Metabolic Contributors to Drought-enhanced Accumulation of Sugars and Acids in Oranges

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ABSTRACT. The nature of sink strength in orange fruit and changes occurring during drought stress were investigated. Potted trees of 'Hamlin' orange [*Citrus sinensis* (L.) Osbeck] grafted on Troyer citrange [*Citrus sinensis* × *Poncirus trifoliata* (L.) Raf.] were irrigated using a microsprinkler system creating either well-watered or water-stressed conditions, as determined by stem water potentials. Fruit were harvested every other week from trees of both well-watered and drought-stressed treatments during the final stage of fruit development when sugars accumulate rapidly. Fruit quality indices and activities of sucrose synthase (SuSy), invertase, sucrose-P synthase, sucrose-P phosphatase, V-ATPase, and V-PPase were measured. Acids and soluble sugar concentrations were elevated in drought-stressed fruit, whereas juice pH decreased in those same fruit. Results indicate that increased sink strength in fruit from stressed trees was accompanied by an increase in SuSy activity and lowered juice pH. The remaining enzymes examined in this experiment showed no changes in activity between control and treated fruit, as was the case for plasmalemma and tonoplast sucrose carriers. Based on the present data, we conclude that SuSy and vacuolar pH are the predominant factors controlling photoassimilate accumulation in orange fruit under enhanced sink conditions brought about by imposition of a mild drought stress.

Movement of assimilated carbon into a particular plant organ is determined by its sink strength, and by the capacity of the plant for photoassimilate production. Sink strength refers to the ability of a particular organ to attract photoassimilates (Ho, 1988). Most plant organs are sinks at some time during development, especially during tissue elongation and expansion, when fixed carbon provides the growing tissue with energy for metabolism and solutes to regulate osmotic potential. In addition, many sinks accumulate carbohydrates, as in the case of storage tissues. Fixed carbon is transported through the phloem mostly in the form of the disaccharide sucrose. For adequate sink strength, cells must cleave sucrose (Ho et al., 1991; Sung et al., 1989) or effectively sequester it from the symplast and into the vacuole (Bennett et al., 1986). Therefore, sink strength is determined by the ability of a sink to metabolize or to store sucrose.

Metabolic conversion and sequestration of sucrose in plant cells can be accomplished through a variety of enzymatic and cellular mechanisms. Two enzymes (invertase, EC 3.2.1.26 and sucrose synthase, SuSy, EC 2.4.1.13) can catalyze the conversion of sucrose to hexoses or to other metabolic intermediates. Invertase catalyzes the irreversible hydrolysis of sucrose to glucose and fructose, whereas SuSy catalyzes the reversible interconversion of sucrose and uridine-5'-diphosphate (UDP) to uridine-5'-diphosphoglucose (UDPG) and fructose. Cleaving sucrose allows the sink to amplify the existing gradient of sugar between the sink and phloem, allowing for continuation of sucrose movement toward the sink cell. In some tissues, sucrose-synthesizing enzymes may also play a part in a cycle of sucrose breakdown and resynthesis that controls the cytosolic sucrose concentration (Wendler et al., 1990). Therefore, related sucrose-synthesizing enzymes that may be involved in sink strength include sucrose-phosphate synthase (SPS, EC 2.4.1.14) and sucrose-phosphate

phosphatase (SPP, EC 3.1.3.24), although their exact functions in a sink organ are uncertain (Wendler et al., 1990). Membrane-bound sucrose carriers at the tonoplast and the plasmalemma may also be important in sink strength, though often their roles are overlooked in sink studies.

An additional mechanism exists for sucrose cleavage in highly acidic fruit. For example, the vacuoles of most citrus (*Citrus* L. sp.) juice cells are highly acidic, with pHs of 3 or lower (Echeverria and Burns, 1989; Sinclair, 1984). Such a low pH permits nonenzymatic acid hydrolysis of sucrose into glucose and fructose in vitro (Wienen and Shallenberger, 1988) and in vivo (Echeverria and Burns, 1989). Hydronium-mediated cleavage is dependent on temperature, pH, sucrose concentration, and water content (Schoebel et al., 1969). In plants, vacuolar pH is determined by the activities of the tonoplast-bound proton-pumping enzymes such as V-ATPase (EC 3.6.1.34) and V-PPase (EC 3.6.1.1). In addition to inducing sucrose hydrolysis, lower vacuolar pH also boosts the efficiency of the sucrose carrier at the tonoplast to sequester sucrose into the vacuole.

During mild drought stress, fruit of several species (including citrus) accumulate higher levels of soluble carbohydrates (Ebel et al., 1993; Yakushiji et al., 1996) without affecting fruit yield. Therefore, controlled drought stress provides an experimental tool to better understand the factors controlling fruit sink strength. Understanding these critical determinants of sink strength could aid in future breeding and molecular work with fruit crops while, more importantly, allowing better understanding of the mechanisms at work in sink strength and stress physiology of plants. The objective of this study was to identify factors involved in increased soluble carbohydrate accumulation in orange (*Citrus sinensis*) fruit in response to drought stress.

Materials and Methods

PLANT MATERIAL. Uniform 3-year-old potted 'Hamlin' orange trees grafted on 'Troyer' citrange (*Citrus sinensis* × *Poncirus trifoliata*) in 26-L pots were purchased in June 1999 from a local nursery. Trees were cultivated outdoors at the University of

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Florida, Citrus Research and Education Center, Lake Alfred. Pots were placed on small boards positioned atop black landscaping cloth to prevent weed growth. Each tree was selected for the presence of 10 to 15 fruit and equivalent canopy size. Pest and disease problems were managed throughout the season as they appeared. A systemic pesticide (AdMire 2F, 1-[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine; Bayer Co., Kansas City, Mo.) was applied to the soil about once a month and the trees were sprayed periodically with oil and soapy water for mite control. The trees were fertilized every 2 weeks with a 20N–8.8P–16.6K water soluble fertilizer (Peters 20–20–20; Spectrum Brands, St. Louis, Mo.). All trees were trimmed and pruned during summer and early fall to develop similar leaf to fruit ratios and fruit sizes before experimentation.

TREATMENTS. As a preliminary trial in late August through early September 1999, five trees were set aside and monitored for drought stress according to specific volumes of water applied each day. Water volumes ranged between 200 and 1000 mL. Stem water potential of these five trees was measured every 2 d using a laboratory assembled Scholander-type pressure chamber. From water potential data and environmental conditions, we found that 1 L·d⁻¹ constituted well-watered plants under all conditions, while 500 mL or less imposed drought stress. Irrigation to drought-stressed trees was modified according to environmental fluctuations such as humid or rainy weather.

Fifty trees were randomly assigned to two groups: control (1 L·d⁻¹) and drought-stress (≈500 mL·d⁻¹) treatments. A microsprinkler system was designed and output was monitored frequently to ensure appropriate water distribution to each tree in both treatments. On occasions, when rainfall was forecasted, plastic bags were placed under the tree pots, then slid up and tied around the trunks to prevent moisture from entering the pots of stressed trees. Holes were cut into the bottom of the bags as a preventive measure should leaking into the bag occur while also limiting buildup of condensation. This was adequate to prevent significant alteration of the soil water status of drought-stressed trees.

The experiment began 25 Oct. 1999 and continued until 24 Dec. 1999, with five trees remaining stressed through January 2000. Water status of treatment and control trees was monitored by measurement of stem water potential every 3 to 7 d using the same pressure bomb described previously. Two leaves from each of five trees were selected for similar solar angle of incidence and canopy position, wrapped in black plastic shortly before sunrise, and then covered with a foil wrap to prevent solar heating. Several hours were allowed for equilibration of the leaves with stem xylem potential before measurement in the pressure chamber.

FRUIT ANALYSIS. Fruit were analyzed every other week beginning in mid-October through the end of December 1999, with one sample in mid-January 2000. Ten fruit were used per treatment per sampling point, divided into two replications of five fruit each. Five fruit were randomly selected for a replicate sampling, one from each of five trees, on the morning data were to be taken. Harvest occurred over 4 d, taking fruit from drought stress or control trees on alternate mornings. Fruit for one sampling point were harvested from the same five control trees, then the same five drought-stressed trees, such that each tree contributed two fruit. After sampling, those trees were excluded from the experiment with the consideration that the sink to source ratio might be altered by fruit picking. Collected fruit were taken immediately from the field to the lab and used for fruit quality analyses and enzyme assays.

Each fruit was weighed and cross-sectioned at the equator.

One half of the fruit was juiced at a time on a hand-held juicer, and 15 mL of this juice was added immediately to 30 mL of a cold stirring buffer containing 0.5 M 3-(N-morpholino)propanesulfonic acid (MOPS, pH 8.0), 1.5% w/v polyvinylpyrrolidone (PVP-40), 250 mM sucrose, 7.5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% w/v bovine serum albumin (BSA), and 14 mM mercaptoethanol. An additional 15 mL of juice was placed in a large centrifuge tube. The remaining half of the fruit was juiced and 15 mL of juice added to 45 mL of stirring, buffered extract. The remaining juice was treated as with the other half, added to the same test tube. The buffer/juice mixture from each fruit (60 mL total) was then filtered through a cheesecloth/nylon mesh into a cold beaker on ice. This procedure was repeated for all five fruit, mixing the buffer/juice solutions together for a total of 300 mL and stirring well after addition of 1 mL of phenylmethylsulfonyl fluoride (PMSF) solution (100 mM PMSF in 95% ethanol, and 5% 2-propanol). The buffered juice extract (pH ≈ 7.2) was used subsequently for enzyme assays and isolation of cell compartment membranes as described below.

Enzyme assays were conducted using subsamples of buffered extract remaining from the membrane isolation procedure (see below). Buffered juice was centrifuged at 77,000 *g*_n for 30 min at 4 °C. After centrifugation, 2.5 mL of the supernatant solution were desalted through a Sephadex PD-10 column preequilibrated with a buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.0), 2 mM dithiothreitol (DTT), and 1 mM MgCl₂. Three milliliters were collected for enzyme assays.

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). Total soluble carbohydrate concentration was measured using an ABBE refractometer (American Optical Corp., Buffalo, N.Y.) at 20 °C after compensation for organic acids. In citrus fruit, ≈90% of the soluble solids is the sum of sugars and organic acids.

MEMBRANE ISOLATION. Isolation and purification of ‘Hamlin’ vesicle membranes was conducted using the protocol described by Echeverria et al. (1997), with a few modifications. The cold juice/buffer solution was adjusted to pH 7.0 with 3.0 N KOH and 250 mL was immediately centrifuged at 113,000 *g*_n for 1.75 h at 4 °C. The pellet containing the microsomal fraction was resuspended in buffer containing 50 mM HEPES (pH 7.6), 100 mM KCl, 150 mM sucrose and 2 mM DTT, and centrifuged at 134,000 *g*_n for 1 h. The resulting pellet was washed and resuspended again into 3 mL of this same buffer. The microsomal fraction was loaded on a sucrose gradient (8%, 17%, 26%, and 38% sucrose) and centrifuged for 45 min at 110,000 *g*_n. Membrane fractions were removed from each sucrose interface into separate tubes and diluted with a buffer solution of 10 mM HEPES (pH 7.6), 20 mM KCl, and 2 mM DTT. These vesicle samples were centrifuged at 113,000 *g*_n for 1 h, resuspended into a storage buffer of 10 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP)/2-(4-morpholino)ethanesulfonic acid (MES, pH 7.5), 250 mM sorbitol, 2 mM DTT, and stored at –80 °C. Protein concentration in vesicles and juice extract was determined using the Coomassie Blue method described by Bradford (1976) and BSA as standard.

SUCROSE SYNTHASE. Sucrose synthase was assayed in the synthetic direction in a solution containing 100 mM HEPES (pH 7.2), 2 mM UDPG, 10 mM fructose, 2 mM MgCl₂, and 100 μL protein extract in a final volume of 500 μL. Aliquots of 100 μL

were taken at 0, 20, 40, and 60 min, mixed with 100 μL of 30% KOH and boiled for 10 min. Sucrose produced was determined following the anthrone method of van Handel (1968). Rates of sucrose breakdown were calculated using the ratio of synthetic/breakdown activity estimated previously for acid lime SuSy of 8:1 (Echeverria, 1992) and corroborated for other *Citrus* cultivars (unpublished data).

SUCROSE PHOSPHATE SYNTHASE. The reaction mixture contained 100 mM HEPES (pH 7.5), 7.5 mM UTPG, 7.5 mM fructose-6-phosphate (F-6-P), 37.5 mM glucose-6-phosphate (G-6-P), 18 mM MgCl_2 , 1.0 mM EDTA, and 100 μL enzyme extract in a final volume of 500 μL . SPS reaction was terminated and analyzed for sucrose and sucrose-6-P (S-6-P) as for sucrose synthase.

SUCROSE PHOSPHATE PHOSPHATASE. Activity of SPP was assayed in a solution containing 100 mM MES (pH 6.5), 10 mM MgCl_2 , 2 mM S-6-P, and 100 μL enzyme extract in a total volume of 500 μL . Aliquots of 50 μL were taken at specific times and mixed with 250 μL of 7.2% SDS to stop the reaction. Released P_i was determined at 850 nm by the method of Chifflett et al. (1988).

INVERTASES. Both acid and alkaline invertases were assayed using either BTP/MES (pH 7.5) or sodium acetate (pH 5.0) buffer. In a total reaction volume of 2.5 mL, 0.5 mL enzyme extract, 100 mM buffer, and 100 mM sucrose were combined and incubated. Aliquots of 500 μL were placed into 1 mL of phosphate buffer (100 mM, pH 7.0), and boiled for 2 min. Glucose was determined at 450 nm by the glucose oxidase method of Kilburn and Taylor (1969).

V-ATPase. V-ATPase activity was determined using 75 μL tonoplast vesicles, 50 mM BTP/MES (pH 7.5), 250 mM sorbitol, 50 mM KCl, BSA at 0.4 $\text{mg}\cdot\text{mL}^{-1}$, 2 mM DTT, 10 μM gramicidin, 3 mM ATP, and 3 mM MgSO_4 adjusted to a total volume of 500 μL . Aliquots of 50 μL were removed from the assay at determined times and product determined as for SPP.

V-PPase. V-PPase activity was assayed as for V-ATPase except that ATP and MgSO_4 were replaced with 1 mM NaPP_i and 1 mM MgSO_4 . Assays for P_i were conducted as with ATPase and SPP.

SUCROSE UPTAKE EXPERIMENTS. Assays for sucrose uptake into either tonoplast or plasmalemma vesicles contained the following: 50 μg tonoplast or plasmalemma vesicle, 50 mM BTP/MES at pH 7.5 or 5.5, 250 mM sorbitol, BSA at 0.4 $\text{mg}\cdot\text{mL}^{-1}$, 2 mM DTT,

and 2 mM ^{14}C -sucrose ($5.18 \times 10^4 \text{ Bq}\cdot\mu\text{mol}^{-1}$) as described by Echeverria et al. (1997). Control experiments contained 100 μM KCl and 10 μM nigericin. Assays were run for 30 min with aliquots (100 μL) taken at 0, 10, 20, and 30 min. Aliquots were removed at the indicated times and washed on prerinsed cellulose nitrate filters (pore size 0.22 μm , 25 mm in diameter, Whatman Intl. LTD., Maidstone, United Kingdom). After vacuum (625 mm Hg) was applied, the vesicles were washed with 5 mL storage buffer (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.). Control vesicle samples with nigericin accumulated low amounts of labeled sucrose which corresponded to the values calculated using vesicle void volume (Echeverria et al., 1997).

STATISTICAL ANALYSIS. Leaf and fruit juice data were subjected to analyses of variance procedures. Student's *t* test (paired) was employed for determining statistical significance with enzyme data when differences in means were recorded. All data were calculated during linear stages of catalytic activity. Data points for enzyme activities were done in triplicate for each experimental sample.

Results

INDUCTION OF DROUGHT STRESS. Reduced irrigation induced differences ($P \leq 0.05$) in stem water potentials after 7 d (Fig. 1). Control trees receiving ample water maintained higher water potentials with generally smaller SES. Meanwhile, measurements from drought-stressed trees indicated development of a consistent stress. However, there was greater variability in stem water potentials and increased sensitivity to environmental conditions in stressed trees coinciding with brief periods of high humidity and rainy weather (days 21 and 45, Fig. 1). Drought-stressed trees remained visibly healthy although leaf curling, a drought stress symptom in citrus, occurred frequently during the hottest hours of the day. At the end of the experiment, drought-stressed trees exhibited a healthy appearance after watering as with controls ($1 \text{ L}\cdot\text{d}^{-1}$).

FRUIT QUALITY. Titratable acidity remained higher in fruit from drought-stressed trees although acids consistently decreased (Fig. 2A) due to a natural decline in acid content during ripening. Also, juice pH remained slightly lower in drought-stressed fruit (Fig. 2B). Differences in juice pH occurred primarily within the first 2 weeks of water restriction and were maintained throughout. The maintenance of a pH difference after 2 weeks is noteworthy, and may indicate formation of a new steady-state response as an adaptation to the altered water conditions.

Soluble sugar concentrations in fruit juice from drought-stressed trees were significantly higher ($P \leq 0.05$) than controls within 14 d (Fig. 3). This difference between the treatments remained constant thereafter. At the end of the experiment, fruit from the mildly stressed trees had a soluble solids concentration of 11.3%, which was nearly 0.5% higher than those from well-watered trees. Fruit fresh weight did not vary between the treatments (data not presented), since picking always occurred early in the morning when fruit were turgid. Therefore, changes seen in total soluble sugars reflect active osmotic adjustment.

SINK-RELATED ENZYMES. Invertase activity was very limited from the beginning of the experimental period. The first three samples showed only barely detectable activity diminishing to levels beyond detection (data not presented). SuSy, the other

Fig. 1. Stem water potential (MPa) of control and water-stressed 'Hamlin' orange trees during the experimental period. Water potentials were significantly different after 7 d, excluding day 21 and 45. Vertical bars represent $\pm$ SE ($n = 10$ leaves).

sucrose-catabolizing enzyme, showed statistical increases in activity ($P \leq 0.05$) in fruit from stressed versus well-watered trees after 28 d (Fig. 4). SuSy activity in fruit from stressed trees remained

higher than controls for the remainder of the experiment.

SPS activities were not different between treatments at any sampling date and remained low when compared to SuSy (Table 1). Also, no changes occurred as the season progressed except for a later increase (80 d). Of the remaining enzymes involved in sink strength (SPP) or tonoplast proton pumps (V-ATPase, V-PPase) none showed treatment differences in mean activity (Table 1). SPP activity increased slowly and steadily during fruit ripening, whereas V-ATPase activity declined during the experiment, especially at 80 d.

Analysis of membrane vesicles for the presence of sucrose transporters indicated the existence of two types of secondary active transport systems for sucrose in juice vesicle cells (Table 2). A sucrose symport was present in the plasmalemma of juice cells. A second transport system was found at the tonoplast, where the ΔpH is used to empower an antiport. In both cases, sucrose was accumulated against a concentration gradient after imposing an artificial ΔpH . However, under a similar imposed transmembrane ΔpH , no differences were obtained between membrane samples from control fruit as opposed to drought-stressed fruit. Therefore, in experiments using equivalent ΔpH , no differences existed in the capacity for sucrose transport in either the plasmalemma or the tonoplast transporters.

Discussion

Several factors are determinants of sink strength (Ho et al., 1991; Warren-Wilson, 1972). However, in most cases, determination of sink strength has been limited to the study of a few enzymes related to sucrose metabolism. Other components such as membrane carriers, H^+ -pumping capacity, and vacuolar pH have not been taken into account. Furthermore, the possible complex interaction between some, or all of these components, has been overlooked for a simplistic view of only sucrose-metabolizing enzymes. In the present study, we took advantage of the previously described effects of water deficit in citrus fruit (Yakushiji et al., 1996, 1998) in an effort to determine those factors involved in increased carbohydrate accumulation in citrus fruit.

Fig. 2. (A) Comparison of titratable acids and (B) juice pH in 'Hamlin' fruit juice extracts during maturation, taken from control and water-stressed trees. Titratable acids were significantly different ($P \leq 0.05$) beginning at 28 d. Differences in juice pH were significant ($P \leq 0.05$) at 14 d and again from 42 d through 80 d. Symbols represent mean values of juice from 10 fruit $\pm$ SE.

Fig. 3. Accumulation of soluble solids in fruit from control and water-stressed trees during maturation $\pm$ SE ($n = 10$). Differences in soluble solid concentrations were significant ($P \leq 0.05$) at 28, 56, and 80 d.

Fig. 4. Activity of sucrose synthase, in control and water-stressed fruit juice extracts. Data represent means $\pm$ SE of four assays, and are significant ($P \leq 0.05$) from 28 d.

Table 1. Activities of the sucrose-metabolizing enzymes [sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP)] and tonoplast proton pumps (V-ATPase and V-PPase) throughout the experiment in well-watered control fruit. Values represent means $\pm$ SE of four experiments and do not include water-stress measurements which were not significantly different ($P \leq 0.05$).

Enzyme	Sampling time (d)					
	0	14	28	42	56	80
SPS ^z	5.1 $\pm$ 0.4	3.2 $\pm$ 0.4	4.5 $\pm$ 0.6	3.2 $\pm$ 0.4	3.7 $\pm$ 0.2	8.1 $\pm$ 0.5
SPP ^y	34.3 $\pm$ 4.4	38 $\pm$ 3.1	36 $\pm$ 4.3	40 $\pm$ 7.6	45.5 $\pm$ 2.1	66.5 $\pm$ 6.3
ATPase ^x	252 $\pm$ 10	225 $\pm$ 20	232 $\pm$ 8	217 $\pm$ 13	219 $\pm$ 5	191 $\pm$ 3
PPase ^x	117 $\pm$ 11	121 $\pm$ 22	114 $\pm$ 6	122 $\pm$ 5	131 $\pm$ 10	128 $\pm$ 15

^zSucrose synthesized, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ fresh weight.

^ySucrose-6-P hydrolyzed, $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ fresh weight.

^xPi hydrolyzed, $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ fresh weight.

Our results confirm that imposition of a mild drought stress by reduced irrigation leads to differences in soluble carbohydrate accumulation in orange fruit. Therefore, the combined data of organic acids and soluble sugar concentrations manifest an increase in total carbon import by fruit under a restricted water regimen. Yakushiji et al. (1996, 1998) reported similar differences in soluble carbohydrate accumulation during drought stresses imposed on 'Satsuma' mandarin trees. The observed increase in sugars reported herein is also in agreement with other deficit irrigation studies in a variety of fruit trees (Goldhamer et al., 1994; Miller et al., 1998; Mills et al., 1996; Yakushiji et al., 1996, 1998), although juice pH was not considered in those studies. Deficit irrigation significantly increased fruit soluble solids without jeopardizing fruit size when drought-stress conditions were imposed towards the end of fruit growth and expansion (stage III of fruit development) (Caspari et al., 1994; Mills et al., 1996). Consequently, active osmotic adjustment may occur during this final stage of fruit development, as observed herein.

Of the enzymes and factors considered as possible indicators of sink strength, only SuSy and lower vacuolar pH were significantly different between fruit from control and treatment trees (Figs. 2B and 4). None of the remaining enzymes or maximal activities of membrane sucrose carriers were significantly affected by the water deficit. The very low activity of acid invertase observed is supported by previous research in citrus fruit, showing its decline at the end of stage II of fruit development (Kato and Kubota, 1978; Lowell et al., 1989), the point at which our research began. Therefore, enzymatic cleavage of sucrose by invertase is an unlikely determinant factor involved in increased carbon import at this stage in fruit development. SPS activity varied slightly during the experiment but followed no particular pattern and showed no treatment differences. SPP, on the contrary, exhibited increasing activity during the experimental period. Consequently, invertase, SPS, and SPP appear to have little effect on changes in the active osmotic adjustment seen during mild drought stress.

Differences in SuSy activity between treatments were seen shortly after imposition of the mild drought stress. Since all trees were provided with the same quantity of water before the start of the experiment, it appears that SuSy may function in osmotic adjustment. SuSy activity declined slightly in well-watered trees, possibly in response to the increased irrigation those trees were receiving (Fig. 4). On the contrary, increasing drought stress on the treated trees significantly increased SuSy activity in those fruit. Similar stress-related/regulated responses in SuSy have been recorded previously by Balibrea et al. (1996), Kleines et al. (1999), and Pelah et al. (1997) in a variety of vegetative and reproductive tissues.

The combined data presented in this communication indicate the presence of a distinctive mechanism responsible for increased carbon import into citrus fruit during drought stress. Such a mechanism may be controlled by multiple alterations to a well-coordinated set of metabolic processes and not to changes in a single factor as is commonly suggested. The initial response to an imposed drought stress was a decline in vacuolar pH (Fig. 2B). The decline in vacuolar pH in juice cells from water-deficit trees could have a large impact on two major factors involved in carbohydrate import.

First, a decline in vacuolar pH would increase the rate of acid-mediated sucrose hydrolysis in the vacuole (Echeverria and Burns, 1989). Acid hydrolysis of sucrose occurs at the low vacuolar pH measured in citrus juice cells which reach levels of ≈ 2.0 to 3.2 (Echeverria and Burns, 1989). In the absence of acid invertases, acid hydrolysis constitutes the only known mechanism for vacuolar sucrose breakdown in juice cells. A decline in juice pH of 0.1 unit (Fig. 2B) constitutes a 25% increase in the vacuolar H^+ concentration. Such a decline in pH alone would result in an increased rate of sucrose breakdown of $\approx 10\%$, using equations and published data from Bates (1942), Echeverria (1991), Echeverria and Burns (1989, 1990), Schoebel et al. (1969), and Wienen and Shallenberger (1988). The actual rate of cleavage, however, is likely to be higher, as the vacuolar pH would be lower *in vivo* than the measured juice pH, perhaps up to 0.5 units lower as demonstrated by Echeverria and Burns (1989). Although we did not characterize sugar composition in the present investigation, sugar analysis data from similar experiments conducted by Yakushiji et al. (1996, 1998) and Graham Barry (Citrus Research and Education Center, personal communication) indicated a near 1:1 ratio increase in glucose and fructose concentrations under similar circumstances, data which support sucrose acid hydrolysis.

Second, the decline in vacuolar H^+ concentration in juice cell vacuoles from stressed trees generates a larger trans-tonoplast pH (ΔpH^+). Increased electrochemical potential gradient improves

Table 2. Maximal activities of sucrose transporters (Sucrose, $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) in 'Hamlin' orange juice vesicles. Values represent the means of three assays $\pm$ SE, after subtracting basal uptake with 10 μM nigericin and 100 μM KCl from total sucrose uptake. Vesicles were energized by imposition of an artificial pH gradient and uptake was carried out for 40 min.

Membrane	0 d	28 d	
		Control	Drought-stressed
Plasmalemma	3.4 $\pm$ 0.6	2.0 $\pm$ 0.8	1.7 $\pm$ 0.6
Tonoplast	4.2 $\pm$ 1.1	3.0 $\pm$ 0.7	2.8 $\pm$ 0.8

is supported by our research, confirming its critical role in sugar metabolism. However, although SuSy is an indicator of sink strength, we conclude that other factors are part of a combined metabolic response to drought stress that leads to increased sink strength.

Literature Cited

- Balibrea, M.E., A.M. Santa Cruz, M.C. Bolarin, and F. Perez-Alfocea. 1996. Sucrolytic activities in relation to sink strength and carbohydrate composition in tomato fruit growing under salinity. *Plant Sci.* 118:47–55.
- Bates, F.J. 1942. Polarimetry, saccharimetry, and the sugars. U.S. Dept. Commerce Govt. Printing Office, Wash., D.C.
- Bennett, A.B., S. Damon, K. Osteryoung, and J. Hewitt. 1986. Mechanisms of retrieval and metabolism following phloem unloading, p. 307–316. In: J. Cronshaw, W.J. Lucas, and R.T. Giaquinta (eds.). *Phloem transport*. Alan R. Liss, New York.

Fig. 5. Proposed model for the process of elevated sink strength in orange juice cells based on the data presented herein. The observed rapid lowering in vacuolar pH brings about an increase in pH mediated sucrose hydrolysis and carrier mediated sucrose transport into the vacuole. Energy to support H⁺ pumping into the vacuole is supplied by increased SuSy (and subsequent ATP generating reactions). Catabolism and compartmentation of sucrose increase soluble solids concentration and widens the sucrose concentration gradient between the juice cell and the transport elements (+ denotes increase in activity or concentration; – denotes decrease in pH).

the catalytic efficiency and transport activity of membrane sucrose carriers (Lalonde et al., 1999). Lack of differences in sucrose uptake by tonoplast vesicles obtained from fruit of control and stressed trees may be due to the fact that uptake was assayed under equal transmembrane Δ pH. Experimental constraints did not allow us to lower the pH of vesicle lumen and explore sucrose uptake under lower vesicular pH (Marsh et al., 2000).

A lower vacuolar pH implies higher rates of H⁺ pumping across the tonoplast which, in the case of oranges, depends exclusively on the V-ATPase (Marsh et al., 2000). Additional ATP necessary for H⁺ pumping into the vacuole (for the rapid decrease in vacuolar pH) could be generated by increased SuSy activity (Fig. 4) and the increased availability of glycolytic intermediates. Changes in SuSy activity also have a direct effect on fruit sink strength by cleaving incoming sucrose from the phloem and maintaining a steeper gradient of sucrose between the cytosol and the translocation stream.

From our results (summarized in Fig. 5), we conclude that a rapid decrease in pH after imposition of drought stress triggers a cascade of events that leads to increases in the rate of total carbon import, as demonstrated previously by (Yakushiji et al., 1996, 1998). The initial drop in vacuolar pH (Fig. 2B) increases sucrose hydrolysis (producing hexoses) while establishing more favorable conditions for increased sucrose transport into the vacuole by the sucrose/H⁺ antiport. Increased rates of sucrose movement into the vacuole in turn could generate a simultaneous increase in sink strength by increasing the sucrose gradient between the juice cells and the phloem-unloading zone. Therefore, it appears that both pH and SuSy are involved, both directly and indirectly, in increasing total carbon import into fruit.

In conclusion, a distinct mechanism for increased sucrose import and osmotic adjustment is present in citrus that has not been reported elsewhere. The importance of SuSy in sink strength

- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Caspari, H.W., M.H. Behboudian, and D.J. Chalmers. 1994. Water use, growth, and fruit yield of 'Hosui' Asian pears under deficit irrigation. *J. Amer. Soc. Hort. Sci.* 119:383–388.
- 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 ATPase. *Anal. Biochem.* 168:1–4.
- Ebel, R.C., E.L. Proebsting, and M.E. Patterson. 1993. Regulated deficit irrigation may alter apple maturity, quality, and storage life. *Hort-Science* 28:141–143.
- Echeverria, E. 1991. Regulation of acid hydrolysis of sucrose in acid lime juice sac cells. *Physiol. Plant.* 81:51–54.
- Echeverria, E. 1992. Activities of sucrose metabolizing enzymes during sucrose accumulation in developing acid limes. *Plant Sci.* 85:125–129.
- 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. K. Burns. 1990. Sucrose breakdown in relation to fruit growth of acid lime (*Citrus aurantifolia*). *J. Expt. Bot.* 41:705–708.
- 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.
- Goldhamer, D.A., G.S. Sibbett, R.C. Phene, and D.G. Katayama. 1994. Early irrigation cutoff has little effect on French prune production. *Calif. Agr.* 48:13–17.
- Ho, L.C. 1988. Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:355–378.
- Ho, L.C., A. Lechamy, and J. Willenbrink. 1991. Sucrose cleavage in relation to import and metabolism of sugars in sink organs, p. 178–186. In: J.L. Bonnemain, S. Delrot, W. Lucas, and J. Dainty (eds.). *Recent advances in phloem transport and assimilate compartmentation*. Quest Editions: Presses Academiques, Nantes, France.
- Kato, T. and S. Kubota. 1978. Properties of invertases in sugar storage tissues of citrus fruit and changes in their activities during maturation. *Physiol. Plant.* 42:67–72.
- Kilburn, D.M. and E.M. Taylor. 1969. Effect of sulfhydryl reagents on glucose determination by the glucose oxidase method. *Anal. Biochem.* 27:555–558.
- Kleines, M., R.C. Elster, M.J. Rodrigo, A.S. Blervacq, F. Salamini, and D. Bartels. 1999. Isolation and expression analysis of two stress-

- responsive sucrose-synthase genes from the resurrection plant *Craterostigma plantagineum* (Hochst.). *Planta* 209:13–24.
- Lalonde, S., E. Boles, H. Hellmann, L. Barker, J.W. Patrick, W.B. Frommer, and J.M. Ward. 1999. The dual function of sugar carriers: Transport and sugar sensing. *Plant Cell* 11:707–726.
- Lowell, C.A., P.T. Tomlinson, and K.E. Koch. 1989. Sucrose-metabolizing enzymes in transport tissues and adjacent sink structures in developing citrus fruit. *Plant Physiol.* 90:1394–1402.
- Marsh, K., P. Gonzalez, and E. Echeverria. 2000. PPi formation by reversal of the tonoplast-bound H⁺-pyrophosphatase from 'Valencia' orange juice cells. *J. Amer. Soc. Hort. Sci.* 125:420–424.
- Miller, S.A., G.S. Smith, H.L. Boldingh, and A. Johansson. 1998. Effects of water stress on fruit quality attributes of kiwifruit. *Ann. Bot.* 81:73–81.
- Mills, T.M., M.H. Behboudian, and B.E. Clothier. 1996. Water relations, growth, and the composition of 'Braeburn' apple fruit under deficit irrigation. *J. Amer. Soc. Hort. Sci.* 12:286–291.
- Pelah D., W. Wang, A. Altman, O. Shoseyov, and D. Bartels. 1997. Differential accumulation of water stress-related proteins, sucrose synthase and soluble sugars in *Populus* species that differ in their water stress response. *Physiol. Plant.* 99:153–159.
- Schoebel, T., S.R. Tannenbaum, and T.P. Labuza. 1969. Reaction at limited water concentration. I. Sucrose hydrolysis. *J. Food Sci.* 34:324–329.
- Sinclair, W.B. 1984. The lemon. Univ. Calif. Press, Oakland.
- Sung, S.S., D. Xy, and C.C. Black. 1989. Identification of actively filling sucrose sinks. *Plant Physiol.* 89:1117–1121.
- van Handel, E. 1968. Direct microdetermination of sucrose. *Anal. Biochem.* 22:280–283.
- Warren-Wilson, J. 1972. Control of crop processes, p. 7–30. In: A.R. Rees, K.E. Cockshull, D.W. Hand, and R.G. Hurd. (eds.). Crop processes in controlled environments. Academic Press, London.
- Wendler, R., R. Veith, J. Dancer, M. Stitt, and E. Komor. 1990. Sucrose storage in cell suspension cultures of *Saccharum* sp. (sugarcane) is regulated by a cycle of synthesis and degradation. *Planta* 183:31–39.
- Wienen, W.J. and R.S. Shallenberger. 1988. Influence of acid and temperature on the rate of inversion of sucrose. *Food Chem.* 29:51–55.
- Yakushiji, H., K. Morinaga, and H. Nonami. 1998. Sugar accumulation and partitioning in Satsuma mandarin tree tissues and fruit in response to drought stress. *J. Amer. Soc. Hort. Sci.* 123:719–726.
- Yakushiji, H., H. Nonami, T. Fukuyama, S. Ono, N. Takagi, and Y. Hashimoto. 1996. Sugar accumulation enhanced by osmoregulation in Satsuma mandarin fruit. *J. Amer. Soc. Hort. Sci.* 121:466–472.