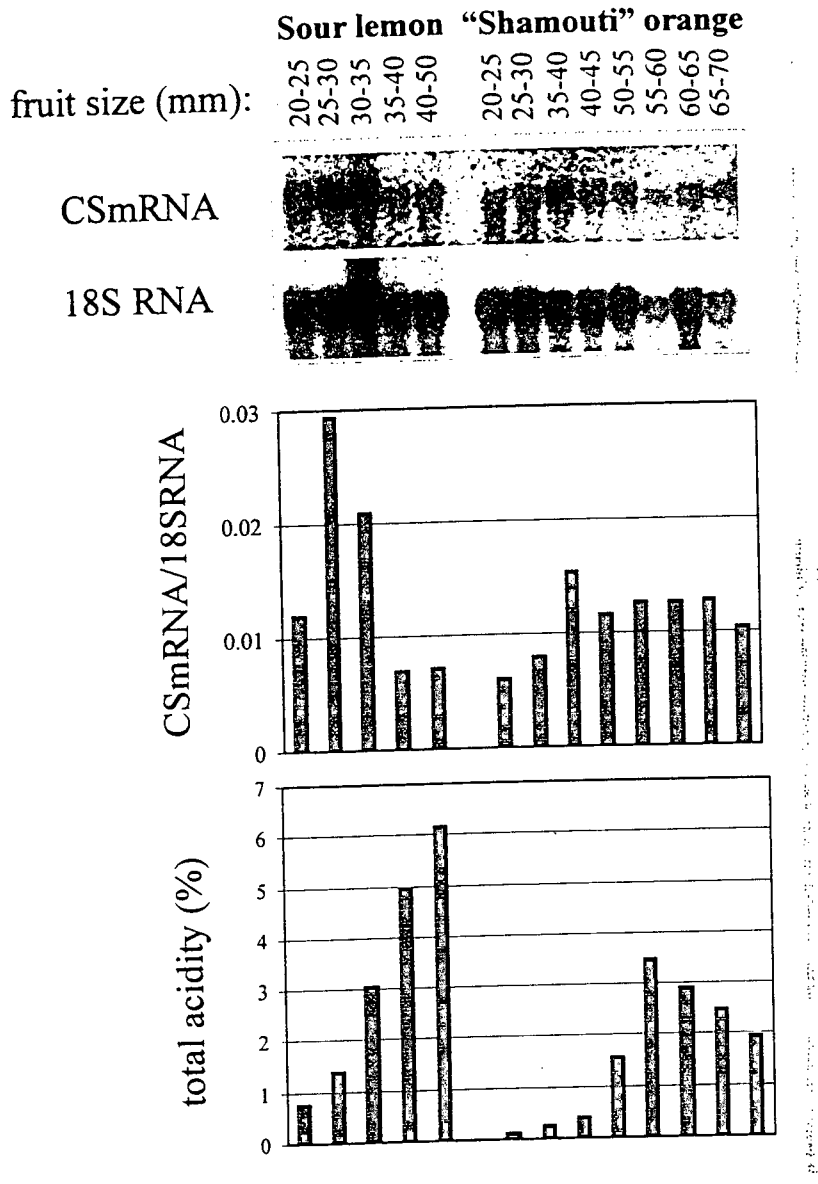
Sour lemon
"Shamouti" orange
fruit size (mm):
20-25
25-30
30-35
35-40
40-50
20-25
25-30
35-40
40-45
50-55
55-60
60-65
65-70
CSmRNA
18S RNA
CSmRNA/18SRNA
0.03
0.02
0.01
0
total acidity (%)
7
6
5
4
3
2
1
0

Fig. 4

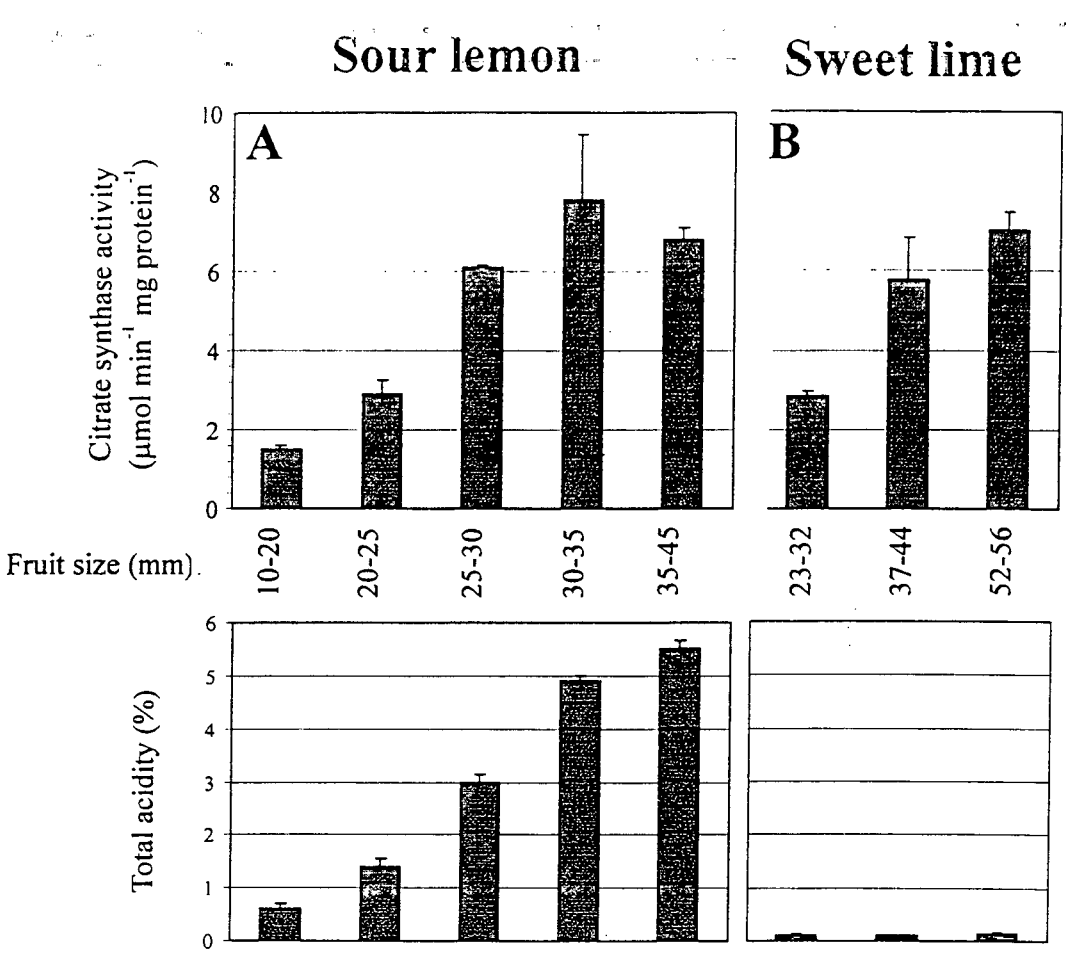
Sour lemon
Sweet lime
A
B
Citrate synthase activity (µmol min-1 mg protein-1)
10
8
6
4
2
0
Fruit size (mm)
10-20
20-25
25-30
30-35
35-45
23-32
37-44
52-56
Total acidity (%)
6
5
4
3
2
1
0

Fig. 5

Appendix 6

Genetic cosegregation using known genes (USA)

In an attempt to identify the gene that corresponds to *acitric*, several genes from the citric acid synthesis pathway were cloned in Dr. Sadka's laboratory and the cloned genes were used as probes in RFLP analyses of high and low acid progeny from segregating populations. The rationale for this test is that if a cloned gene corresponds to *acitric* and has polymorphic restriction fragments, then all low-acid progeny should inherit only a restriction fragment derived from 2240 pummelo and all high acid progeny should have at least one restriction fragment derived from the high-acid parent or grandparent. These cosegregation tests did not identify the gene altered by the *acitric* mutation, but they did show that *acitric* does not code citrate synthase, NAD- or NADP-isocitrate dehydrogenase, malic dehydrogenase, or some aconitase enzymes. The mitochondrial aconitase gene was not cloned or tested and remains a possible candidate gene.

Differential Display (USA)

Differential display and restriction fragment differential display were used to compare patterns of gene expression in juice vesicles of developing fruit of high and low acid genotypes derived from pummelo 2240 and other citrus varieties. Although conventional differential display revealed some differentially expressed fragments, additional investigation did not confirm these patterns of expression - the common problem of too many "false positives" was found. Restriction fragment differential display was then used in an attempt to overcome this problem. However, with this technique we were not able to obtain repeatable fragment profiles from replicate RNA samples.