Developmental Transition Between Sucrose Uptake and Sucrose Efflux Systems in the Vacuole of Red Beet Hypocotyl Cells

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Additional index words. *Beta vulgaris*, sucrose mobilization, vacuole, beet development

Abstract. The mechanisms of sucrose uptake into the vacuole and sucrose efflux from the vacuole were studied using tonoplast vesicles from red beet at two distinct developmental stages. Vesicles from both developing and mobilizing hypocotyls (sucrose uptake and efflux, respectively) accumulated sucrose against a concentration gradient. However, higher rates and maximal levels of sucrose accumulation were obtained with tonoplast from developing hypocotyls. ATP-dependent sucrose efflux was more pronounced in vesicles from mobilizing hypocotyls. Despite the apparent overlapping, the data indicate that the physiological mechanisms for sucrose uptake and sucrose efflux are separated in time and governed by the developmental state of the cell. Chemical name used: adenosine 5'-triphosphate (ATP).

In a recent report (Echeverria and Gonzalez, 2000), we established the presence of an ATP-dependent mechanism for sucrose efflux from the vacuole of mobilizing hypocotyl cells from sprouting red beet (*Beta vulgaris* L.) plants. The system is directly energized by ATP, and transports sucrose from the vacuole to the cytosol in response to cellular metabolic demands. The existence of an ATP-dependent sucrose efflux mechanism in storage vacuoles where sucrose uptake is indirectly energized by ATP (Briskin et al., 1985; Getz, 1991; Keller, 1992) appears contradictory, and could potentially lead to a futile cycle of sucrose uptake and efflux.

In an analogous system, both synthesis and breakdown of fructans in storage cells of Jerusalem artichoke (*Helianthus tuberosus* L.) tubers occur in the vacuole (Darwen and John, 1989; Frehner et al., 1984). In this case, a developmental separation between enzymes of synthesis and degradation was observed. Frehner et al. (1984) demonstrated that fructan-fructan fructosyl transferase [FFT, Enzyme Commission (EC) 2.4.1.100; enzyme catalyzing fructan polymerization] was never present in the vacuole concurrently with fructan exohydrolase (FEH, EC 3.2.1.80; enzyme of fructan depolymerization). Therefore, although both synthesis and degradation of fructans take place within the vacuole, an energy consuming futile cycle is prevented by ontogenetic separation of the two processes.

During our previous study (Echeverria and Gonzalez, 2000), we noted that the capacity for sucrose efflux from tonoplast vesicles varied relative to the time red beet hypocotyls were allowed to mobilize sucrose under natural conditions. This observation was acknowledged as

an indication that the system for sucrose efflux may develop in response to continuous demands for sucrose and possibly is not a permanent characteristic of the tonoplast. In the present study, we analyzed the capacity for sucrose uptake and efflux in the vacuole of red beet hypocotyl cells at two very distinctive stages, i.e., sucrose accumulation and sucrose efflux.

Materials and Methods

Red beet plants were grown from seeds in 20-L pots during Spring 1998. The plants were kept in a greenhouse under natural light and temperature, and watered when necessary. During stages of rapid sucrose accumulation (accumulating hypocotyl), several plants were taken for tonoplast isolation. The remaining plants were allowed to grow until the end of the year. At this point, all plants were removed from the ground, leaves cut off, and hypocotyls placed in a refrigerator at 4 °C for 2 months to simulate dormancy. Prior to replanting, stored hypocotyls were surface sterilized with 0.5% sodium hypochlorite for 5 min and then placed in individual pots back in the greenhouse. During sprouting (mobilizing hypocotyl), leaves began to develop rapidly, but were excised after final size was reached. In this way, development of leaves was dependent exclusively on mobilized sucrose.

Tonoplast vesicles were isolated as previously reported (Echeverria and Gonzalez, 2000), a method modified from Bennett et al. (1984). Peeled hypocotyl (100 g) was chopped into smaller pieces and homogenized in a Waring blender with 180 mL of buffer containing 250 mM sucrose, 70 mM Tris-HCl buffer (pH 8.0), 4 mM DTT, 3 mM Na₂EDTA, 0.5% PVP-40, and 0.1% BSA. The homogenate was filtered through four layers of cheesecloth and centrifuged for 25 min at 10,000 g.

The supernatant was collected and centrifuged for 45 min at 80,000 g. After resuspension of the microsomal pellet in a buffer containing 250 mM sucrose, 10 mM Tris/MES (pH 7.0), 2 mM DTT, and 150 mM KCl, the resulting suspension was re-centrifuged at 80,000 g for 45 min. A buffer containing 250 mM sucrose, 10 mM Tris/MES (pH 7.0), and 2 mM DTT was used for a final resuspension of the microsomal fraction. This was layered on a discontinuous sucrose gradient of 16%, 26%, 34%, and 40% sucrose in 10 mM Tris/MES and 2 mM DTT at pH 7.0. The tonoplast fraction was collected from the 16% to 26% interface after centrifugation of the gradient for 2 h at 80,000 g.

Fractions containing purified tonoplast vesicles were diluted with an equal volume of 10 mM Tris/MES pH 7.0 and 2 mM DTT before centrifugation at 80,000 g for 40 min. The final pellet was resuspended in 1 mL of storage buffer containing 250 mM sorbitol, 10 mM MES/KOH, and 2 mM DTT at pH 5.5. Tonoplast vesicles were stored at -80 °C until needed. Procedures were conducted at 4 °C.

Loading of sucrose into purified tonoplast vesicles was performed using stock solutions of 20 mM [¹⁴C]-sucrose at a radiological concentration of 51.8 × 10³ Bq·μmol⁻¹. Prior to uptake studies under artificial pH gradient, the tonoplast vesicles were equilibrated in storage buffer at pH 5.5 by applying two freeze/thaw cycles. Tonoplast vesicles (50 μg) were incubated at 30 °C in a medium containing 250 mM sorbitol, 50 mM buffer HEPES (pH 7.5), 2 mM DTT, and 1 mg·mL⁻¹ BSA. Gramicidin was added at 10 μM when required. Uptake was started by the addition of radiolabeled sucrose at 2 mM final concentration and stopped at appropriate times by pipetting 100 μL aliquots into pre-rinsed cellulose nitrate membrane filters (Whatman, Maidstone, England; pore size 0.22 μm, 25 mm diameter). After applying vacuum (at 625 mm Hg) to wash the excess incubation media, the vesicles were rinsed with 5 mL of cold storage buffer at pH 7.5.

Radioactivity retained by the vesicles was determined by liquid scintillation spectroscopy after immersing the filter discs in 5 mL of Scintiverse BD SX 18-4 (Fisher Scientific, Pittsburgh). Results are given as the mean value of at least three independent experiments from separate preparations performed in duplicate.

ATP-dependent sucrose efflux experiments were conducted as described by Echeverria and Gonzalez (2000). Efflux from sucrose loaded vesicles (at 30 min) was initiated by the addition of ATP/Mg at 2 mM (ATP/Mg is the true substrate for the ATP-dependent sucrose transporter and formed by adding ATP-Na and MgSO₄ at equal concentrations). Aliquots of the solution were sampled at different times, filtered as described above, and the remaining sucrose determined by scintillation spectroscopy.

Results and Discussion

Tonoplast vesicles isolated from red beet hypocotyls at two distinct developmental stages accumulated sucrose against a concentration gradient, albeit at different rates (Fig. 1). Higher

Table 1. Comparison of sucrose uptake and efflux capabilities in tonoplast vesicles isolated from red beet hypocotyl during phases of sucrose accumulation (maximum photosynthesis) and during mobilization (i.e., sprouting). In addition, the activities of bafilomycin-insensitive sucrose-mobilizing ATPase are in tonoplast preparations from both stages of development are presented. Sucrose efflux from loaded vesicles was induced by 2 mM ATP/Mg. Experiments were conducted in duplicate and values given as the average. Duplicate experiments did not vary over 12%.

Tonoplast from	Sucrose uptake (nmol-mg-prot ⁻¹)	Sucrose efflux (nmol-mg-prot ⁻¹)	% Total efflux (nmol-mg-prot ⁻¹)	Total ATPase (nmol-min ⁻¹ -mg-prot ⁻¹)	% ATPase activity in presence of 10 μ M bafilomycin
Accumulating hypocotyls	32.4	9.72	24	363.1	3
Mobilizing hypocotyls	13.9	10.28	74	229.7	34

rates of sucrose uptake were obtained by tonoplast vesicles isolated from hypocotyl cells at stages of rapid sucrose accumulation. Not only the initial rates of accumulation were higher, but the final steady state was also significantly greater. When control values (uptake by diffusion in the presence of 10 μ M gramicidin) were taken into account, final sucrose accumulation (9 mM) for tonoplast from accumulating hypocotyls was twice as high as those from mobilizing hypocotyls (4 mM; Fig. 1). The above results indicate that the tonoplast from sucrose accumulating tissue possesses a higher capacity for sucrose uptake than tonoplast from mobilizing tissue.

Upon addition of ATP/Mg to sucrose loaded vesicles, total sucrose efflux appeared only slightly higher in tonoplast vesicles from mobilizing hypocotyl cells (Table 1). However, total amount of accumulated sucrose was different in the vesicle populations from accumulating and mobilizing cells and, therefore, not comparable by absolute values. When compared based on percentage of the accumulated sucrose, efflux was about three times higher in tonoplast vesicles from mobilizing cells than those from accumulating cells (Table 1), demonstrating that the capacity for ATP-dependent sucrose efflux increased significantly during mobilization. Taken together, the above data demonstrated a developmental shift in sucrose uptake and efflux systems as the storage tissue transforms itself from a sink to a source of sucrose.

Although efflux was more pronounced in mobilizing cells and uptake in accumulating cells, both systems appeared to occur in vesicles from all beets. Furthermore, efflux of sucrose in the presence of ATP/Mg never reached control levels, even in tonoplast vesicles from beets allowed to mobilize for over 2 months (Table 1). Three reports should be given special consideration at this point. First, Ross and Davies (1985) noted that in sprouting potato (*Solanum tuberosum* L.) tubers, mobilization of reserves (starch in this particular case) initially occurred in parenchyma cells located around existing phloem regions. Their observations show that not all cells within the sprouting tuber are simultaneously participating in the efflux process, and therefore, efflux systems may not be evenly developed throughout all cells in the storage organ. Second, in the protein storage vacuoles of barley (*Hordeum vulgare* L.) aleu-

rone cells, carriers and enzymes necessary for protein breakdown and export are also present during development (Bethke et al., 1998). These polypeptides are synthesized in the developing grain and, in vivo, activated during imbibition. Third, mobilizing cells from red beet hypocotyl are morphologically different from dormant cells (Echeverria, 2000). Under light microscopy, cells from sucrose accumulating and dormant tissue showed a smooth and clear layer of cytoplasm around the large central vacuole. In contrast, a portion of the cells from mobilizing hypocotyls contain a much larger cytosol : vacuole ratio and are overwhelmed with an array of diminutive vacuoles believed to be involved in sucrose export to the apoplast (Echeverria, 2000). In considering the above observations, it is apparent that various populations of cells at different developmental and metabolic stages exist within the hypocotyl. For example, during sink stages, some cells participate in the uptake process, whereas others are involved in efflux (phloem and phloem parenchyma). The direction of carbon flow reverses during sprouting. Consequently, experiments conducted with vesicles extracted from the entire organ will always contain population of vesicles at different developmental states and of different anatomical origin, resulting in apparent overlapping of the systems (Leigh, 1997). This conclusion is supported by experiments (see Table 1) where the ATP-dependent sucrose efflux system (represented by the bafilomycin-insensitive vacuolar ATPase; Echeverria and Gonzalez, 2000) is shown to rapidly increase in mobilizing beet hypocotyl cells, but to be virtually absent from sucrose accumulating cells.

The life cycle of many long-term storage cells involves two very distinctive phases. A first phase is dominated by a process of massive reserve accumulation that terminates at the onset of dormancy. During the second phase, the energy reserves are utilized for internal consumption and in many cases for external transport. In red beet plants, the hypocotyl becomes the overwintering storage tissue in which cells accumulate as much as 220 mM sucrose (Leigh et al., 1979). The data reported here demonstrates that the physiological mechanisms for sucrose uptake and efflux are separated in time and governed by the developmental state of the cell.

Fig. 1. Sucrose uptake by tonoplast vesicles from red beet hypocotyls isolated from beets during stages of sucrose accumulation (●) and sucrose mobilization (□) as compared with control samples in the presence of 10 μ M gramicidin (○). The experiments were conducted at least three times. Vertical bars represent $\pm$ SD.

Literature Cited

- Bennett, A., S.D. O'Neill, and R.M. Spanswick. 1984. H⁺-ATPase activity from storage tissue of *Beta vulgaris*. I. Identification and characterization of an anion sensitive H⁺-ATPase. *Plant Physiol.* 74:538-544.
- Bethke, P., S.J. Swanson, S. Hillmer, and R.L. Jones. 1998. From storage compartment to lytic organelle: The metamorphosis of the aleurone protein storage vacuole. *Ann. Bot.* 100:399-412.
- Briskin, D.P., W.R. Thornley, and R.E. Wyse. 1985. Membrane transport in isolated vesicles from sugarbeet taproot. II. Evidence for a sucrose/H⁺ antiport. *Plant Physiol.* 78:865-870.
- Darwin, C.W. and P. John. 1989. Localization of enzymes of fructan metabolism in vacuoles isolated by a mechanical method from tubers of Jerusalem artichoke (*Helianthus tuberosus* L.). *Plant Physiol.* 89:658-663.
- Echeverria, E. 2000. Vesicles-mediate transport between the vacuole and the plasmalemma. *Plant Physiol.* 123:1-10.
- Echeverria, E. and P.C. Gonzalez. 2000. ATP-induced sucrose efflux from red beet tonoplast vesicles. *Planta* 211:77-84.
- Frehner, M., F. Keller, and A. Wiemken. 1984. Localization of fructan metabolism in the vacuoles isolated from protoplasts of Jerusalem artichoke tubers (*Helianthus tuberosus* L.). *J. Plant Physiol.* 116:197-208.
- Getz, H.P. 1991. Sucrose transport into tonoplast vesicles of red beet roots is linked to ATP hydrolysis. *Planta* 185:261-268.
- Keller, F. 1992. Transport of stachyose and sucrose by vacuoles of Japanese artichoke (*Stachys sieboldii*) tubers. *Plant Physiol.* 98:442-445.
- Leigh, R.A. 1997. Solute composition of vacuoles. *Advances Bot. Res.* 25:171-194.
- Leigh, R.A., T. aP Rees, W. Fuller, and J. Banfield. 1979. The localisation of acid invertase and sucrose in the vacuoles of storage roots of beetroots. *Biochem. J.* 178:539-547.
- Ross, H.A. and H.V. Davies. 1985. A microscopic examination of starch depletion in tubers of cv. Maris Piper during sprouting. *Potato Res.* 28:113-118.