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Transport of 1-kestose across the tonoplast of Jerusalem artichoke tubers

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Abstract

The capacity for 1-kestose uptake into the vacuole of fructan storing Jerusalem artichoke tubers was investigated. 1-kestose serves both as building block for fructan initiation and as a fructose donor for chain elongation. Tonoplast vesicles were isolated from actively storing tubers, and their vesicles were capable of transporting sucrose in a manner indicative of a sucrose/H^+ antiport. Under similar conditions, 1-kestose was not taken up by vesicles energized by either a pH jump or in the presence of ATP. When added together at 2 mM, sucrose uptake was not affected by the presence of 1-kestose. The data argues against the possible synthesis of 1-kestose in the cytosol and subsequent transport to the vacuole. The data also presents definite evidence for the existence a mechanism for sucrose accumulation in fructan storing vacuoles.

Keywords: Fructan synthesis; Tonoplast transport; Fructan initiation; Vacuole

1. Introduction

The initial step in the synthesis of fructans in higher plants involves the enzymatic transfer of a fructosyl unit from a sucrose donor to another sucrose acceptor forming the trisaccharide 1-kestose. This step is catalyzed by sucrose:sucrose fructosyl transferase (1-SST; EC 2.4.1.99), a vacuolar enzyme isolated and purified from a wide variety of tissues (Pollock and Suzuki, 1986; Praznik et al., 1990; Angenent et al., 1993; Koops and Jonker, 1996; Van den Ende et al., 1996). Although demonstrated both in vivo (Cairns et al., 1989; Hellwege et al., 1997) and in vitro (Bancal and Gaudillere, 1993; Cairns, 1993; Penson and Cairns, 1994; Lüscher et al., 1996; Van den Ende and Van Laere, 1996; Itaya et al., 1997; Vijn et al., 1998), synthesis of 1-kestose by 1-SST has remained ambiguous in light of the enzyme's unusually high K_m for sucrose which varies from 75 mM up to over 500 mM depending on the tissue investigated and assay conditions (Chatterton et al., 1988; Cairns and Ashton, 1994). For example, in developing Jerusalem artichoke tubers, the K_m for sucrose has been estimated to be approximately 500 mM (Koops and Jonker, 1996). This value is considerably higher than the measured vacuolar sucrose concentration of 0.3 mM (Frehner et al., 1984), a condition that would render 1-SST virtually ineffective. It should be noted, however, that the validity of kinetics systems with two equal substrates has been questioned (Koops and Jonker, 1996).

Recent reports of in vitro fructan synthesis (Bancal and Gaudillere, 1993; Cairns, 1993, 1999; Penson and Cairns, 1994; Van den Ende and Van Laere, 1996; Itaya et al., 1997) also carried the inherent inefficiencies of 1-SST as sucrose concentrations of 50–800 mM were required. The in vivo synthesis of 1-kestose by transformed potato tubers (Hellwege et al., 1997) was facilitated by the higher vacuolar sucrose concentration and by long term incubation. Based on these observations, his results, and those of others, Cairns (1999) suggested that 1-kestose may be synthesized elsewhere and transported into the vacuole where elongation of fructan chains takes place. Cairns hypothesis is consistent with that presented earlier by Pontis (1995) in which he proposed that 1-kestose may be synthesized in the cytosol and transported to the vacuole.

In the present study, we investigated the capacity of tonoplast enriched vesicles from developing Jerusalem artichoke tubers to actively transport the trisaccharide

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1-kestose in reference to its capacity for sucrose transport. Some form of active transport of 1-kestose into the vacuole would be necessary since the location of 1-kestose in cells of Jerusalem artichoke tubers is almost exclusively vacuolar (Frehner et al., 1984). Our results indicate that 1-kestose is not transported across the tonoplast by either 1-kestose/H^+ exchange or directly energized by an ATP driven mechanism. Tonoplast vesicles, however, transported sucrose against a concentration gradient in a manner indicative of a sucrose/H^+ antiport.

2. Results and discussion

2.1. Purity and competence of tonoplast vesicles

Isopycnic separation of microsomal fractions from fructan accumulating Jerusalem artichoke tubers in a discontinuous sucrose gradient of 16, 26, 34 and 40% sucrose, resulted in four distinct membrane layers. When tested against antisera to V-ATP*ase*, a much stronger immunological reaction was obtained with the lowest density membrane fraction (top of 16% sucrose layer, Fig. 1). The antibodies specific to sub-unit 31 kDa of tonoplast V-ATP*ase* (Betz and Dietz, 1991; Dietz et al., 1995) reacted with a protein of a molecular mass of approximately 31 kDa. The stronger immunological reaction of the lowest density layer indicate a substantial enrichment with vacuolar membrane. V-ATP*ase* activity from similar membrane samples was inhibited 90% by bafilomycin-A and NO_3^- (Table 1). This acute inhibition by NO_3^- and bafilomycin-A, and the reduced effect by vanadate (17%), are consistent with properties

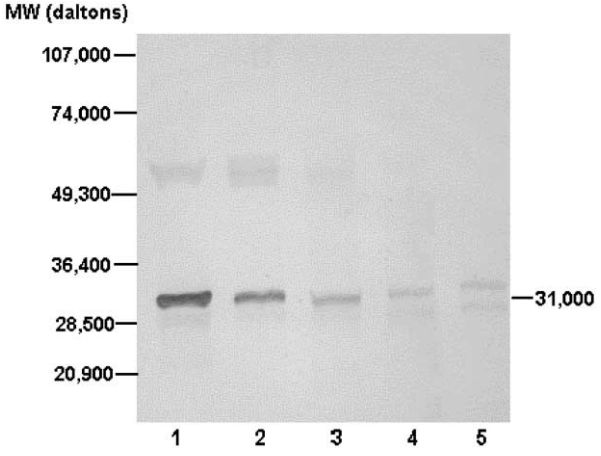

Fig. 1. Immunolocalization of V-ATP*ase* in different membrane fractions from Jerusalem artichoke tubers using antibodies against subunit E. Membrane samples were collected from the interfaces sucrose discontinuous gradients: lane 1, layer on top of 16% sucrose; lane 2, 16/26% sucrose interface; lane 3, 26/34% sucrose interface; lane 4, 34/40% sucrose interface; lane 5, crude extract. All sample lanes contained equal protein (7.5 μg protein).

exhibited by membranes of tonoplast origin. Additional experiments using fluorescin-labeled concanavalin-A (which binds to glycoproteins of the plasmalemma) confirmed a very low contamination with plasmalemma (Fig. 2). Fluorescence in lowest density layers was minimal when compared to intense fluorescence in the highest density layers (Fig. 2) demonstrating that membranes collected from the lowest density fraction were virtually free of plasmalemma markers. Therefore our tonoplast vesicle preparations showed enough features to allow the study of membrane transporters.

Incubation of tonoplast vesicles with ATP/Mg resulted in the formation of a pH gradient as evidenced by the decrease in absorbance of acridine orange at 495 nm (Fig. 3). After steady state was reached, addition of 10 μM gramicidin resulted in total collapse of the pH gradient as expected from the action of the protoionophore. The presence of bafilomycin-A and NO_3^- from the start of the reaction inhibited the formation of a pH gradient. The above data demonstrate that fractions collected from the top of the 16% sucrose layer were: (1) highly enriched with tonoplast, (2) of high degree of purity and, (3) competent for transport (uptake) experiments.

2.2. Uptake experiments

Energization of tonoplast vesicles prior to sucrose or 1-kestose loading (by a suspected sucrose-[saccharide]/H^+ antiport) could be accomplished by two distinct methods (i.e. pre-incubation in Mg/ATP or PP*i*, and by generating an artificial pH gradient). An artificial pH gradient was established by incubating tonoplast vesicles with a lumen pH of 5.5 in an experimental sucrose solution at pH 7.5. The isolated vesicles had been previously equilibrated in a buffer solution at pH 5.5. The difference in pH between the vesicle lumen and the incubating solution of 2 pH units constituted the proton motive force used to generate sucrose uptake. Uptake experiments were performed with vesicles energized by either a pH jump or by adding ATP as indicated in the text.

Artificially energized vesicles (by a pH jump) accumulated sucrose at rates higher than de-energized controls (+10 μM gramicidin, Fig. 4). Accumulation was rapid at first, but rates declined gradually until maximum accumulation occurred at ~30 min. At steady state, the amount of sucrose accumulated was approximately 14.2 nmol sucrose/mg protein which, considering an average vesicle volume of 3 μl/mg protein, indicates a final sucrose concentration within the vesicles of approximately 4.7 mM.

1-Kestose, at 2 mM, was not accumulated above control levels in vesicles pre-energized either with a pH jump or in the presence of ATP. The slower rates of 1-kestose movement into control de-energized tonoplast

Table 1
ATP*ase* activity in the lowest density membrane fraction from Jerusalem artichoke tubers[a]

	ATPase activity (nmol min^{-1} mg $protein^{-1}$)		
	Total activity	+ Bafilomycin-A + KNO_3	+ Vanadate
Tonoplast fraction	139.7±10.4	15.3±2.7	102.5±9.8

[a] The membrane sample was collected on top of the 16% sucrose layer from a discontinuous sucrose gradient of 16, 26, 34 and 40%. All samples contained 10 M gramicidin. Effectors were added at the following concentrations: bafilomycin-A, 30 nM; nitrate, 50 mM; vanadate, 100 μM. Activities are the average of three experiments ± s.d.

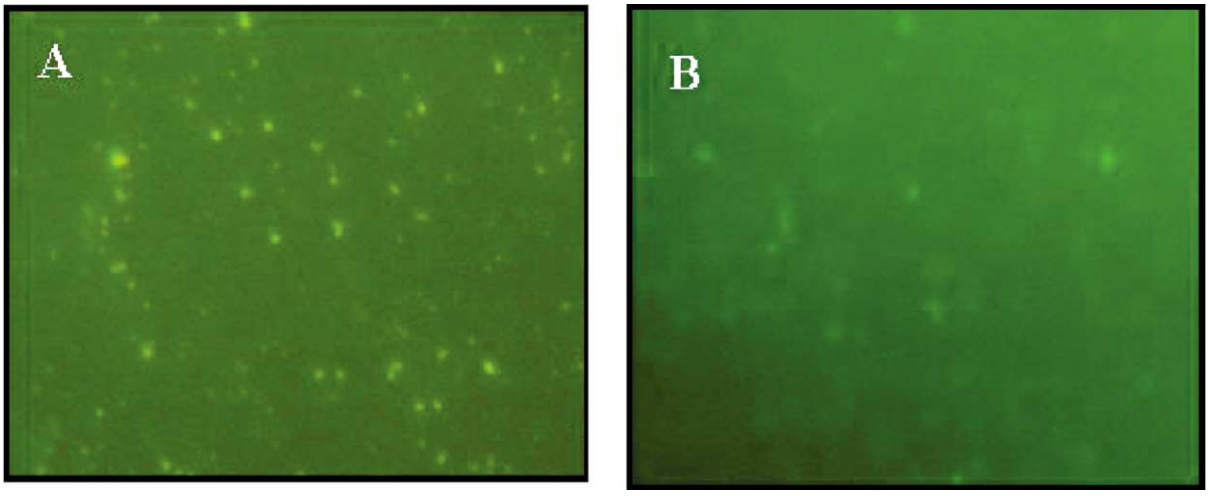

Fig. 2. Fluorescent micrographs of membrane vesicles collected from the different interfaces of the sucrose gradient after incubation with 1 mg/ml FITC-tagged Concanavalin-A (Sigma C-7642) for 1 h at 4 °C. Membrane samples in (A) were collected from the 34/40% sucrose interface of the density gradient. Sample (B) corresponds to the lightest membrane fraction collected from the top of the 16% sucrose layer (see Table 1 for enzymatic characteristics).

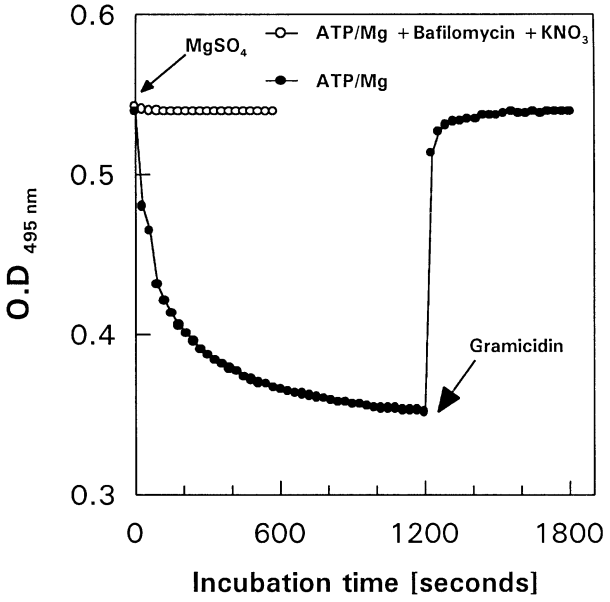

Fig. 3. ATP-dependent quenching of acridine orange absorbance in control samples (●), and in the presence of 30 nM bafilomycin-A and 50 mM NO_3^- (○). Experimental mixture contained 2.5 mm ATP and 50 mm KCl and reaction started by the addition of 2.5 mm $MgSO_4$. Gramicidin (10 μM) was added to control the sample at indicated time.

vesicles (+10 μM gramicidin, Fig. 4) when compared to sucrose, are most likely due to its larger molecular size and consequently lower rates of diffusion.

When vesicles (with internal pH of 7.5) were pre-incubated in ATP and sucrose added subsequently, accumulation of the disaccharide occurred but at rates much below those observed with an established pH jump (data not shown). On the contrary, 1-kestose did not accumulate above control rates with ATP in the presence or absence of 10 μM gramicidin (Fig. 4).

Sucrose uptake was coupled to H^+ transport, as evidenced by the decrease quenching of quinacrine fluorescence during sucrose accumulation (Fig. 5). To better illustrate H^+-coupled sucrose uptake, vesicles were energized with ATP to demonstrate both the formation of a pH gradient and the H^+/sucrose exchange. Due to the lipophilic nature of quinacrine and to its rapid crossing of the membrane, vesicles with internal pH of 5.5 do not show pH gradient formation. Vesicles resuspended at pH 7.5 were incubated with ATP for approximately 30 min and allow to reach steady state (Fig. 5). At 30 min, an ATP trap of hexokinase and mannose was added and allowed to incubate for 5 min. The ATP trap depletes all available ATP and consequently inactivates the V-ATP*ase*. When sucrose was

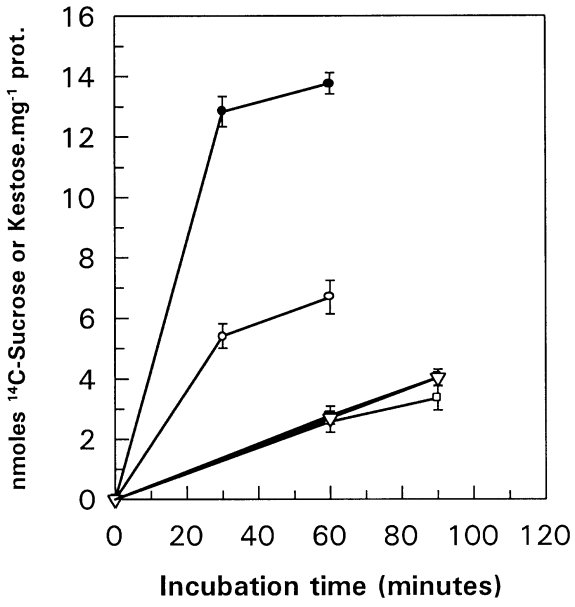

Fig. 4. Uptake of sucrose (●) and 1-kestose (□) by tonoplast vesicles from Jerusalem artichoke tubers. Control samples for sucrose (○) and 1-kestose (■) contained gramicidin from the initial sampling time. A separate sample contained 1-kestose, ATP and no gramicidin (▽). Vesicles were energized by a pH jump of 2 units. Vesicles (with a lumen pH of 5.5 equilibrated by three freeze/thaw cycles) were incubated in a medium of pH 7.5 (see Materials and Methods). Both sucrose and 1-kestose were supplied at a final concentration of 2 mM and radiological concentrations of 51.8 and 22.3×10^3Bq mol^{-1}, respectively.

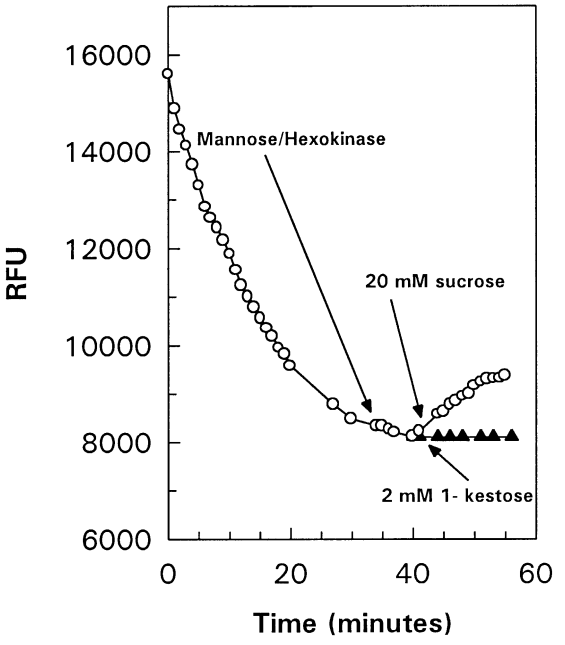

Fig. 5. Changes in H^+ gradient by tonoplast vesicles from Jerusalem artichoke tubers in the presence of ATP/Mg and 10 μM quinacrine. Mannose/hexokinase (ATP trap) were added to deplete the remaining ATP and stop H^+/ATP*ase* activity. Sucrose and 1-kestose were added after equilibrium and changes in H^+ gradient followed for additional 20 min. RFU; relative fluorescence units.

added at this time, a decrease in the fluorescence quenching of quinacrine was evident indicating the efflux of H^+ as sucrose is taken up. This data is in accordance with a sucrose/H^+ antiport previously reported for red beet hypocotyl (Briskin et al., 1985; Getz, 1991) and Japanese artichoke tonoplast vesicles (Keller, 1992; Greutert and Keller, 1993). Under similar conditions, addition of 1-kestose did not induce a decrease in fluorescence quenching indicating the lack of an electrogenic transport mechanism across the tonoplast.

Concentration dependence of sucrose transport followed Michaelis–Menten kinetics with an estimated K_m of 2.8 mM and V_{max} of 3.1 nmoles/mg prot/min (Fig. 6). The estimated K_m for sucrose uptake into tonoplast vesicles from developing Jerusalem artichoke tuber was very similar to that for red beet hypocotyl tissue of 1.2 mM (Getz, 1991). Higher K_m's for sucrose uptake of 7 and 25 mM have been reported for sugar cane internode cells and developing tubers of Japanese artichokes (*Stachys sieboldii*) (Williams et al., 1990; Greutert and Keller, 1993), respectively.

In competition experiments, the presence of 1-kestose did not reduce [^{14}C]-sucrose uptake at equal concentrations. Uptake of sucrose in the presence of 1-kestose proceeded at similar rates as when added alone (data not shown) demonstrating a high degree of specificity by the sucrose carrier to its substrate.

The data presented in this communication argues against the possibility of 1-kestose synthesis in the cytosol and subsequent active transport across the tonoplast prior to become the fructosyl donor for fructan polymerization. Three pieces of evidence support this contention. First, energized tonoplast vesicles from developing Jerusalem artichoke tubers did not accumulate [^{14}C]-1-kestose in spite of actively accumulating sucrose. The

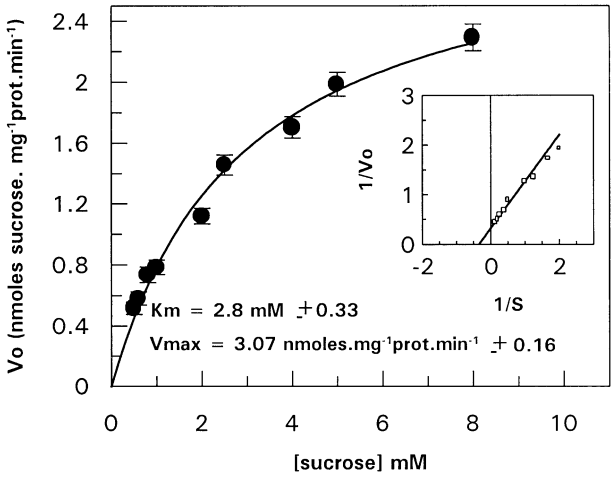

Fig. 6. Concentration dependence of sucrose uptake in tonoplast vesicles from Jerusalem artichoke tubers. Each point is the average of 4 experiments with standard deviation not exceeding 12%. Lineweaver–Burk plot shown in inset.

lack of 1-kestose accumulation was noted in tonoplast enriched samples energized either by a pH jump or in the presence of ATP. Accumulation of sucrose occurred against a concentration gradient in a manner very similar to that of red beet hypocotyl cells (Briskin et al., 1985; Getz, 1991; Echeverria and Gonzalez, 2000). Second, 1-kestose did not induce a decrease in the fluorescence quenching of quinacrine as was the case for sucrose (Fig. 4). Third, sucrose uptake was not reduced by 1-kestose when both were added at the same concentration. The surprising discernment between 1-kestose and sucrose demonstrates a high degree of specificity for the sucrose carrier by being able to discriminate between two very similar molecules. In the case of Japanese artichoke, the same carrier is able to transport, in addition to sucrose, raffinose, and stachyose (Keller, 1992; Greutert and Keller, 1993).

It should be mentioned that our tonoplast fractions, despite being virtually free of plasmalemma contamination, contained measurable amounts of golgi marker UDPase. However, since no transport of 1-kestose was observed, the presence of golgi clearly had no effect on the results, nor it modify the conclusions.

The search for alternative means for 1-kestose synthesis and its transport to the vacuole stems from the puzzling in vitro inefficiency of 1-SST, the commonly accepted 1-kestose synthesizing pathway. This is despite the fact that genetically modified tissues with 1-SST have been able to synthesize 1-kestose (Hellwege et al., 1997; Vijn et al., 1998). Synthesis of 1-kestose in the cytosol and transport into the vacuole offered an appealing possibility in light of the capacity of sucrose carriers to transport other related molecules (Keller, 1992; Greutert and Keller, 1993). Our experiments demonstrate that, under present conditions, tonoplast from rapidly fructan accumulating Jerusalem artichoke tubers were not capable of transporting 1-kestose from the cytosol by a direct or indirect ($\Delta\mu H^+$) ATP-dependent mechanism, a conjecture earlier made by Martinoia and Ratajczak (1997). The present data, however, do not rule out other means of 1-kestose synthesis and transport into the vacuole, a possibility alluded by the results of Cairns (1999), or the possibility of microcompartmentation or enzymatic association with the tonoplast which would increase the enzymatic efficiency of 1-kestose synthesis. Such possibility has been recently outlined by Guerrand et al. (1999) which noted a strong association between 1-SST and the tonoplast.

3. Experimental

3.1. Plant material

Jerusalem artichoke tubers (*Helianthus tuberosus*) were a gift from Mr. Jerry Smith, Neshkoro, WI. Tubers were planted at the Citrus Research and Education Center in Lake Alfred, FL during spring of 1999 and harvested in early October of the same year, at a time of rapid rates of fructan synthesis. Artichoke plants were fertilized on a monthly basis and irrigated when necessary.

3.2. Tonoplast isolation

Tonoplast vesicles from Jerusalem artichoke tubers were prepared by following the procedure described by Bennett et al. (1984) for red beet hypocotyls with the following modifications. Peeled tubers (120 g) were chopped into smaller pieces and homogenized in a Waring blender with 185 ml of buffer containing 150 mM sucrose, 70 mM Tris–HCl buffer (pH 8.0), 4 mM DTT, 3 mM Na_2EDTA, 1.5% PVP-40 and 0.5% BSA, and 1 mM PMSF. The homogenate was filtered through four layers of cheesecloth and centrifuged for 25 min at $10{,}000\times g$. The supernatant was collected and centrifuged for 1 h at $112{,}000\times g$. 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 134,000 *g* for 1 h. 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 16/26% interface after centrifugation of the gradient for 1.5 h at $47{,}000\times g$.

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 $80{,}000 \times g$ for 40 min. The final pellet was re-suspended in 1 ml of storage buffer containing 250 mM sorbitol, 10 mM Tris/MES 7.0 (or MES/KOH, pH 5.5 depending on the experiments), and 2 mM DTT at pH. Tonoplast vesicles were stored at -80 °C until needed. Procedures were conducted at 4 °C.

3.3. Simultaneous measurement of ATPase activity and formation of pH gradient

Simultaneous measurements of ATP*ase* activity and H^+ pumping capacity were performed as described by Palmgren (1990). Reactions were started by the addition of 2.5 mM $MgSO_4$ to the reaction mixture containing 50 μg tonoplast vesicle protein. ATP*ase* activity was determined by following the oxidation of NADH at 340 nm. pH gradient formation was measured by observing the change in absorbance of acridine orange (10 μM) at 495 nm. All reactions were carried out at 30 °C in a Shimadzu UV-160 (Kyoto, Japan). For experiments of Fig. 4, quinacrine (10 μM) was used instead of acridine

orange and changes in fluorescence followed with excitation/emission of 400/510.

3.4. Synthesis of [^{14}C]-1-kestose

Kestose was synthesized by incubating in a total volume of 0.4 ml, 80 μmol sucrose (6.25 μCi μmol^{-1}), 20 μmol acetate buffer (pH 5.0), and chicory 1-SST (prepared as indicated below). The reaction was carried overnight at 30 °C and stopped by boiling in a water bath for 2 min. After centrifugation at 12,000×*g* for 2 min, the supernatant was applied to a BioGel column P-2 (>400 mesh) 2 × 250 cm previously equilibrated with water at pH 8.0. Elution was performed with the equilibrating solution at 8 ml h^{-1}. Fractions of 2 ml each were collected and radioactivity in aliquots of each fraction were determined by liquid scintillation spectroscopy. Three eluted peaks corresponded to 1-kestose, sucrose, and glucose previously determined with commercial standards. The trisaccharide containing peaks were pooled and lyophilized. The powder was dissolved in water and analyzed for fructose and radioactivity.

3.5. Preparation of 1-SST

1-SST from chicory roots was prepared according to Van den Ende et al. (1996). Chicory roots (1.8 g) from one-month-old plants were peeled and powdered in liquid nitrogen. A homogenate was prepared by stirring the powder in 3.6 ml of a buffered solution containing 100 mM acetate buffer (pH 5.0), 1 mM PMSF, 1 mM β-mercaptoethanol, and 10 mM $NaHSO_3$ for 1 h at 0 °C. The homogenate was filtered through two layers of cheesecloth. The filtrate was centrifuged at 180,000 × *g* for 15 min. The supernatant was brought to 30% saturation with ammonium sulfate. The precipitate formed was removed by centrifugation and discarded. The ammonium concentration of the supernatant was increased to 60% saturation and the precipitate collected after centrifugation at 20,000 × *g* for 20 min. The pellet was re-suspended in the minimum volume of 20 mM acetate buffer (pH 5.0), 0.1 mM PMSF, and 1 mM β-mercaptoethanol and dialyzed against the same buffer. The dialyzate was use as source of 1-SST.

3.6. Uptake experiments

Sucrose or 1-kestose uptake into purified tonoplast vesicles was performed using stock solutions of 20 mM [^{14}C]-sucrose, and 4 mM [^{14}C]-1-kestose at radiological concentrations of 51.8 × 10^3 Bq μmol^{-1} and 22.3 × 10^3 Bq μmol^{-1}, respectively. Prior to uptake studies under artificial pH gradient, the tonoplast vesicles were equilibrated in storage buffer at pH 5.5 by applying three freeze/thaw cycles. Tonoplast vesicles (approximately 200 μg tonoplast protein) were incubated at 30 °C in a medium containing 250 mM sorbitol, 50 mM buffer HEPES (pH 7.5), 2 mM DTT, 0.4 mg ml^{-1} BSA in a final volume of 550 μl. Gramicidin was added at 10 μM when required. Uptake was started by the addition of radiolabeled sucrose or 1-kestose 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, PA). Results are given as the mean value of at least three independent experiments from separate preparations performed in duplicate.

3.7. Immunodetection

One-dimensional SDS-PAGE of membrane fractions was prepared on 12% polyacrylamide gels as described by Laemmli (1970). A total of 7.5 μg of membrane protein were loaded in each well. The proteins from the SDS gels were electrotransferred to 0.2 μm supported nitrocellulose membrane (BioRad) for 1 h at 100 V and 4 °C while stirring in a "Mini-Trans-Blot" electrophoretic transfer cell (BioRad). The blots were blocked with 5% non-fat dry milk in TBS solution (25 mM Tris/HCl pH 7.5, 150 mM NaCl) for 30 min. Afterwards, the membrane blots were incubated overnight in TBS containing 5% dry milk with polyclonal antibodies against a V-ATP*ase* sub-unit 31 from barley leaf (courtesy of Dr. Karl Joseph Dietz). A second antibody (AP anti-rabbit IgG, Sigma) followed for 3 h. Final stain was achieved with NBT/BCIP solution for approximately 1 h.

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