J. AMER. SOC. HORT. SCI. 120(3):510–514. 1995.

In Vitro [^{14}C]Citrate Uptake by Tonoplast Vesicles of Acidless *Citrus* Juice Cells

Camilo Canel[1], Julia N. Bailey-Serres[2], and Mikeal L. Roose[3]
Department of Botany and Plant Sciences, University of California, Riverside, CA 92521-0124

Additional index words. Citrus maxima, citrate transport, organic acid accumulation, citrus fruit

Abstract. **The acidless phenotype of the pummelo 2240 [Citrus *maxima* [Burro.] Merrill] is caused by a mutation affecting a key element of the citric acid accumulation pathway. To test the functionality of the tonoplast citrate transport mechanism, we obtained a tonoplast-enriched membrane fraction from juice tissues of acidless fruit by centrifugation through a discontinuous Ficoll gradient. The isolated tonoplast vesicles incorporated radioactively labeled citrate at a higher rate than vesicles from similarly fractionated high-acid fruit juice. Uptake of [^{14}C]citrate occurred against a concentration gradient was stimulated by nitrate-sensitive ATP hydrolysis, but not by hydrolysis of PPi, and was not affected by the ionophore nigericin. Uptake was not inhibited by malate and only slightly by isocitrate. We did not find evidence of a defective citrate transport mechanism at the tonoplast of juice cells of acidless fruit. We propose that citric acid accumulation in the fruit of citrus is mediated by a carrier that uses energy produced during hydrolysis of ATP to transport citrate into the vacuole actively and specifically.**

Organic acid accumulation in the vacuole of juice cells of citrus fruit is a developmentally regulated process (Clements, 1963; Ting and Vines, 1966), the degree and timing of which varies greatly among species and varieties, and is highly susceptible to environmental conditions (Vandercook, 1977). Despite the observed variation, citric acid usually constitutes >90% of the titratable acid in citrus juice, followed by malic acid at ≈9%, and small quantities of isocitric, malonic, succinic, and other acids. Typically, citric acid content increases very rapidly ≈100 days after pollination, reaches a maximum of 1% to *2%* on a fresh weight basis, or 50 to 100 mM, and then decreases slowly as the fruit matures (Sinclair, 196 1). While hypothetical models have been proposed to account for the formation of the accumulating citric acid (Vandercook, 1977), there are no reports about the mechanism of citrate transport across the tonoplast of citrus juice cells.

The acidless pummelo 2240 has been used in crosses with citrus varieties of intermediate and high acid levels (Soost and Cameron, 196 1). Pummelo 2240 carries a gene, *acitric,* that causes very low acidity in homozygous individuals and reduced acidity in heterozygotes without affecting other traits. In genotypes that are not homozygous for *acitric,* acid content is a quantitative trait. Total titratable acidity of pummelo 2240 fruit juice does not increase beyond 0.08% to 0.10% (Soost and Cameron, 1961); results from HPLC analysis indicate that citric acid concentration in the juice of pummelo 2240 is ≈10 times lower than in normal fruit. A population of selected F_2 individuals (population B) derived from the cross between pummelo 2240 and a mandarin hybrid of intermediate-high acid level (1.65% titratable acid) is available at the Univ. of California, Riverside, Agricultural Experiment Station (Cameron and Soost, 1977). These individuals were chosen as representative of the high- and low-acid types of a larger segregating population, having acidity levels similar to those of their high- and low-acid grandparents. The low-acid individuals of population B are homozygous for *acitric,* while the high-acid individuals are either homozygous for the normal gene or carry one copy of *acitric.* We have used population B to try to identify *acitric* and characterize the mutation at the physiological and genetic levels. Disruption of organic acid metabolism in pummelo 2240 may result from one of the following: 1) reduced biosynthetic capacity, 2) increased organic acid utilization and degradation, or 3) the presence of a dysfunctional transport mechanism. In this study, we addressed the latter possibility by testing the capacity of tonoplast vesicles isolated from juice tissues of acidless fruit to incorporate [^{14}C]citrate and investigated the energy dependence and the substrate specificity of the process.

Materials and Methods

Plant material. Immature fruit 8 to 10 cm in diameter were collected from five trees—low-acid 6B3-5, 6B3-8, 6B3-15, and high-acid 6B3-11, 6B3-20— belonging to F_2 population B, which was derived from a cross between pummelo 2240 *(Citrus maxima* [Burro.] Merrill) and Kinnow mandarin *(Citrus reticulate* Blanco, hybrid King x Willowleaf). Fruit were collected during August to mid-October, 3 to 4 h after dawn, and kept at room temperature (RT) (20 to 22C) until dissected, usually within 1 h.

Juice fractionation. The fractionation procedure was adapted from that used to isolate tonoplast vesicles from kiwifruit (Chedhomme and Rona, 1985). Discontinuous Ficoll (Sigma, St. Louis) gradients were prepared in 30-ml glass centrifuge tubes. Gradients consisted of 3-ml steps of 5%, 8%, and 12% (w/v) Ficoll in vesicle dilution buffer (VDB): 25 mM Hepes-Tris (pH 7.3) and 450 mM mannitol. Fruit juice was squeezed out of chunks of juice tissue through two layers of cheesecloth and filtered through one layer of Miracloth (Calbiochem, La Jolla, Calif.) into chilled sterile flasks. Gradients were layered with 6 to 9 ml of juice, and centrifuged using a swinging bucket rotor (JS - 13.1, Beckman, Palo Alto, Calif.) at 3600 rpm, 10C, for 30 min in a centrifuge (J2-21; Beckman).

Membranes partitioned at the two interfaces were recovered separately with Pasteur pipettes, mixed with 5 vol of VDB, pelleted by centrifugation as described-above, and carefully resuspended in VDB. The pellet faction was washed once with 5 ml of VDB, and resuspended in the same buffer. The concentration of the samples was then adjusted to 1 g protein/liter by dilution in VDB containing

Received for publication I Aug. 1994. Accepted for publication 14 Dec. 1994. This article is a portion of a doctoral thesis submitted by Camilo Canel. We thank the California Citrus Research Board for financially supporting this work, The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

[1]Postdoctoral research assistant.
[2]Assistant professor.
[3]Associate professor.

1 mM DTT and 0.1 g BSA/liter. Protein concentration was determined by the Bradford method (Bradford, 1976) using commercially available reagents (US Biochemical, Cleveland) and BSA as standard. Unless otherwise stated, vesicle preparations were kept on ice until used.

Enzyme assays. The activities of ATPase, pyrophosphatase (PPase), and latent uridine diphosphatase (UDPase) were measured spectrophotometrically (Lambda 3B spectrophotometer; Perkin-Elmer, Norwalk, Corm.) as the Pi released after 20 min of incubation at 37C by addition of ammonium molybdate in the presence of ferrous ions. To calculate the amount of Pi in the solution, net absorbance at 750 nm was divided by the slope of a calibration curve generated with dilutions of 6 mM potassium phosphate (pH 7). Reactions contained 60 µg of membrane protein in a volume of 0.3 ml. ATPase assays were carried out in 30 mM Tricine-Tris (pH 7.3), 50 mM KCl, 0.1 mM NH_4MoO_4, 4 mM $MgSO_4$, and 4 mM ATP (disodium salt). When present, the concentrations of ATPase inhibitors were 1 mM NaN_3, 50 mM KNO_3, or 1 mM Na_3VO_4. Vanadate stocks were prepared as described by O'Neill and Spanswick (1984). PPase activity was assayed in 50 mM Hepes-Tris (pH 7.5), 50 mM KNO_3, 5 mM NH_4Cl, 3 mM $MgSO_4$, 0.5 mM NH_4MoO_4, 0.5 mM NaN_3, 1 mM NaP_2O_7. UDPase assays were performed in 30 mM Mes-Tris (pH 6.5), 50 mM KCl, 3 mM $MgSO_4$, and 3 mM UDP; activity was calculated as the difference between samples containing 0.03% (v/v) Triton X- 100 and those without detergent. Reactions were stopped by addition of 0.3 ml of ice-cold 10% (w/v) TCA, 0.3% (w/v) SDS, placed on ice for 5 min and spun down in a microcentrifuge for 3 rein; 0.54 ml of supernatant was mixed with 0.36 ml of Taussky-Shorr reagent (Taussky and Shorr, 1953); absorbance at 750 nm was measured after incubation at RT for 5 min.

[^{14}C]Citrate uptake assay. Vesicles were resuspended in VDB supplemented with 4 mM $MgSO_4$, 1 mM DTT, and 0.1 g BSA/liter, at a concentration of 1.2 g protein/liter. After allowing the suspension to reach RT, ATP was added to a concentration of 4 mM. [1,5–^{14}C]Citric acid (80 Ci/mol), (NEN Research Products, Boston) was added either immediately following addition of ATP or, when measuring the effect of preincubation with ATP, after 15 min. Final volume was adjusted by addition of VDB; samples were thoroughly but carefully mixed using a plastic pipette tip and incubated at RT with occasional gentle shaking.

Sample volume in time-course experiments was 0.6 ml; a 95-µl aliquot was removed every 10 rein, layered on 5% (w/v) Ficoll in VDB in a 1.5-ml microfuge tube, and centrifuged at 5000 rpm for 9 min. Pelleted vesicles were carefully rinsed once with 1 ml of VDB, dissolved in 90 µl of 2% (v/v) NP-40, mixed with 1 ml of Scinti-Verse II scintillation fluid (Fisher, Pittsburgh), and counted in a scintillation counter (LS 3801; Beckman). Sample volume in one-time-point experiments was 0.1 ml; 95 µl was removed after 30 min to be centrifuged, washed, and counted as described above.

Statistical analyses. Analysis of variance was computed using the GLM procedure of SAS with independent experiments treated as blocks in a RCB model. Mean separation used the protected LSD test. In cases where several treatments were compared with a single control, Dunnett's test was also computed; the conclusions arrived at using the LSD and the Dunnett's tests were identical.

Results and Discussion

Characterization of membrane fractions. The juice sacs of citrus fruit consist of a tough, cuticle-covered epidermis enclosing many tightly packed, thin-walled, and very large juice cells (Schneider, 1968). Most of the volume of the juice cells is occupied by the vacuole, which ruptures and releases the juice when the tissue is squeezed. The isolation method used here was expected to yield small vesicles that form upon revesicularization of sections of the tonoplast around the released vacuolar contents.

Several combinations of Ficoll gradient steps of various concentrations were tested to determine those conditions that produced the desired enrichment of tonoplast vesicles. Hydrolysis of ATP by membrane-bound H^+-translocating ATPases under various inhibitory conditions is typically used to establish the subcellular origin of membrane fractions. Tonoplast ATPases are sensitive to nitrate and insensitive to azide and vanadate, while mitochondrial and plasmalemma ATPases are inhibited by azide and vanadate, respectively (Sze, 1985). PPase is a positive marker for the tonoplast, while latent UDPase activity is associated with dictyosomal membranes (Blumwald, 1987; Quail, 1979). Inhibition of ATP hydrolysis by nitrate was highest in membranes recovered at the interface between 8% and 12% Ficoll (12% fraction). More than one-half of the latent UDPase activity was detected in the fraction recovered from the interface between 5% and 8% Ficoll (8% fraction). Most of the PPase activity was recovered in the 8% and 12% fractions. No other significant differences in marker enzyme activities were detected among the three fractions. Fractionation of juice squeezed from high- and

Table 1. Marker enzyme activities in membrane fractions isolated from juice of high- and low-acid genotypes. Enzyme activity in gradient fractions (nmol Pi/mg protein per h)[z,y]

Genotype	Treatment	8%	12%	Pellet
	ATPase activity[x]			
High acid	4 mM ATP (control)	536 ± 32 a	496 ± 37 a	495 ± 19 a
	+50 mM nitrate	460 ± 31 a	267 ± 36 b	436 ± 21 a
Low acid	4 mM ATP (control)	549 ± 33 a	477 ± 20 a	540 ± 18 a
	+50 mM nitrate	482 ± 26 a	231 ± 35 b	455 ± 48 a
	+1 mM azide	537 ± 35 a	479 ± 11 a	467 ± 25 a
	+1 mM vanadate	514 ± 37 a	466 ± 21 a	469 ± 71 a
	UDPase activity			
Low acid	3 mM UDP	1153 ± 117 a	558 ± 51 b	271 ± 116 b
	PPase activity[w]			
Low acid	1 mM PPi	2385 ab	3322 a	1138 b

[z]Values are means and SE of three independent experiments.
[y]Mean separation by LSD (5%); 91 for ATPase and 343 for UDPase.
[x]ATPase activity was compared within fractions and across fractions only within treatments.
[w]Ln-transformed data were analyzed to equalize variances. Reverse-transformed means are shown.

low-acid fruit produced a similar distribution of nitrate-sensitive ATPase activity. In summary, the gradient consisting of 5 %, 8%, and 12%-Ficoll steps yielded a tonoplast-rich fraction, which also contained some dictyosomal membranes, but showed no evidence of contamination by plasmalemma or mitochondrial membranes (Table 1).

The tonoplast-enriched fraction contained only ≈0.2 mg of protein, which represented 2% to 3% of the protein content of 6 ml of unfractionated juice. The 8% fraction and the pellet contained ≈0.2 and 0.1 mg of protein, respectively. Both genotypes yielded approximately equal amounts of vesicle protein. Light microscopic examination of aliquots of the tonoplast-enriched fraction established the presence of numerous round vesicles of no more than 10pm in diameter. Since the juice cells of the source fruit have a diameter of ≈100 µm, and most of their volume is occupied by a single large vacuole, the tonoplast vesicles in the fraction cannot be intact vacuoles; rather, they must have formed by revesicularization of the ruptured tonoplast, as expected.

The ATPase activity of the fractionated tonoplast membranes was sensitive to the isolation and storage conditions. Vesicles treated with 0.03% (v/v) Triton X- 100 had only 15% of the ATPase activity of untreated vesicles, indicating that membrane integrity was required for ATP hydrolysis. Resuspension of the vesicles in VDB without the stabilizing agents BSA and DTT decreased their capacity to hydrolyze ATP to 32% of the control sample and to 19% when kept at RT for 1 h. To simulate the conditions to which high-acid vesicles are exposed during isolation, low-acid vesicles were resuspended in juice supernatant from high-acid fruit (pH 2.8) and refractionated. The hydrolytic capacity of the vesicles exposed to low pH was significantly reduced; this reduction was not accompanied by a change in nitrate sensitivity, indicating that the low pH does not differentially affect a particular ATPase (Table 2). It is probable that exposure to acidic conditions affects high-acid vesicles similarly; however, the observed level of nitrate-sensitive ATP hydrolysis in the two types of vesicles was not significantly different (Tables 1 and 2), which raises the possibility that, under isolation conditions not involving exposure to low pH, vesicles from high-acid fruit would show a higher ATPase specific activity. The high- and low-acid genotypes being compared are not near-isogenic; a somewhat higher ATPase activity in vesicles from high-acid fruit may reflect genetic variation that is not related to the disruption of acid metabolism.

Uptake of [^{14}C]citrate by tonoplast vesicles. We incubated the fractionated tonoplast vesicles with radioactively labeled citrate to investigate whether a deficiency in the citrate transport mechanism was the cause of the acidless phenotype of pummelo 2240, in which case we expected the vesicles isolated from the juice of low-acid fruit to be unable to incorporate the label or do so at a drastically reduced level compared with vesicles from high-acid fruit. Vesicles recovered in the tonoplast-enriched fraction of low-acid juice incorporated [^{14}C]citrate in a concentration- and time-dependent manner. The rate of citrate uptake was greatest during the first 15 to 25 min of incubation and increased as the applied concentration of radioactive citrate increased. Uptake occurred well beyond the point at which the internal [^{14}C]citrate concentration equaled that of the assay medium. For an estimated total vesicle volume of no more than 2 µl after centrifugation through the 5% Ficoll cushion, internal citrate concentration reached levels at least 4 to 5 times higher than the applied concentration after 25 min of incubation. Since only a fraction of the vesicles in the assay mix are of tonoplast origin, the final internal citrate concentration in the tonoplast vesicles is actually much higher. Only that radioactivity associated with intact vesicles was recovered and counted since membranes from fractions treated with the detergent NP-40 did not penetrate the Ficoll cushion.

Table 2. Effect of exposure to low pH on ATP hydrolysis by tonoplast vesicles from low-acid genotypes.

Genotype	Treatment	ATPase activity [z] (nmol Pi/mg protein per h)	Percent of control
Low acid	No exposure (control)	431 ± 45 a	100
	+50 mM nitrate	221 ± 36 bc	51
	Exposure to pH 2.8[y]	228 ± 38 b	53
	+50 mM nitrate	128 ± 6 c	30
High acid	No exposure (control)	382 ± 40 a	100
	+50 mM nitrate	206 ± 30 bc	54
LSD (5%)		114	

[z]Values are means and SE of two independent experiments.
[y]Membranes were resuspended in high-acid juice supernatant and refractionated.

Vesicles obtained from the juice of high-acid fruit incorporated between 50% and 75% as much radioactivity as vesicles from low-acid fruit (Table 3). Presumably, in vivo citrate transport occurs normally in the juice cells of high-acid fruit; thus, we hypothesize that the reduced uptake by tonoplast vesicles from high-acid fruit may be due to their already high internal concentration of citric acid. Alternatively, it is possible that contact with the acidic medium during isolation may have affected the functionality of the transport mechanism; the 47% reduction in ATP hydrolysis observed when low-acid vesicles were exposed to high-acid juice supernatant is within the range of values by which citrate uptake is lower in high-acid vesicles. The stimulator effect of ATP hydrolysis served to establish that citrate uptake was related to the presence of tonoplast vesicles in the membrane fraction. When ATPase inhibitors were added, azide and vanadate had little or no effect, whereas nitrate, specific inhibitor of tonoplast ATPase activity, reduced uptake by 53% (Table 4).

Energy dependence of citrate uptake. Exclusion of ATP from the assay medium greatly reduced citrate uptake, affecting high- and low-acid vesicles similarly (Tables 3 and 4). Hydrolysis of ATP has also been shown to stimulate citrate uptake in studies of tonoplasts isolated from the sap of the rubber tree, *Hevea brasiliensis* (Marin et al., 1981), and barley mesophyll (Rentsh and Martinoia, 199 1); in contrast, it did not affect the rate of uptake by tomato fruit tonoplasts (Oleski et al., 1987). The proton gradient generated by the tonoplast H^+-translocating ATPase has been proposed to drive citrate uptake into latex vacuoles of *H. brasiliensis* (Marin et al., 1981). If this was the case in citrus juice cells, incubation of the vesicles with ATP could be expected to energize the vesicles and increase the rate of citrate uptake. Rather, we observed that when ATP was added 15 min before the addition of [^{14}C]citrate, uptake decreased by 17% and 22% in low- and high-acid vesicles, respectively (Table 3). Depletion of the ATP pool is not a satisfactory explanation for the reduced incorporation of labeled citrate because at a measured rate of 450 nmol·mg^{-1}·h^{-1}, the ATPases in the assay mixture hydrolyze only about 3'%. of the available ATP during the 15-min preincubation period. Inhibition by the products of ATPhydrolysis can also be ruled out, since the presence of 1 mM ADP or 1mM phosphate did not decrease citrate uptake (Table 3).

The lack of stimulation by pre-incubation with ATP suggested that ATP hydrolysis and citrate transport may occur simultaneously, as part of a primary active transport mechanism where ATP hydrolysis is directly coupled to citrate transport. Alternatively, the failure to observe a stimulator effect may have resulted

Table 3. Energy requirements of citrate uptake by tonoplast vesicles isolated from low- and high-acid genotypes.

Genotype	Treatment	Citrate uptake[zy] (nmol·mg^{-1} protein per h)	Percent of control
		Effect of preincubation	
Low acid	Control	11.44 ± 0.71 a	100
	No ATP	2.99 ± 0.46 e	26
	Preincubated[x]	9.48 ± 0.71 b	83
High acid	Control	8.01 ± 0.15 c	100[w]
	No ATP	2.81 ± 0.31 e	35
	Preincubated	6.22 ± 0.28 d	78
LSD (5%)		1.34	
		Test of electrochemical gradient	
Low acid	Control	12.63 ± 0.41 a	100
	No ATP	3.76 ± 0.24 d	30
	1 mM PPi, no ATP	5.95 ± 0.13 c	47
	50 mM KCl	11.04 ± 0.39 b	87
	15 μM nigericin, 50 mM KCl	12.61 ± 0.66 a	100
	15 μM nigericin, no KCl	12.91 ± 0.66 a	102
	1 mM ADP	12.58 ± 0.47 a	100
	1 mM PO_4	12.80 ± 0.03 a	101
LSD (5%)		0.78	

[z]Values are means and SE of two to four independent experiments.
[y]Unless otherwise stated, reactions contained 0.1 mM [^{14}C]citrate and 4 mM ATP.
[x]Incubated with 4 mM ATP at RT for 15 min before addition of [^{14}C]citrate.
[w]Uptake was 70% of that by low-acid control.

from dissipation of the proton gradient in the absence of [^{14}C]citrate. To elucidate whether a transmembrane potential or proton gradient played a role in citrate transport, the effects of an alternative energy source and of including ionophores was tested (Table 3). Inorganic pyrophosphate (PPi), the hydrolysis of which can also generate a proton motive force across the tonoplast, was used instead of ATP as the energy source. Stimulation of citrate uptake in the presence of 1 mM PPi was only 25% of that produced by 4 mM ATP; similar] y poor stimulation was observed with 0.5 mM PPi. In addition, the inclusion of chloride ions, which have been shown to dissipate the transmembrane potential in some systems (Bennett and Spanswick, 1983), reduced the stimulator effect of ATP by only 18%. The ionophore nigericin, which catalyzes an electroneutral H^+/K^+ exchange, did not affect citrate uptake either in the presence or absence of potassium ions (Table 3). These results indicate that during the developmental stage examined in this study, most, if not all, citrate transport across the tonoplast occurs via the primary active transport pathway; if a transtonoplast proton gradient is involved in citrate transport in the expanding juice cells of immature citrus fruit, its contribution to total juice acidity is very small.

Substrate specificity of citrate uptake. The substrate specificity of the ATP-dependent transport of citrate across the tonoplast of low-acid fruit vesicles was investigated by adding excess unlabeled organic acids to the assay mix (Table 4). No reduction in "C incorporation was observed when unlabeled malate was added; unlabeled isocitrate produced a small decrease in uptake, which was not statistically significant. Unlabeled malate concentrations of 2 and 2.5 mM also failed to inhibit citrate uptake. On the other hand, excess unlabeled citrate saturated the uptake mechanism, reducing the incorporation of the label by 63%.

Experiments using [^{14}C]malate indicate that a process similarly dependent on ATP hydrolysis operates in the tonoplast of citrus juice cells for malate uptake. However, the observed specificity for citrate suggests that malate and citrate follow separate paths across the tonoplast. The high specificity of the citrate carrier in citrus juice cells is consistent with the high citric acid content of normal fruit, and suggests that citric acid accumulation in citrus fruit does not necessarily depend on increased citrate synthesis and availability relative to other organic acids.

We have shown that tonoplast vesicles isolated from juice cells of acidless fruit born by individuals homozygous for the low-acidity gene, *acitric*, incorporate [^{14}C]citrate. Citrate uptake by low-acid vesicles is characterized by high specificity and occurs against a concentration gradient; these features indicate that their citrate transport mechanism functions normally. This is further evidenced by the observation that low-acid vesicles incorporate

Table 4. Effect of ATPase inhibitors and unlabeled organic anions on citrate uptake by tonoplast vesicles isolated from low-acid genotypes.

Treatment	Citrate uptake[zy] (nmol·mg^{-1} protein per h)	Percent of control
	Effect of ATPase inhibitors	
Control	10.29 ± 0.28 a	100
No ATP	3.09 ± 0.46 c	30
50 mM nitrate	4.82 ± 0.28 b	47
1 mM azide	9.40 ± 0.51 a	91
1 mM vanadate	9.82 ± 0.45 a	95
LSD (5%)	1.38	
	Effect of unlabeled organic anions	
Control	10.58 ± 0.87 a	100
No ATP	3.90 ± 0.17b	37
0.5 mM citrate	3.87 ± 0.17b	37
0.5 mM malate	10.41 ± 0.98 a	98
0.5 mM isocitrate	9.54 ± 0.48a	90
LSD (5%)	1.32	

[z]Values are means and SE of two or three independent experiments.
[y]Unless otherwise stated, reactions contained 0.1 mM [^{14}C]citrate and 4 mM ATP.

labeled citrate at an equal or higher level than their high-acid counterparts.

Because citric acid accumulation in the fruit of citrus is developmentally regulated, it is still possible that the mutation disrupting normal citrate metabolism in pummelo. 2240 is related to the transport process by affecting the timing of expression of the genes that code for the elements involved. Accumulation of citric acid begins during the early stages of fruit development when it would be impractical to isolate tonoplast vesicles due to the small size and low juice content of the fruit. Our work provides a functional assay to detect inhibition of tonoplast ATPase activity and citrate transport by monoclinal antibodies to be raised-against juice-cell tonoplast proteins (Vivekananda et al., 1988), thus constituting the initial step towards the identification of these elements and the study of their expression.

Literature Cited

Bennett, A.B. and R.M. Spanswick. 1983. Optical measurements of A pH and AY in corn root membrane vesicles: Kkinetic analysis of Cl^- effects on a proton-translocating ATPase. J. Membrane Biol. 71 :95–1 07.

Blumwald, E. 1987. Tonoplast vesicles as a tool in the study of ion transport at the plant vacuole. Physiol. Plant. 69:73 1–734.

Bradford, M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Cameron, J.W. and R.K. Soost. 1977. Acidity and total soluble solids in Citrus hybrids and advanced crosses involving acidless orange and acidless pummelo. J. Amer. Sot. Hort. Sci. 102: 198–201.

Clements, R.L. 1963. Organic acids in citrus fruit. II. Seasonal changes in the orange. J. Food Sci. 29:281–286.

Chedhomme, F. and J-P. Rona. 1985. Isolation and electrical characterization of tonoplast vesicles from the Kiwi fruit *(Actirridia chinernis).* Physiol. Plant. 67:29–36.

Marin, B., J.A.C. Smith, and U. Litttge. 1981. The electrochemical proton gradient and its influence on citrate uptake in tonoplast vesicles of *Hevea brasilierzsis.* Planta 153:486493.

Oleski, N., P. Mahdavi, and A.B. Bennett. 1987. Transport properties of the tomato fruit tonoplast. H. Citrate transport. Plant Physiol. 84:997–1000.

O'Neill, S.D. and R.M. Spanswick. 1984. Effect of vanadate on the plasma membrane ATPase of red beet and corn. Plant Physiol. 75:586–591.

Quail, P.H. 1979. Plant cell fractionation. Annu. Rev. Plant Physiol. 30:425-484.

Rentsh, D. and E. Martinoia. 1991. Citrate transport into barley mesophyll vacuoles—Comparison with malate-uptake activity. Planta 184:532–537.

Soost, R.K. and J.W. Cameron. 1961. Contrasting effects of acid and nonacid pummelos on the acidity of hybrid citrus progenies. Hilgardia 30:351-357.

Schneider,'H. 1968. The anatomy of citrus fruit, p. 1–8 1, In: W. Reuther, L.D. Batchelor, and H.J. Webber (eds.). The citrus industry. II. Univ. of California Press, Berkeley.

Sinclair, W.B. 1961. Organic acids and buffer properties, p. 161–1 90. In: W.B. Sinclair (cd.). The orange, its biochemistry and physiology. Univ. of California Press, Berkeley.

Sze, H. 1985. H^+-translocating ATPases: Advances in membrane vesicles. Annu. Rev. Plant Physiol. 36: 175–208.

Taussky, H.H. and E. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202:675–685

Ting, S.V. and H.M. Vines. 1966. Organic acids in the juice vesicles of Florida 'Hamlin' orange and 'Marsh Seedless' grapefruit. J. Amer. Sot. Hort. Sci. 88:291–297.

Vandercook, C.E. 1977. Organic acids, p. 209–227. In: S. Nagy, P.E. Shaw, and M.K. Veldhius (eds.). Citrus fruit technology. Avi Publ. Co., Westport, Corm.

Vivekananda, J., C.F. Beck, and D.J. Oliver. 1988. Monoclinal antibodies as tools in membrane biochemistry. J. Biol. Chem. 263:4782-4788.