*Proc. Fla. State Hort. Soc.* 118:43-48. 2005.

# ACCUMULATION OF SUCROSE AND OTHER SOLUBLE SOLIDS IN CITRUS JUICE CELLS

ED ETXEBERRIA* AND PEDRO GONZALEZ
*University of Florida, IFAS*
*Citrus Research and Education Center*
*700 Experiment Station Road*
*Lake Alfred, FL 33850*

*Additional index words. Citrus reticulata*, Brix, endocytosis, sugar accumulation, juice sacs, vacuole

*Abstract.* **To investigate the accumulation of sugars and other soluble solids into *Citrus* juice cells, we analyzed vesicle sink strength and sucrose uptake in Murcott mandarin (*Citrus reticulata* Blanco) fruit tissue. Plasmalemma and tonoplast vesicles isolated from fruits (juice sacs) collected at different developmental stages were analyzed for their transport capabilities. There was an active transport mechanism for sucrose in energized plasmalemma vesicles. Unexpectedly, tonoplast vesicles were shown to lack active transport mechanism of sucrose into the vacuole. *Citrus* juice cells were shown to incorporate membrane impermeable dyes into their vacuoles in the presence of sucrose. High definition confocal microscopy revealed the co-localization of membrane impermeable markers in cytoplasmic vesicles and the formation of vesicles at the plasmalemma. In addition, cells were able to take up artificial nano-particles. The data provide evidence for an endocytic system of transport that allows direct transport of sucrose**

*Corresponding author; e-mail: eje@crec.ifas.ufl.edu

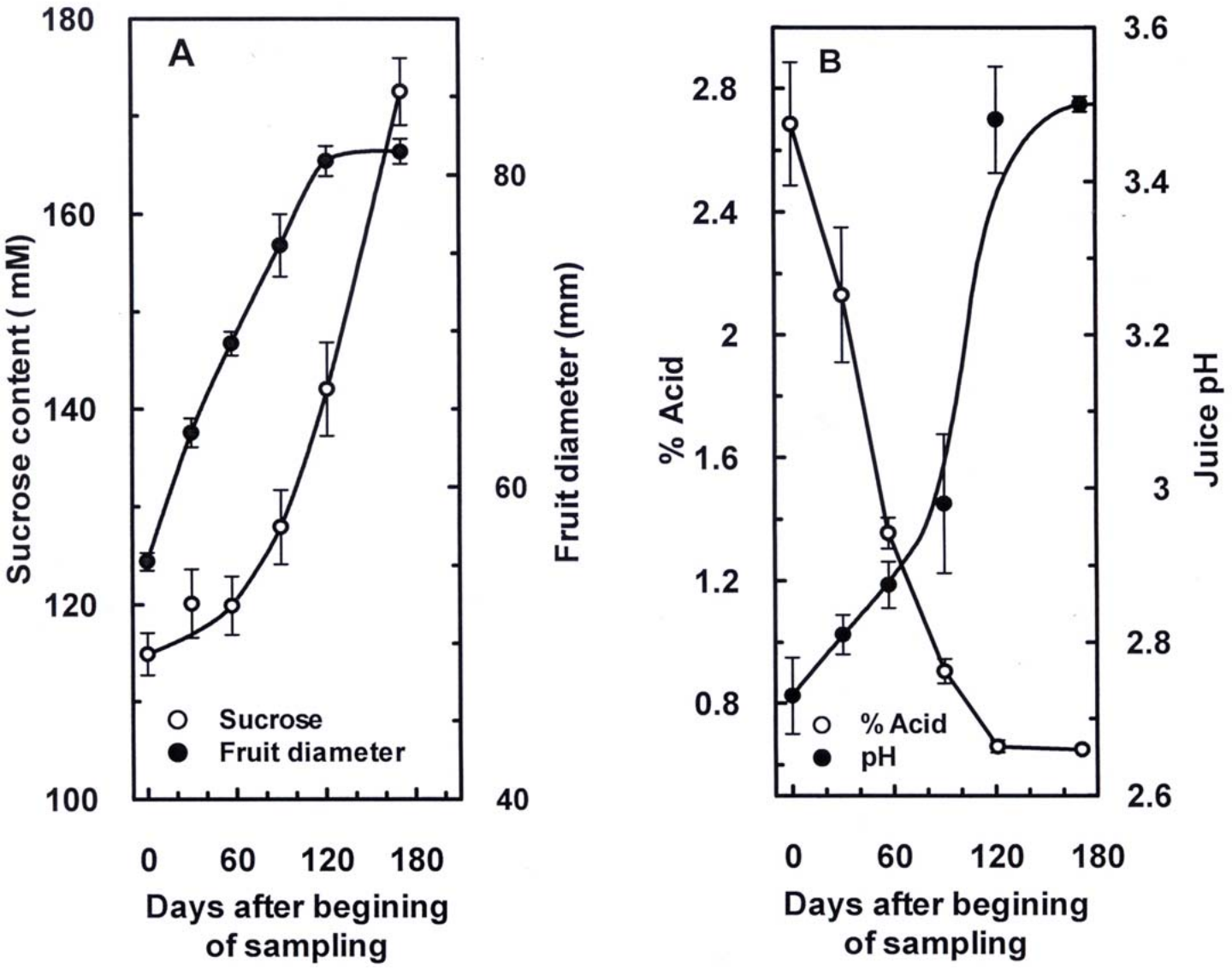


Fig. 1. Changes in sucrose content and fruit diameter (A), and percent acid and juice pH (B) in 'Murcott' mandarin juice cells throughout development. Initial fruit sampling was carried out in 6 Aug. 2002 and terminated in 22 Jan. 2003. Each symbol represents 3 replicate samples. Bars indicate standard deviation.

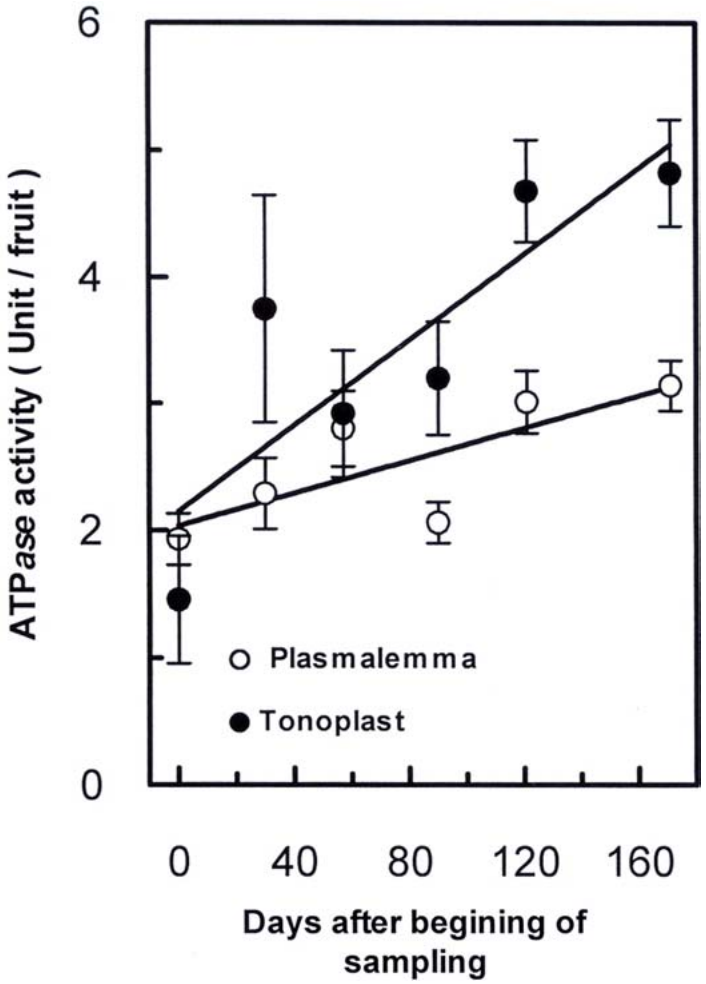


Fig. 2. Activities of V-ATP*ase* and P-ATP*ase* in isolated tonoplast and plasmalemma samples from 'Murcott' mandarin during development. Initial fruit sampling was carried out in 6 Aug. 2002 and terminated in 22 Jan. 2003. One unit is defined as the production of 1 µmol of product per min. Each symbol represents 3 replicate samples. Bars indicate standard deviation.

**from the apoplast to the vacuole in *Citrus* juice cells. Having identified the factors regulating sucrose uptake and solute accumulation, we can proceed to design experiments aimed at increasing the rate of sugar uptake resulting in higher Brix.**

Sugars, like most other soluble solids in *Citrus* fruits, originate in the leaves and are transported to the fruit through an extensive vascular system. After reaching the juncture with the juice sacs at the fruit segment wall, these solutes are released from the phloem terminals and into the extracellular space (Koch and Avigne, 1990). A concentration/osmotic gradient drives solids movement through the remaining distance until reaching the periphery of the juice cells. At this point, sugars need to be taken up by juice cells to be utilized as energy source, for conversion into other juice components and for accumulation that translates to higher Brix.

The mechanisms of sugar uptake into juice cells (and other storage cells) is essential in determining the final concentration of solids at maturity. The existence of protein carriers, transporters, and channels, located at the various cell membranes, has indirectly led to the notion that external solutes cross individual membranes before final passage into the storage vacuole. However, a growing volume of evidence indicates that transport of solutes (including sugars) from the cell exterior into the cell vacuole is not always through individual channels or transporters (MacRobbie, 1999). Some data suggest the presence of alternate or parallel transport mechanisms (Fernie et al., 2000; Hajirezaei et al., 2000; Haug and Shi, 1991). In fact, in cultured storage cells, a vesicle mediated mechanism for sugar transport has been recently documented (Etxeberria et al., 2005a).

To further our understanding into how sugar accumulates in *Citrus* juice cells, we investigated several known mechanism and factors involved in solute accumulation using 'Murcott' mandarin (*Citrus reticulata* Blanco). The data indicate that sugars and other soluble components enter the juice cells by a system comprised of solute filled vesicles with extracellular nutrients comparable to that reported for other storage cells (Etxeberria et al., 2005a). This will provide a tool to improve soluble solid content in *Citrus* fruits.

## Materials and Methods

*Plant material.* 'Murcot' mandarin (*Citrus reticulata* Blanco), were collected monthly between 6 Aug. 2002 and the end of Jan. 2003 from groves located at the UF/IFAS Citrus Research and Education Center in Lake Alfred, Fla. A total of 30 fruit were used per sample for each collection date. Collected fruit were taken immediately to the laboratory and used for fruit analyses, enzyme extraction, and membrane isolation. Five fruit were used for membrane isolation, and five for extraction of soluble enzymes. The experiments were run in triplicates of 10 fruit each.

*Enzyme extraction.* Each fruit was weighed and cross-sectioned at the equator. From each half of 5 fruit, 1 mL juice was mixed with 1 mL of cold buffer as described by Etxeberria et al., 2005b. All aliquots were combined and the buffered juice centrifuged at 77,000 $g_n$ for 30 min at 4 °C. After centrifugation, 2.5 mL of the supernatant were desalted through a Sephadex PD-10 and 3 mL were collected for enzyme assays. Samples were run in triplicates.

Juice pH and titratable acids were measured individually for each of the five unbuffered fruit-juice extracts. Juice pH (15 mL) was measured using a pH meter (model HI-9219, Hannah Instruments, Inc., Woonsocket, R.I.) and titrated to an end point pH of 8.0 using standard alkali solution (0.3125 N NaOH). For sucrose analysis, juice samples were clarified by centrifugation and sucrose concentration determined by the anthrone method of Van Handel (1968).

*Plasmalemma and tonoplast isolation.* Plasmalemma and tonoplast were isolated following the procedure described by Etxeberria and Gonzalez (2003), a method that combines the principles of sucrose density gradient and aqueous two-phase partition. Approximately 150 mL of juice cell extract from 5 fruit were squeezed directly into 150 mL of homogenization buffer and centrifuged at 10,000 $g_n$ for 10 min to eliminate debris. The supernatant was centrifuged for 30 min at 40,000 $g_n$, and the pellet set aside for plasmalemma purification by aqueous two phase partitioning. The remaining supernatant was centrifuged at 100,000 $g_n$ for an additional 60 min, and the pellet used for tonoplast purification by sucrose density gradient (Etxeberria and Gonzalez, 2003).

*Enzyme assays.* Total ATP*ase* activity was determined using 25 µg tonoplast or plasmalemma vesicle protein, in a total volume of 500 µL (Etxeberria et al., 2005b). Aliquots of 50 µL were removed from the assay at determined times and the product P*i* determined as described by Chifflett et al. (1988).

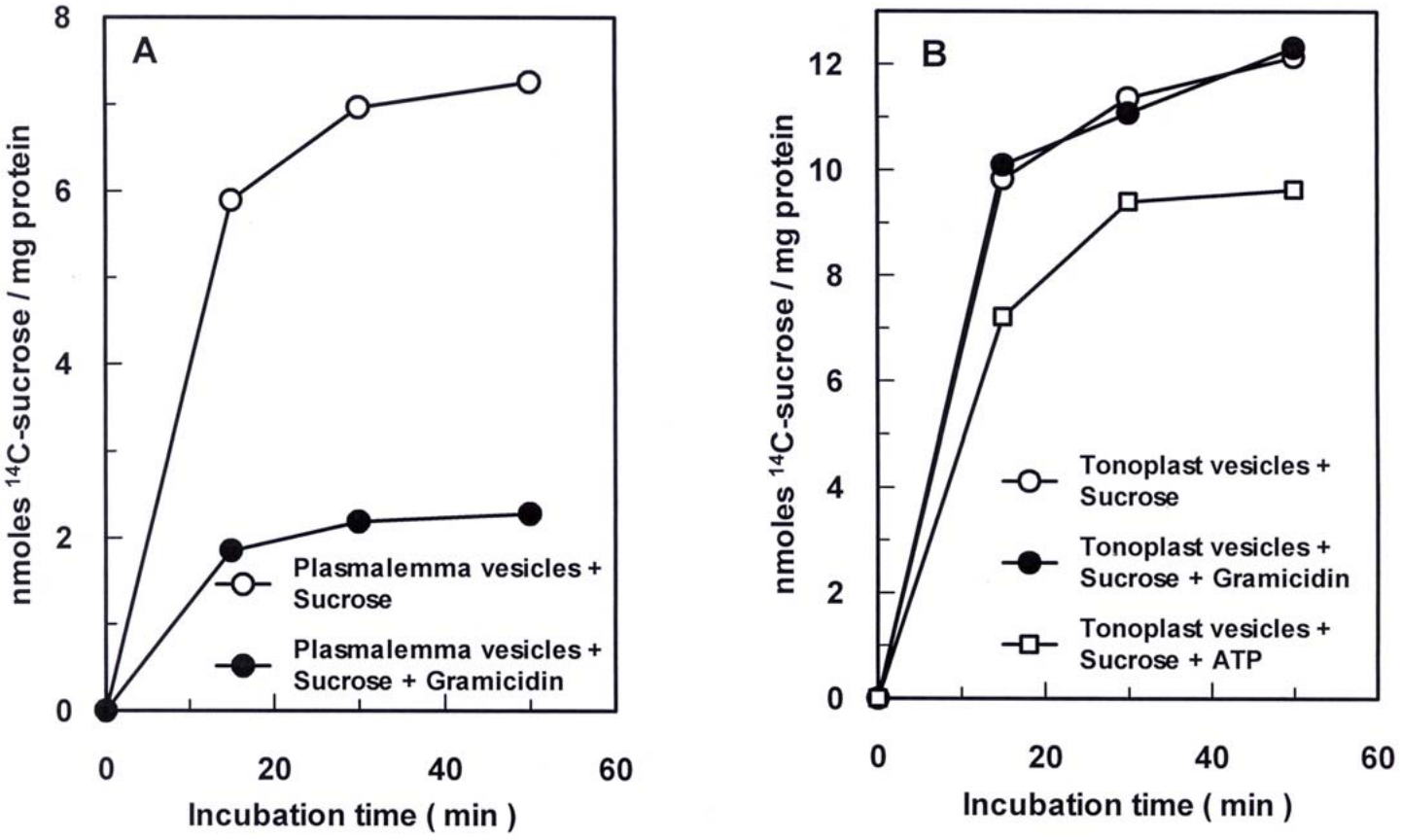


Fig. 3. $^{14}$C-sucrose uptake into purified plasmalemma vesicles (A) and tonoplast vesicles (B) from 'Murcott' mandarin. Vesicles were energized using a pH jump (Bush, 1989) of 2 units and incubated in the presence of 2.5 mM Mg/ATP or 10 µM gramicidin (control). Fig. 3 is a representative result from an experiment replicated three times.

*Sucrose uptake experiments.* The capacities of tonoplast and plasma membrane to take up sucrose were assayed in artificially energized vesicles as described by Echeverria et al. (1997). Radioactivity retained in the vesicles was determined by scintillation spectroscopy after placing the filter discs in 5 mL of ScintiVerse BD SX 18-4 (Fisher Scientific, Pittsburgh, Pa.).

*Tissue incubation in fluorescent probes.* Juice sacs (containing juice cells) from fruit at the rapid stage of sucrose accumulation, were excised carefully and cut in halves. Ten halves were incubated in 3 mL of a buffered solution containing 250 mM sucrose and 1 mg mL$^{-1}$ d-TR (dextran MW 3,000 tagged with Texas Red, Molecular Probes, Eugene, Ore.) or 100 µM Alexa 488 (Molecular Probes, Eugene, Ore). After approximately 24 h incubation in the dark at room temperature, the solution was drawn and protoplasts prepared for better visualization (Etxeberria et al., 2005b). The released protoplasts were viewed under a Leica microscope equipped with appropriate filters.

## Results and Discussion

*Fruit growth, sugar accumulation and acid content.* Sucrose accumulation in 'Murcott' mandarin commenced at a time of rapid fruit expansion (Fig. 1A) and increasing juice pH (Fig. 1B), which directly reflects vacuolar $H^+$ concentration (Echeverria and Burns, 1989; Echeverria et al., 1992). During the experimental period starting in early Aug. 2002, sucrose content increased from approximately 115 to 170 mM at the end of Jan. 2003. Concurrently, average fruit diameter expanded from 55 to 82 cm (Fig. 1A). Acidity declined approximately 60% with a concomitant raise in pH from 2.7 to 3.5 during the same period (Fig. 1B). Although organic acids are known to be utilized as an energy source and as intermediates for the synthesis of other organic compounds at later stages of development (Murata, 1977), most of the noted decline in acidity and corresponding increase in pH reported here were likely the result of utilization (Murata, 1977) and dilution, given the concurrent increase in vacuolar V-ATP*ase* $H^+$ pumping activity (Fig. 2).

*Membrane-bound $H^+$ pumps.* Whereas tonoplast-bound V-ATP*ase* activity more than doubled throughout development, plasmalema P-ATP*ase* increased over 50% during the same period (Fig. 2). The increase in the cell's potential capacity to acidify both the apoplast and vacuole by their respective $H^+$ pumps is in conformity with the existence of $H^+$-energized carriers at both membranes.

*Sucrose accumulation by isolated vesicles.* When energized plasmalemma vesicles were incubated in the presence of sucrose, uptake was significantly higher than control samples which contained 10 µM gramicidin to dissipate the energization (Fig. 3A). There was no sucrose uptake in vesicles treated with ATP plus gramicidin (data not shown), eliminating the possibility of a direct ATP-energized sucrose transport system. This implies the existence of an active transport system of sucrose from the apoplast to the cell cytosol.

In *Citrus* juice cells, sucrose is accumulated in the vacuole against a concentration gradient (Echeverria and Valich, 1988), a condition that would also require an active sucrose

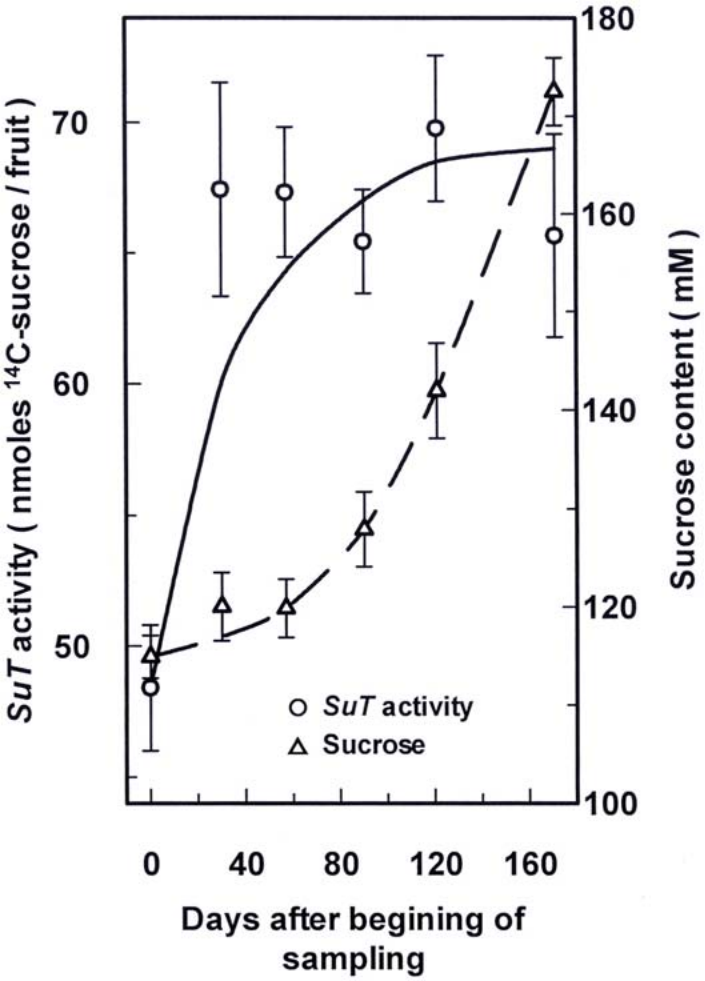


Fig. 4. Plasmalemma sucrose transport activity from 'Murcott' mandarin juice cells throughout development (solid line). Initial fruit sampling was carried out in 6 Aug. 2002 and terminated in 22 Jan. 2003. Dotted line represents the parallel increase in fruit sucrose content. Each symbol represents 3 replicate samples. Bars indicate standard deviation.

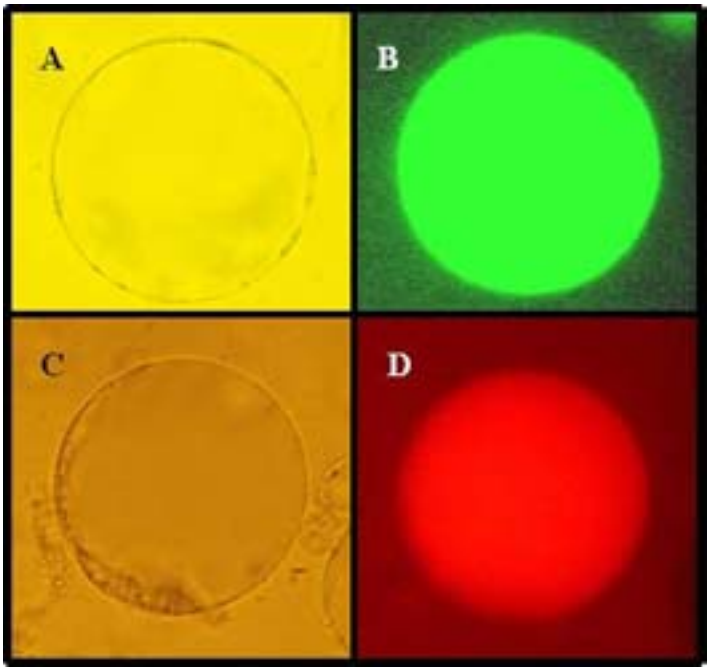


Fig. 5. Light (A, B) and fluorescent micrographs (C, D) of juice cells after incubation with the membrane impermeable markers d-TR (C) and in Alexa-488 (D). Protoplasts were prepared for observation after for juice sacs were incubated in 250 mM sucrose and the respective fluorescent marker for 24 h. Notice the fluorescence of Alexa-488 and d-TR in the vacuole.

transport mechanism at the tonoplast. When corresponding transport experiments were conducted with tonoplast vesicles, however, no increase in sucrose uptake was measured above control samples (Fig. 3B). In fact, addition of ATP alone resulted in lower a accumulation of sucrose (Fig. 3B). The lack of an active sucrose transport system was unexpected, given the accumulation of sucrose against a concentration gradient in the vacuole (Echeverria and Valich, 1988) and the presence of sucrose carrier at the plasmalemma (Fig. 3A). This suggests a separate mechanism for sucrose and other solids accumulation.

The possibility of a tonoplast-bound active sucrose transport system present only at some critical stages of sucrose accumulation was tested using tonoplast samples collected from fruit at different developmental stages. At no time during fruit development there was sucrose uptake into tonoplast vesicles either artificially energized or with added ATP. On the contrary, in plasmalemma samples, electrogenic sucrose transport was present at all stages and parallel sucrose accumulation (Fig. 4).

*Uptake of membrane impermeable dyes and nano-particles.* The possibility of a direct route between the apoplast and the vacuole was investigated by incubating juice sacs (containing

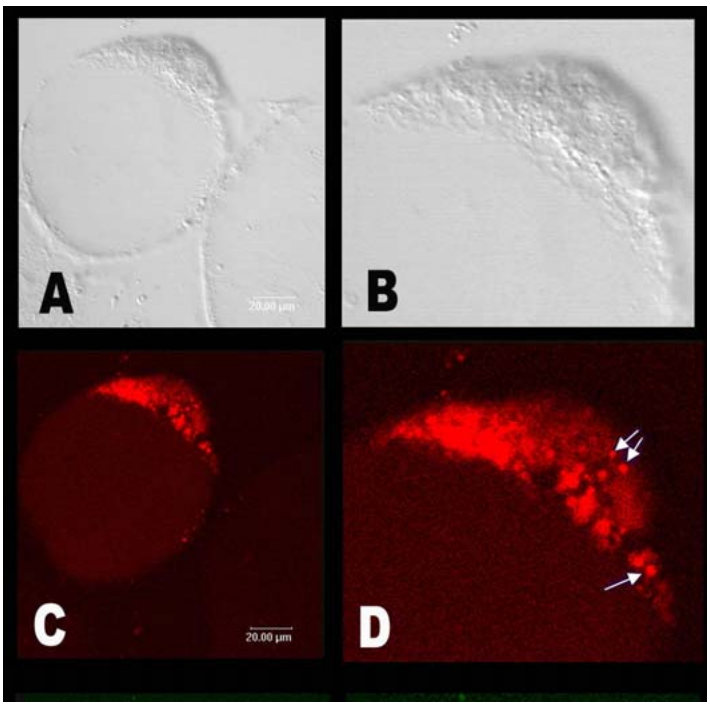


Fig. 6. Light and confocal fluorescent micrographs of a *Citrus* juice cell after 2 h incubation with sucrose and the membrane impermeable markers d-TR and Alexa-488. A and B represent light images of the cell in two magnifications. C and D correspond to images taken under appropriate filter for d-TR. Images in E and F are taken under filter for Alexa-488. Arrows indicate some of many intracellular vesicles containing both d-TR and Alexa-488 simultaneously. Note that both dyes appear together indicating that cells take up solutes from the outside in bulk.

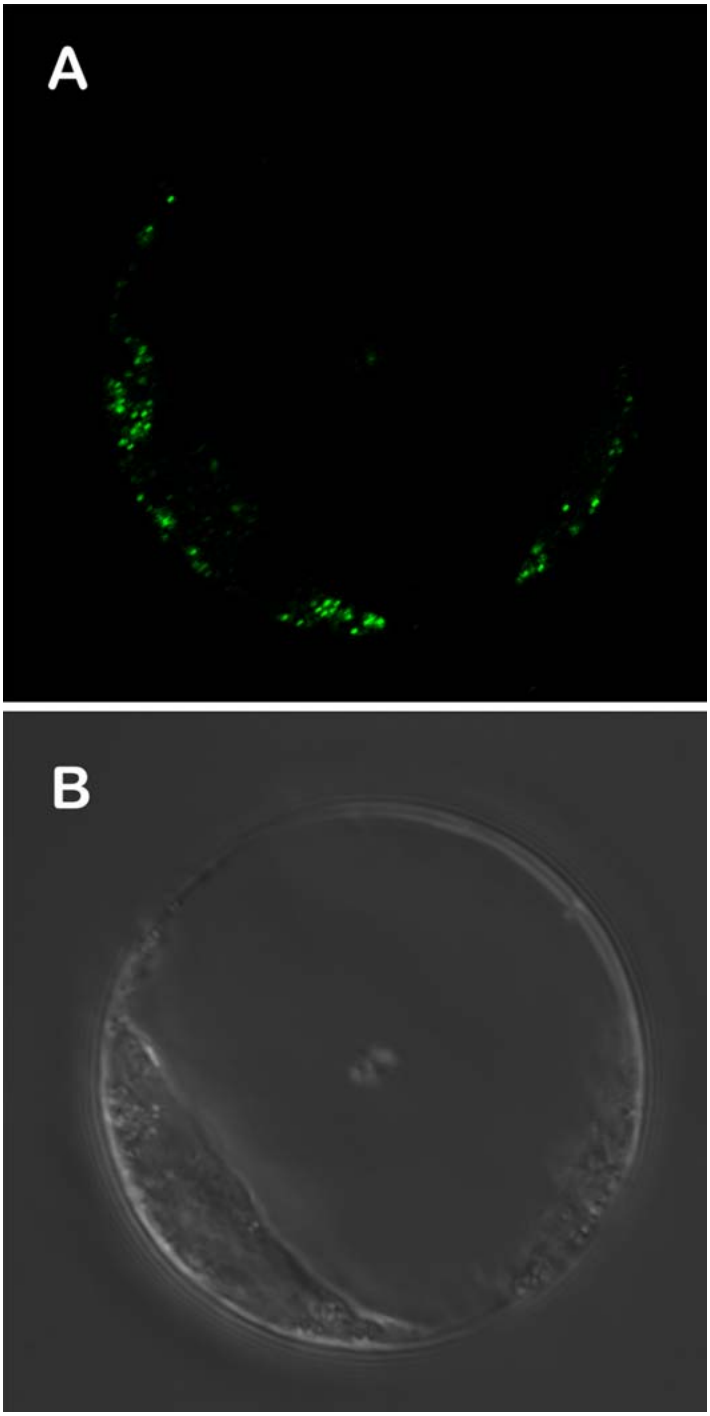


Fig. 7. High definition confocal microscopy of *Citrus* juice cells after 2 h incubation in incubation medium containing $10^{14}$ CdSe/ZnS quantum dots. Cell was observed under appropriate filter for fluorescin. The uptake of large particles demonstrate the indiscriminate uptake of external solutes by structures other than channels or transporters.

juice cells) overnight in a buffered solution of 250 mM sucrose and the fluorescent membrane-impermeable dyes d-TR (Horn et al., 1992), Alexa-488 (Emans et al., 2002), or quantum dots (Hew et al., 1999). After 24 h of incubation, protoplasts were prepared for better visualization. Microscopic examination of protoplasts revealed a highly fluorescent vacuole indicating transport and accumulation within the vacuolar compartment of both dyes (Fig. 5). The vacuole, which occupies most of the cell volume, is highly fluorescent in both cases, whereas the cytosol is seen as a non-fluorescent layer located at one side of the cell. Given the inability of these compounds to traverse biological membranes, the results of Fig. 5 support the existence of an alternative mechanism of direct solute transport from the apoplast to the vacuole for storage.

Closer examination using high definition confocal microscopy of juice cells incubated for 2 h in a solution containing Alexa-488, d-TR, and sucrose, provides additional evidence for an endocytic mechanism for sucrose transport into the vacuole of juice cells. In Fig. 6 (arrows), both Alexa-488 and d-TR are internalized into distinct cytoplasmic compartments within the cytosol. At this time, fluorescence of both dyes appear together within individual compartments whereas no fluorescence was observed in the cytosol. Topographic confocal membrane analysis of the plasmalemma of a juice cell during sucrose accumulation further substantiates the presence of an endocytic transport mechanism by demonstrating the formation of vesicles of characteristic size of approximately 70 nm (Thiel et al., 1998).

The ability of juice cells to take up external solutes was confirmed using artificial quantum dots (20 nm). After 2 h incubation, fluorescence was evident in cytosolic vesicles (Fig. 7) demonstrating the indiscriminate uptake of external fluids containing nutrients.

Taken together, the data presented in this communication demonstrate the existence of an active sucrose carrier at the plasmalemma of *Citrus* juice cells (Fig. 3A). The plasmalemma-bound sucrose carrier mediates transport of sucrose from the apoplast into the cytosol. However, accumulation of sucrose into the vacuole appears not to be carrier mediated. Instead, the data provide evidence for the existence of a non-selective vesicle mediated mechanism of solute transport consistent with properties of an endocytic system similar to those reported for parenchyma cells of maize root cortex (Baluška et al., 2004), for tobacco (*Nicotiana Tabacum* L.) cultured cells (Emans et al., 2002) and sycamore cultured cells (Etxeberria et al., 2005a). An endocytic system can transport sucrose from the apoplast directly to the vacuole bypassing the cytosol as evidenced by the accumulation of d-TR and Alexa-488 in the vacuole (Fig. 5) and by high resolution confocal microscopy or similar events (Figs. 6 and 7). Having identified a transport system involving vesicles that carry solutes in bulk, we can now focus in finding ways to accelerate the process in order to increase soluble solids and advance fruit maturity throughout the rapid accumulation of Brix.

## Literature Cited

Baluška, F., J. Samaj, A. Hlacvacka, J. Kendrick-Jones, and D. Vilkamann. 2004. Actin-dependent fluid-phase endocytosis in inner cortex cells of maize root apices. J. Exp. Bot. 55:463-473.

Chifflett, S., A. Torriglia, A. Chisea, and S. Tolosa. 1988. A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: Application to lens AT-P*ase*. Anal. Biochem. 168:1-4.

Echeverria, E. and J. K. Burns. 1989. Vacuolar acid hydrolysis as a physiological mechanism for sucrose breakdown. Plant Physiol. 90:530-533.

Echeverria, E. and J. Valich. 1988. Carbohydrate and enzyme distribution in protoplasts from 'Valencia' orange juice sacs. Phytochemistry 27:73-76.

Echeverria, E., J. K. Burns, and H. Felle. 1992. Compartmentation and cellular conditions controlling sucrose breakdown in mature acid lime fruits. Phytochemistry 31:4091-4095.

Echeverria, E., P. C. Gonzalez, and A. Brune. 1997. Characterization of proton and sugar transport at the tonoplast of sweet lime (*Citrus limmetioides*) juice cells. Physiol. Plant. 101:291-300.

Etxeberria E., E. Baroja-Fernandez, F. J. Muñoz, and J. Pozueta-Romero. 2005a. Sucrose inducible endocytosis as a mechanism for nutrient uptake in heterotrophic plant cells. Plant Cell Physiol. 46:474-481.

Etxeberria, E. and P. Gonzalez. 2003. Simultaneous isolation of tonoplast and plasmalemma from *Citrus* juice cells by combining density gradient and aqueous two phase partitioning. HortScience 39:174-176.

Etxeberria E., P. C. Gonzalez, and J. Pozueta-Romero. 2005b. Sucrose transport into the vacuole of *Citrus* juice cells: Evidence for an endocytic transport system. J. Am. Soc. Hort. Sci. 130:269-274

Emans, N., S. Zimmermann, and G. R. Fischer. 2002. Uptake of a fluorescent marker in plant cells is sensitive to brefeldin A and wortmannin. Plant Cell 14:71-86.

Fernie, A. R, J. W. Riesmeier, J. W. Martini, S. Ramalingan, L. Willmitzer, and R. N. Trethewey. 2000. Consequences of the expression of a bacterial glucokinase in potato tubers, both in combination with and independently of a yeast derived invertases. Australian J. Plant Physiol. 27:827-833.

Hew, W. S. R., A. J. Robertson, R. Ross, and D. Hopwood. 1999. The study of the process of fluid-phase endocytosis in cervical squamous cells using fluorescent microspheres. Cytopathology 10:375-382.

Hajirezaei, M. R., Y. Takahata, R. N. Trethewey, L. Willmitzer and U. Sonnewald. 2000. Impact of elevated cytosolic and apoplastic invertases activity on carbon metabolism during potato tuber development. J. Exp. Bot. 51:439-445.

Horn, M. A., P. F. Heinstein, and P. S. Low. 1992. Characterization of parameters influencing receptor-mediated endocytosis in cultured soybean cells. Plant Physiol. 98:673-769.

Haug, A. and B. Shi. 1991. Biochemical basis of aluminum tolerance in plant cells. Dev. Plant Soil Sci. 45:839-845.

Koch, K. E. and W. T. Avigne. 1990. Postphloem, non-vascular transfer in citrus: Kinetics, metabolism, and sugar gradients. Plant Physiol. 93:1405-1416.

MacRobbie, E. A. C. 1999. Vesicle trafficking: a role in trans-tonoplast ion movements? J. Exp. Bot. 50:925-934.

Murata, T. 1977. Studies on the postharvest physiology and storage of citrus fruit. J. Japanese Soc. Hort. Sci. 46:283-287.

Thiel, G., M. Kreft, and R. Zorec. 1998. Unitary exocytotic and endocytotic events in *Zea mays* L. coleoptile protoplasts. Plant J. 13:117-120.

Van Handel, E. 1968. Direct microdetermination of sucrose. Anal. Biochem. 22:280-283.