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Sucrose-inducible Endocytosis as a Mechanism for Nutrient Uptake in Heterotrophic Plant Cells

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The capacity of plant heterotrophic organs to transport and accumulate incoming nutrients (mostly in the form of sucrose) directly impacts their final size, crop productivity and nutritional value. Endocytosis as a mechanism for nutrient uptake in heterotrophic cells was investigated using suspension culture cells of sycamore (*Acer pseudoplatanus* L.) and the endocytic inhibitors wortmannin and LY294002. Time course analysis of sucrose uptake in intact walled cells revealed a two-phase process involving an initial 90 min wortmannin- and LY294002-insensitive sucrose uptake period, followed by a prolonged phase of rapid sucrose accumulation which was greatly inhibited by the two endocytic inhibitors. Walled cells were assessed for their capacity to incorporate the fluorescent endocytosis marker lucifer yellow-CH (LY) in the presence or absence of sucrose. Rates of sucrose and LY accumulation were virtually identical, as was their response to wortmannin. In addition, LY incorporation increased as a function of external sucrose concentration. When sucrose was substituted by other sugars or amino acids, uptake of LY greatly diminished, indicating that sucrose itself is the primary signal of endocytosis. Microscopic observations revealed the formation of vesicles containing LY and its eventual accumulation on the vacuole when sucrose was present in the incubation medium. These results demonstrate the existence of a sucrose-inducible endocytic process as a viable mechanism for solute transport into the vacuole of storage cells.

Keywords: *Acer pseudoplatanus* — Endocytosis — Metabolite uptake — Photoassimilate transport — Sucrose uptake — Vacuole.

Abbreviations: ADH, alcohol dehydrogenase; dTR, dextran–Texas red; LY, lucifer yellow-CH; LY294002, 2-(4-morpholynyl-)-8-phenyl-4H-1 benzopyran-4-one.

Introduction

Heterotrophic plant organs such as fruits, seeds, tubers, roots, etc., directly or indirectly sustain life on earth, as they constitute major portions of human diets in addition to being essential food to wild fauna. As storage sink organs, they can import as much as 80% of the carbon that is photosynthetically assimilated by autotrophic plant parts. Their ability and strength to transport and store incoming nutrients (mostly in the form of sucrose) directly impact their metabolism, overall productivity, final size, and nutritional value. A thorough understanding of the mechanism(s) involved in the uptake of nutrients by heterotrophic cells is critically important for an efficient design of experimental approaches aimed at improving crop yield and productivity.

In many heterotrophic plant organs, sucrose and other nutrients are delivered to the cells through the apoplast (Patrick 1997). From the apoplast, a substantial amount of photoassimilates is transported to the vacuole, which serves as a temporary reservoir. To achieve successful transport into the storage vacuole, a minimum of five sucrose transport activities along the translocation path have been predicted (Lalonde et al. 1999). The existence of carriers, transporters and channels, located both at the plasmalemma and tonoplast, has indirectly led to the notion that external solutes to be accumulated in the vacuole evidently diffuse through the cytosol before final passage across the tonoplast. Sucrose transporters have been localized at the tonoplast (Getz 1991, Keller 1992) and at the plasmalemma of storage cells (Manning et al. 2001, Rosche et al. 2002); however, they have never been localized in both membranes of a single cell type.

There is a growing volume of evidence indicating that metabolite transport from the apoplast into the plant vacuole cannot always be reconciled solely by solute movement through individual channels or transporters, but in some instances, the data suggest the presence of alternative or parallel transport mechanisms (Haug and Shi 1991, MacRobbie 1999, Fernie et al. 2000, Hajirezaei et al. 2000). The anomalies described for ion accumulation, sucrose transport and subsequent metabolism, are more consistent with a transport process comprised of solute-filled vesicles than with a static system of protein carriers mediating single molecules. Supporting this view, there is now compelling evidence about the occurrence of endocytosis in plant cells (Hubner et al. 1985, Villanueva et al. 1993, Battey et al. 1999, Baluška et al. 2002, Baluška et al. 2004). Furthermore, a recent report by Emans et al. (2002)

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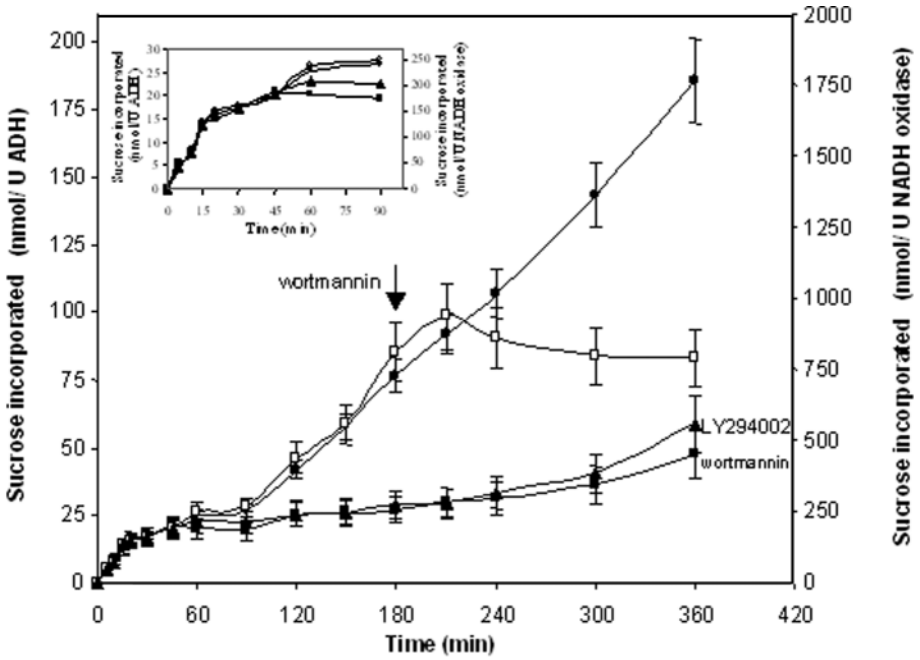

Fig. 1 Time course of the sucrose content in sycamore cells cultured with 50 mM sucrose in the presence or absence of wortmannin or LY294002. Cells were cultured in 'starving medium' for 24 h prior to addition of sucrose. Endocytic inhibitors were added either from the beginning or after 3 h of culture. Inset: close up of sucrose accumulation during the initial 2 h incubation. For all treatments, 4-day-old cultured cells were placed in starving medium for 24 h to maximize sucrose uptake. After addition of sucrose, cell aliquots were taken at the indicated times, washed, extracted in ethanol, and analyzed for sucrose by ion chromatography. Sucrose content was based on both ADH and NADH oxidase activities as cytosolic and plasmalemma markers, respectively. None of these activities was affected by either wortmannin or LY294002 (not shown). Results are given as the mean ± SE of four analyses per time.

using a dual system of membrane-impermeable fluorescent dyes, and by Baluška et al. (2004) using root cortex cells, carried the implication that external solutes may be transported directly into the vacuole. These results are in agreement with early work where different membrane-impermeable markers were eventually observed to accumulate in the vacuole (Hillmer et al. 1986, Hillmer et al. 1989, Lazzaro and Thompson 1992).

Amongst the many distinctive vesicle-mediated intracellular transport systems, endocytosis is recognized as a nutritional mechanism in hepatocytes (Anderson et al. 1992), squamous cells (Hew et al. 1999), slime molds (Hacker et al. 1997), fungi (Mendgen et al. 1995) and algae (Ginzburg et al. 1999). In clear contrast, endocytosis in plant cells has been addressed mostly as a means to regulate the recycling of membrane and wall components (Battey et al. 1999, Marcote et al. 2000, Baluška et al. 2002), whereas its effectiveness as a mechanism of nutrient uptake has been questioned (Oparka et al. 1993).

Recognizing that endocytosis is a common activity to all eukaryotes, we investigated the possible occurrence of endocytosis acting as a means of nutrient uptake in heterotrophic plant cells. Towards this end, we employed the well-characterized cultured cells of sycamore (*Acer pseudoplatanus* L.) (Rébeillé et al. 1985), the reliable fluorescent marker of fluid phase endocytosis lucifer yellow-CH (LY; Roszak and Rambour 1997, Wiederkehr et al. 2001, Baluška et al. 2004, Yano et al. 2004) and the potent endocytic inhibitors wortmannin (Emans et al. 2002) and 2-(4-morpholinyl-)-8-phenyl-4H-1 benzopyran-4-one (LY294002) (Vlahos et al. 1994). Collectively, our results demonstrate that uptake and accumulation of external solutes into the storage vacuole of heterotrophic cultured cells are largely mediated by an endocytic mechanism which is induced by sucrose.

Results

Overall sucrose uptake is strongly inhibited by endocytosis inhibitors

If nutrients were transported, at least in part, by endocytosis, uptake of extracellular sucrose would be expected to diminish considerably or be completely repressed by endocytosis inhibitors. To test this possibility, a time course analysis of sucrose incorporation was performed in cultured cells of sycamore previously starved for 24 h prior to re-introduction of sucrose both in the presence and in the absence of either wortmannin or LY294002. These structurally different compounds have been shown to inhibit the early endocytic process in a multitude of plant and animal species by interfering with both phosphatidylinositol 3-kinase and phosphatidylinositol 4-phosphatase (Li et al. 1995, Brunskill et al. 1998) but without affecting plasmalemma-bound sugar transporters (Khayat et al. 1998).

Judicious examination of sucrose uptake revealed an initial 90 min period in which sucrose content rose sharply but leveled off at about 20 nmol sucrose U^{-1} alcohol dehydrogenase (ADH; about 250 nmol sucrose U^{-1} NADH oxidase, Fig. 1, inset). This period was wortmannin and LY294002 insensitive. Most importantly, after 90 min of culture, the rate of sucrose uptake rapidly increased, resulting in a final sucrose accumulation of approximately 200 nmol U^{-1} ADH (about 1,800 nmol U^{-1} NADH oxidase) after 6 h of incubation. This second phase was highly repressed by wortmannin and LY294002, in agreement with the existence of an endocytic transport of sucrose into the cells. Strongly substantiating these observations is the complete cessation of sucrose uptake when wortmannin was added at a time when sucrose had already

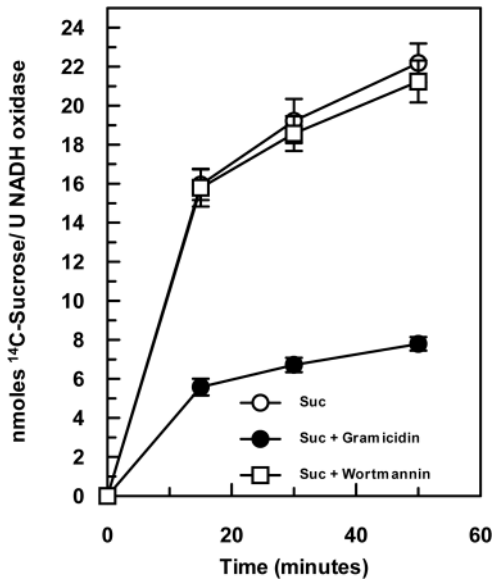

Fig. 2 Uptake of sucrose into plasmalemma vesicles is not affected by wortmannin. Vesicles from 4-day-old cultured cells of sycamore previously energized by a pH jump were incubated with 50 mM [^{14}C]sucrose in the presence or absence of 33 μM wortmannin and subjected to analysis of [^{14}C]sucrose uptake by scintillation spectroscopy. Control vesicles contained 10 μM gramicidin to prevent energization. Sucrose content is also presented based on the plasmalemma NADH oxidase marker enzyme. Results are given as the mean ± SE of three analyses per time.

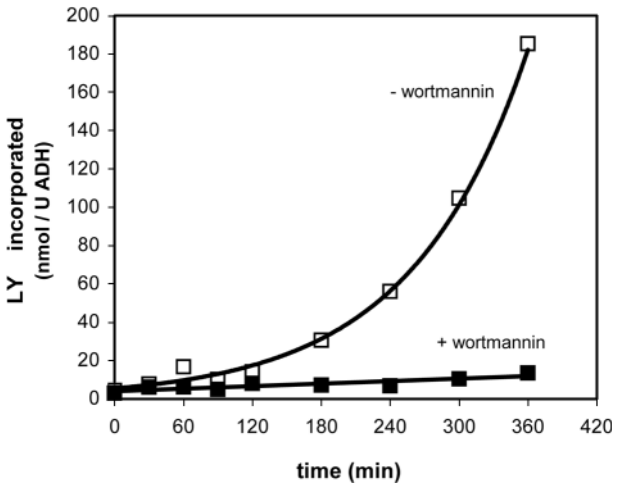

Fig. 3 Time course analysis of the LY uptake by sycamore cultured cells after simultaneous addition of 1 mM LY and 50 mM sucrose in the presence or absence of 33 μM wortmannin. Four-day-old cells were pre-incubated without sucrose for 24 h. After addition of LY and sucrose, cells were taken at the indicated times, protoplasts prepared, and LY analyzed fluorometrically. LY content was based on the cytosolic marker ADH as a means to standardize the amount of living cells in the aliquot samples. Results are given as the mean of four analyses per time.

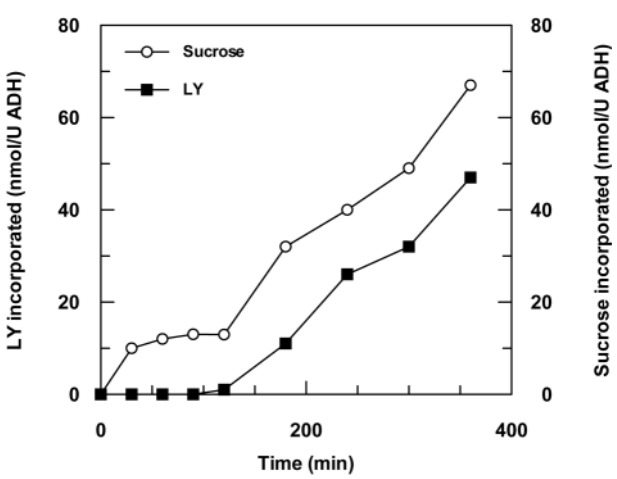

Fig. 4 Time course of sucrose (open circles) and LY (closed squares) content in sycamore cells cultured in a solution containing equimolar amounts of both sucrose and LY. Cells were cultured in 'starving medium' prior to addition of sucrose. After the simultaneous addition of 1 mM LY and 1 mM sucrose, aliquots were taken at the indicated times and extracted in ethanol for sucrose analysis. Protoplasts were prepared for LY and ADH analysis. LY and sucrose content are based on the cytosolic marker ADH as a means to standardize the amount of living cells in the aliquot samples. Results are given as the mean of four analyses per time.

begun to accumulate rapidly (Fig. 1). The nearly complete inhibition of sucrose uptake by wortmannin within an hour is highly significant inasmuch as an equal concentration of the inhibitor did not suppress electrogenic sucrose uptake into energized plasmalemma vesicles purified from cells grown under similar conditions (Fig. 2). In clear contrast, sucrose uptake into energized vesicles treated with the protoionophore gramicidin was significantly reduced, suggesting the presence of a wortmannin- and LY294002-insensitive sucrose/H^+ symport system. Although the concentrations of wortmannin and LY294002 reported in Fig. 1 are of 33 and 5 μM respectively, similar inhibition patterns were obtained at concentrations ranging from 10 nM to 10 μM in both cases (data not shown).

Together, these results indicate that, despite the occurrence of wortmannin- and LY-insensitive sucrose carriers at the plasmalemma, transport of a large portion of external sucrose into heterotrophic cells during prolonged cultured conditions is mediated by a process with distinctive characteristics of endocytotic transport.

Sucrose-inducible endocytic incorporation of membrane-impermeable dyes

To substantiate the aforementioned result, which suggested that external sucrose is taken up in great part by endocytosis, we proceeded to trace the incorporation of the membrane-impermeable fluorescent endocytic marker LY into the cells. Although few early studies using the inorganic anion transport inhibitor probenecid suggested that internalization of LY might also occur in a non-vesicular manner (Oparka et al.

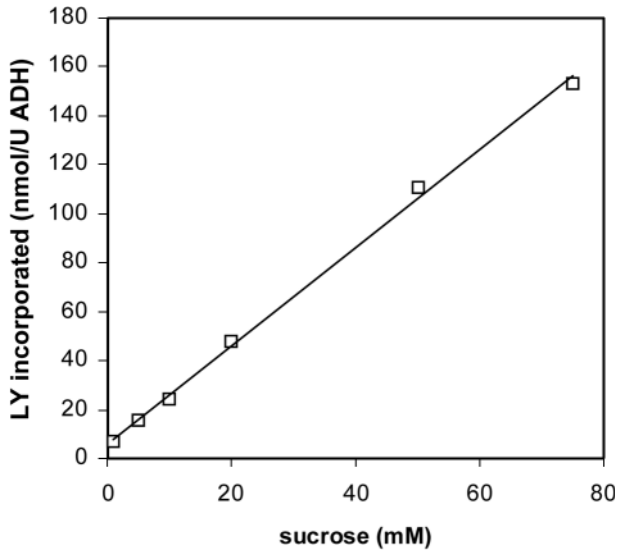

Fig. 5 Uptake of LY as a function of increasing sucrose concentration. After 24 h of culture in starving medium, sycamore cultured cells were incubated for 3 h at a constant LY concentration of 1 mM and the indicated sucrose concentrations. LY is based on the cytosolic marker ADH as a means to standardize the amount of living cells in the aliquot samples.

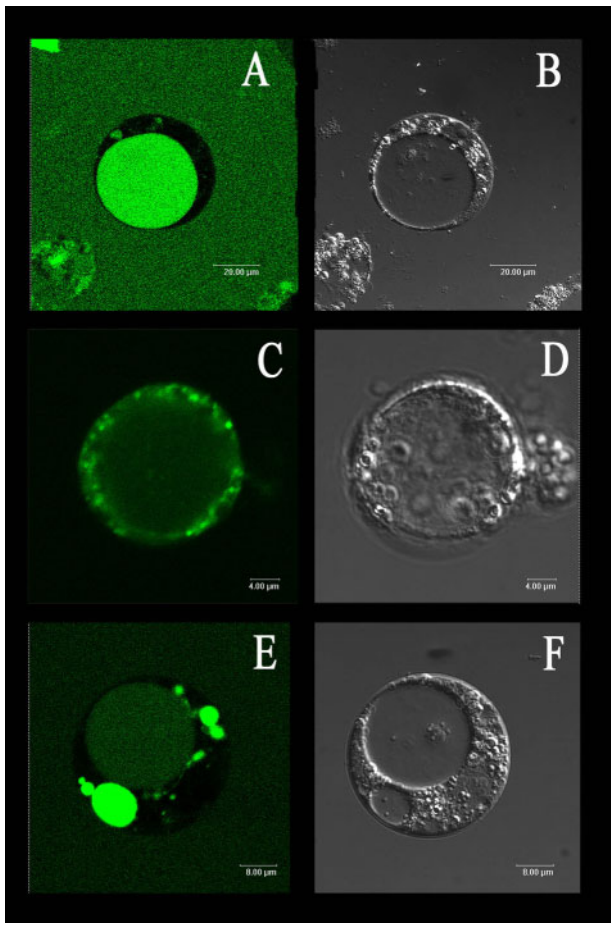

Fig. 6 Confocal fluorescence microscopy analysis of LY distribution in protoplasts isolated from sycamore cells cultured with both 50 mM sucrose and 1 mM LY during (A) 24 h, (C) 1 h and (E) 3 h incubation. The corresponding light micrographs are (B), (D) and (F), respectively.

1991, Cole et al. 1991, Ballatori et al. 1999), recent studies have demonstrated decisively the usefulness of LY as a fluid phase marker in plant cells (Owen et al. 1991, Roszak and Rambour 1997, Wiederkehr et al. 2001, Baluška et al. 2004, Yano et al. 2004).

Incorporation of LY during sucrose accumulation was followed fluorometrically. As illustrated in Fig. 3, not only did LY uptake follow a pattern very similar to that of sucrose after 90 min of culture (see Fig. 1), but inclusion of wortmannin (or LY294002, not shown) also substantially inhibited LY accumulation. The similar patterns of LY and sucrose accumulation during prolonged incubation times, and the similar inhibitory effect exerted by wortmannin and LY294002, strongly suggest that sucrose and LY share a common transport mechanism consistent with endocytosis.

To evaluate the relative contribution of endocytosis in relation to extracellular solute import, incorporation of both sucrose and LY was followed in cells incubated with nearly equimolar amounts of each compound. The rationale of this experiment was that, if endocytosis plays a minor role compared with carrier-mediated sucrose transport in the overall process of sucrose uptake, the amount of sucrose incorporated by the cells should be much larger than that of LY. As presented in Fig. 4, however, both the rates and total amount of sucrose and LY incorporated by the cells during prolonged culture conditions were very similar, further indicating that endocytosis plays a decisive role in the uptake of extracellular solutes.

The conspicuous similarities in the accumulation patterns of sucrose and LY prompted further analysis of their transport relationship. When LY accumulation was measured as a factor of increasing external sucrose concentrations, an increasing linear response was obtained (Fig. 5). Considering that the concentration of LY in the incubation medium remained constant, the increase in its accumulation can only be interpreted as an increment in the endocytic capabilities parallel to sucrose concentration. These results not only demonstrate that extracellular solutes are transported non-selectively by a system consistent with endocytic uptake and transport, but that the endocytosis itself is induced by sucrose and its cargo capabilities proportionally to the external sucrose concentration.

Microscopic observations of vacuolar incorporation of impermeable dyes

Among its many physiological functions, the vacuole serves as a temporary reservoir for sucrose in plant cells (Nishimura and Beevers 1978). When extracellular sucrose is omitted under experimental conditions, vacuolar sucrose is consumed rapidly. Conversely, when sucrose is added to the

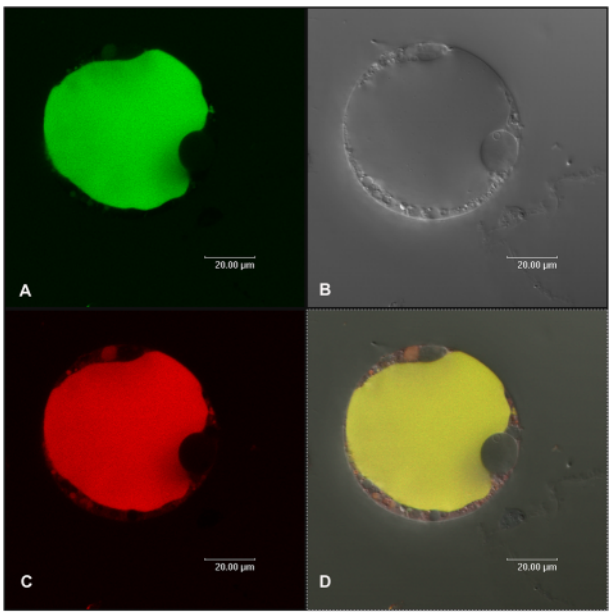

Fig. 7 *Citrus* juice cells after incubation for 12 h in a buffered solution containing 250 mM sucrose and two membrane-impermeable dyes (Alexa-488 at 100 µM and dTR at 1 mg ml^{-1}). These fluorescent soluble probes vary considerably in molecular size and charge. Protoplasts were prepared following incubation, and the juice cells observed under fluorescent Nomarski microscopy with appropriate filters (Leica TCS SL). (A), (C) and (D) show the same cell under appropriate filters for Alexa-488, dTR, and both Alexa-488 plus dTR, respectively.

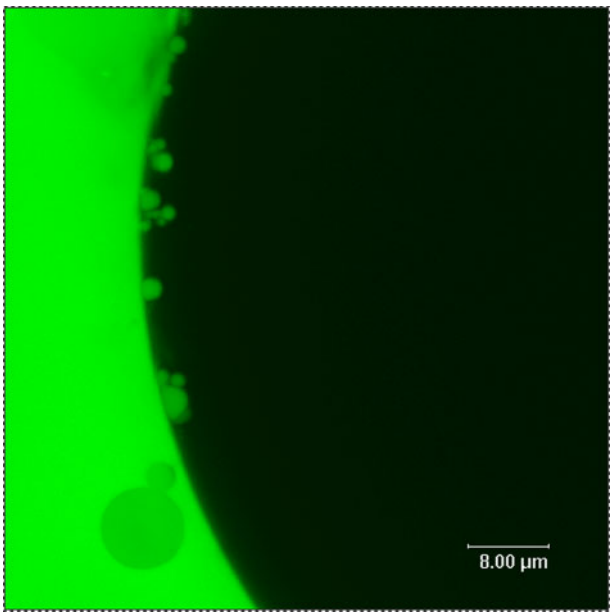

Fig. 8 *Citrus* juice cell protoplast prepared after 2 h incubation in a buffered solution containing 250 mM sucrose and the endocytic probe 100 µM Alexa-488. Cells were observed under the appropriate filter in a Leica TCS SL confocal microscope. Endocytic vesicles containing fluorescing Alexa-488 are seen at the periphery of the darker vacuolar background. Vacuole remains dark given the short time of incubation. The cytosolic layer is not clearly delimited due to the high fluorescent background.

nutrient medium, vacuolar sucrose increases (Rébeillé et al. 1985).

If both sucrose and the membrane-impermeable LY were transported together into the vacuole by the same non-selective endocytic mechanism, fluorescence should be observed emanating from the vacuole of cultured cells. Incubation of starved cultured cells with sucrose and LY (plus or minus wortmannin) for 24 h produced contrasting and notable results when observed under high definition confocal microscopy. As shown in Fig. 6A, a strong fluorescence labeled the central vacuole after 24 h of incubation, clearly visible when compared with corresponding light micrographs (Fig. 6B).

Shorter periods of incubation revealed seemingly intermediate stages of the endocytic pathway. After 1 h incubation, numerous fluorescent vesicles could be observed in the cytosol (Fig. 6C, D). These small vesicles become less numerous with time and, at 3 h incubation, larger structures become apparent (Fig. 6E, F). The presence of wortmannin completely abolished incorporation of LY in the vacuoles, as was the case in control samples incubated in LY without sucrose (data not shown).

Sucrose-inducible incorporation of membrane-impermeable markers into the vacuole via endocytosis was not limited to LY or to sycamore cultured cells. As shown in Fig. 7, for instance, *Citrus* juice cells simultaneously accumulated two distinct membrane-impermeable dyes [Alexa-488 and dextran–Texas red (dTR)] into the vacuole. Incubation of *Citrus* juice cells with the endocytic marker Alexa-488 (Emans et al. 2002) for only 2 h failed to produce fluorescent vacuoles. Instead, fluorescence was prominent in small peripheral vesicles (approximately 50–100 nm) located in the cytosol (Fig. 8).

Endocytic uptake of extracellular solutes is preferentially triggered by sucrose

In higher plants, phloem sap translocated from autotrophic to heterotrophic cells contains numerous metabolites in addition to sucrose. Among other sugars translocated, glucose and fructose are the most universal. Amino acids also constitute an important component of the phloem sap. To examine for the specificity of the endocytosis process, experiments were carried out in which LY uptake was traced in the presence of other naturally occurring sugars and amino acids as well as sugars not found in the phloem sap. From the data in Table 1, it is evident that LY incorporation in the presence of other metabolites constituted only a small fraction of that observed in the presence of sucrose. The fact that other disaccharides, such as maltose and trehalose, or the combination of glucose and fructose, did not induce endocytic uptake of LY at levels comparable with sucrose is a particularly remarkable result which demonstrates that endocytic uptake of external solutes in the present system is a process induced predominantly by sucrose.

Table 1 Lucifer yellow uptake by sycamore cultured cells incubated with various sugars and amino acids

Compound added (50 mM)	LY incorporated	
	nmol U^{-1} ADH	% with respect to sucrose
Sucrose	124.6	100.0
Glucose	9.3	7.5
Fructose	9.4	7.5
Glucose + fructose	5.8	4.6
Maltose	11.2	9.0
L-Glutamine	12.0	9.6
Trehalose	10.1	8.1
Mannose	5.6	4.5
L-Asparagine	6.0	4.8

Except for maltose and mannose, the remaining metabolites are commonly found in plant phloem saps. LY uptake in the presence of sucrose was considered as 100%.

The lack of LY incorporation into cells with any carbon source other than sucrose (Table 1), and its inhibition parallel to sucrose in the presence of wortmannin (Fig. 3) strongly argues against the possibility of LY entering the cell through membrane channels (Cole et al. 1991, Oparka et al. 1991). Furthermore, fluorescence was never observed in the cytosol, demonstrating that in our studies, LY was taken up exclusively by endocytosis, as recently demonstrated by Baluška et al. (2004) working with maize roots and by Yano et al. (2004) with tobacco cultured cells.

Discussion

The data presented in this communication demonstrate for the first time that, essentially identically to other organisms (Anderson et al. 1992, Mendgen et al. 1995, Bright et al. 1997, Hacker et al. 1997, Ginzburg et al. 1999, Hew et al. 1999), extracellular nutrients can be transported into the plant vacuole by endocytosis. Furthermore, the data also indicated that sucrose itself functions as a signal molecule that triggers the endocytic mechanism. Extracellular sucrose sensors implicated in regulation of multiple processes such as endocytosis have been suggested to occur in plants (Low and Chandra 1994, Lalonde et al. 1999). To our knowledge, this is the first report providing evidence demonstrating the occurrence in plants of a sucrose-sensing mechanism related to nutrient transport via endocytosis.

Our conclusions are supported by four major observations. First, sucrose uptake was almost completely suppressed in the presence of two well-described and structurally different endocytic inhibitors, even at times when plasmalemma sucrose transporters were unaffected (Fig. 1, 2). Secondly, membrane-impermeable dyes were transported in parallel with sucrose (Fig. 3–5) and eventually accumulated in the vacuole consistent with non-selective endocytic uptake (Fig. 6, 7). Thirdly, cellular structures consistent with the endocytic transport machinery are clearly visible when viewed under high resolution confocal microscopy (Fig. 6–8). Fourthly, uptake of the external LY marker occurred preferentially in the presence of sucrose, whereas other naturally occurring compounds triggered a fraction of LY incorporation compared with sucrose (Table 1). Therefore, endocytosis must also be considered as a means for uptake of extracellular nutrients in plants and not exclusively as a mechanism for membrane recycling.

It is crucial to point out that uptake of extracellular nutrients, such as sucrose, by endocytosis is not in direct conflict with transport through membrane-bound carriers given that cell homeostasis can be better maintained if both mechanisms operate in parallel. 'Reserve' sucrose to be accumulated in the vacuole is transported in bulk through a mechanism that bypasses the cytosol, whereas 'transitory' sucrose immediately needed by the cytosolic metabolism is transported by plasmalemma-bound carriers and funneled directly into the corresponding catalytic activities. In this manner, the highly regulated cytosol is not disrupted by the constantly changing flow of metabolites arriving from source cells. Tonoplast-associated sucrose carriers (Lalonde et al. 1999) probably operate in the fine regulation of cytosolic sucrose concentration and in the export of vacuolar sucrose at times of high demands (Etxeberria and Gonzalez 2003).

Materials and Methods

Plant material

Sycamore (*A. pseudoplatanus* L.) cells were grown in 250 ml of culture medium as described by Frehner et al. (1990), in continuously agitated 500 ml flasks (200 rpm, 28°C). Citrus fruits (*Citrus limettioides*) were collected from the orchards at the University of Florida, Citrus Research and Education Center in Lake Alfred, FL, U.S.A.

Isolation of plasmalemma vesicles

Plasmalemma samples were isolated from 4-day-old cultured cells following the procedure described elsewhere (Etxeberria and

Gonzalez 2004) that combines the principles of sucrose density gradient and aqueous two-phase partition. Cultured cells (approximately 100 g) were rinsed with culture medium and homogenized in a Waring blender with 150 ml of a buffered solution containing 70 mM MOPS/KOH (pH 8.0), 0.5% PVP-40, 4 mM dithiothreitol (DTT), 3.5 mM EDTA, 250 mM sucrose, 0.1% bovine serum albumin (BSA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). After squeezing through two layers of cheesecloth, the homogenate was centrifuged at 10,000×*g* for 10 min to eliminate debris. The supernatant was centrifuged for 30 min at 40,000×*g* and the pellet set aside for plasmalemma purification by aqueous two-phase partitioning. The resulting vesicles were virtually free of cytoplasmic contamination and from other endomembrane systems.

Sucrose uptake experiments

For analyses of uptake into walled cells, 4-day-old cultured cells were thoroughly washed with 'starving medium' (culture medium without sucrose) and placed back in 250 ml of the same starving solution. After 24 h starvation, cells were transferred to culture medium containing 50 mM sucrose in the presence or absence of 33 μM wortmannin (W-1628, Sigma-Aldrich, St Louis, MO, U.S.A.; Emans et al. 2002) or 5 μM LY294002 (L-9908, Sigma-Aldrich; Vlahos et al. 1994). Cell aliquots were taken at the indicated times, washed with starving medium, filtered through Miracloth, re-suspended in 10-fold (v/w) 95% ethanol and centrifuged for 10 min at 10,000×*g*. The resulting supernatant was evaporated to a minimum volume and re-suspended with water. Sucrose was determined by HPLC with pulsed amperometric detection on a DX-500 Dionex system fitted to a CarboPac PA1 column (Baroja-Fernández et al. 2003).

For uptake of sucrose into plasmalemma vesicles, membrane vesicles were artificially energized by a pH jump (Bush 1989) and incubated with 50 mM [^{14}C]sucrose in the presence or absence of 33 μM wortmannin. Uptake of [^{14}C]sucrose was carried out as described by Echeverria et al. (1997) and analyzed by scintillation spectroscopy using a Koulter–Beckman, LS-6000 SC counter.

Preparation of protoplasts

Protoplasts from sycamore cultured cells were prepared using a cell wall hydrolytic solution as described by Frehner et al. (1990). For the preparation of *Citrus* juice cell protoplasts, juice sacs were incubated in a cell wall hydrolytic solution similar to that used for sycamore cells but without agitation. After 3 h, juice sacs were removed from the hydrolytic solution and protoplasts released by gently squeezing the juice sacs.

Uptake of membrane-impermeable dyes

Four-day-old walled sycamore cells were starved for 24 h. At this time, the culture medium was replaced with fresh medium containing pre-determined sugar concentrations and 1 mM LY (Molecular Probes, Eugene, OR, U.S.A.), and aliquots subsequently were taken at the indicated times. Depending on the experiment, 33 μM wortmannin or 5 μM LY294002 were also added. To avoid a background of LY unspecifically bound to cell walls, protoplasts were prepared as described above. From the protoplast preparation, separate aliquots were taken and analyzed for enzyme activity (see below) and LY content. LY was analyzed fluorometrically in a Fluoroskan Neonat (Labsystems, Hampshire, U.K.) with an excitation/emission of 425/550, respectively.

For the analysis of endocytic marker uptake into *Citrus* juice cells, juice sacs were dissected from sweet lime, cut in half and incubated in 100 mM MES (pH 5.6), 0.5 mM $CaCl_2$, 250 mM sucrose, 2 mM DTT, 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, and 100 μM Alexa-488 with or without 1 mg ml^{-1} dTR (mol. wt 3,000 Da; Molecular Probes, Eugene, OR, U.S.A.). Protoplasts were then prepared as described above.

Microscopy

Microscopy on both sycamore and *Citrus* juice protoplasts was performed with a Leica TCS SL confocal microscope (Leica, Heidelberg, Germany).

Enzyme assays

Both sucrose and LY uptake values are presented based on the cytosolic marker ADH (EC 1.1.1.1) as a means to standardize the data based on the amount of live cells. Uptake measurements are also referred to the plasmalemma marker NADH oxidase (EC 1.6.99.3). ADH and NADH oxidase were measured as described by Widholm and Kishinami (1988) and Morre and Morre (1998), respectively. One unit (U) is defined as the amount of enzyme necessary to produce 1 μmol of product per min.

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