Journal of Experimental Botany, Vol. 56, No. 417, pp. 1905–1912, July 2005
doi:10.1093/jxb/eri185 Advance Access publication 23 May, 2005

RESEARCH PAPER

Existence of two parallel mechanisms for glucose uptake in heterotrophic plant cells

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Received 24 December 2004; Accepted 7 April 2005

Abstract

The implied existence of two mechanisms for glucose uptake into heterotrophic plant cells was investigated using the fluorescent glucose derivative 2-NBDG (2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose), two membrane impermeable fluorescent markers (3000 mol. wt. dextran-Texas Red (d-TR) and Alexa-488), hexose carrier and endocytic inhibitors (phloridzin and wortmannin-A, respectively), and fluorescent and confocal microscopy. Both phloridzin and wortmannin-A significantly reduced the uptake of 2-NBDG into sycamore cultured cells, which was confirmed by fluorescent microscopy. Phloridzin prevented 2-NBDG uptake exclusively into the cytosol, whereas the wortmannin-A effect was more general, with 2-NBDG uptake into the vacuole being the more affected. Simultaneous incubation of cells in the membrane-impermeable fluorescent probes Alexa-488 and d-TR for 24 h resulted in co-localization of the labelling in the central vacuole and other endosomal compartments. Cytoplasts, cells devoid of vacuoles, were instrumental in demonstrating the transport of 2-NBDG by separate uptake mechanisms. In cytoplasts incubated simultaneously in 2-NBDG and d-TR for 2 h, a green fluorescent cytosol was indicative of transport of hexoses across the plasmalemma, while the co-localization of 2-NBDG and d-TR in internal vesicles demonstrated transport via an endocytic system. The absence of vesicles when cytoplasts were pre-incubated in wortmannin-A authenticated the endocytic vesicular nature of the co-shared 2-NBDG and d-TR fluorescent structures. In summary, uptake of 2-NBDG occurs by two separate mechanisms: (i) a plasmalemma-bound carrier-mediated system that facilitates 2-NBDG transport into the cytosol, and (ii) an endocytic system that transports most of 2-NBDG directly into the vacuole.

Key words: Cytoplasts, endocytosis, hexose symporter, photoassimilate transport, vacuole.

Introduction

In sink tissues, photoassimilates are transported to storage cells either through the symplasmic continuum, when adequate plasmodesmata connections exist between storage cells (symplastic route), or movement can occur through the apoplast (apoplastic route). The symplastic route seems to be the predominant pathway in most tissues investigated, whereas the apoplastic step is largely associated with sinks containing different genomes (such as seeds) and those which accumulate high concentrations of soluble sugars (Patrick, 1997). In the apoplastic route, sugars (and other metabolites) can serve as metabolic signals, and their transport is often facilitated by the presence of cell wall-bound invertases. Movement of sugars through the apoplast is inherently dependent on efficient uptake mechanisms to retrieve solutes into the storage cells for both utilization and storage.

Recent demonstrations of a vesicle-mediated endocytic mechanism for solute uptake in heterotrophic plant cells (Etxeberria *et al.*, 2005*a*, *b*) indirectly imply the existence of at least two separate pathways for sugar uptake. In

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Abbreviations: 2-NBDG, 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; d-TR, dextran (mol. wt. 3000)-Texas Red.

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one mechanism, plasmalemma-bound electrogenic carriers transport sugars and H^+ simultaneously from the apoplast into the cytosol. These sugar symporters are highly specific and are energized by the electrochemical potential gradient established by the plasmalemma-bound H^+/ATPase (Martinoia *et al.*, 2000). The second uptake mechanism is vesicle-mediated, and transports solutes non-selectively from the apoplast into the vacuole, bypassing the cytosol. The endocytic pathway has recently been described as a sucrose-inducible mechanism of nutrient uptake which operates in cultured cells of sycamore (*Acer pseudoplatanus*; Etxeberria *et al.*, 2005*a*), *Citrus* juice cells (*Citrus sinensis*; Etxeberria *et al.*, 2005*b*), and sufficient evidence exists to suggest it also operates in turnip (*Brassica rapa* L.) storage roots (E Etxeberria, unpublished results). As pointed out by Etxeberria *et al*. (2005*a*), these two distinct uptake mechanisms of solute transport are not exclusive, but may operate simultaneously in supplying nutrients to different cell compartments. 'Reserve' sucrose to be accumulated in the vacuole is transported in bulk through a mechanism that bypasses the cytosol, whereas 'transitory' sucrose that is immediately needed by cytosolic metabolism is transported by plasmalemma-bound carriers and funnelled directly into catalytic activities. In this manner, the highly regulated cytosol is not disrupted by the constantly changing flow of metabolites arriving from source cells.

Plasmalemma-bound sugar transporters have been identified in various storage cells (Lalonde *et al.*, 1999) and are divided in two sub-families. The disaccharide transporter, which catalyses primarily sucrose transport in phloem elements (Lemoine, 2000), has also been demonstrated in storage cells of grapes (Manning *et al.*, 2001), celery petioles (Noiraud *et al.*, 2000), in *Arabidopsis* male gametophytes (Schneidereit *et al.*, 2003), and other storage tissues (Meyer *et al.*, 2004), whereas monosaccharide transporters, which mediate transport of a variable range of monosaccharides, have been characterized in a myriad of cell types including storage organs such as olive fruit (Oliveira *et al.*, 2002), carrots (Krook *et al.*, 2000), and suspension cultures of *Phaseolus vulgaris* (Botha and O'Kennedy, 1998). In both instances, sugars are transported into the cytosol where they are channelled directly into various catalytic activities, and as often believed, stored in the vacuole by corresponding tonoplast-bound carriers.

Fluid phase endocytosis in plant cells has recently been documented (Emans *et al.*, 2002; Etxeberria *et al.*, 2005*a*, *b*; Baluška *et al.*, 2004; Yano *et al.*, 2004) and its non-selective nature demonstrated using a combination of membrane-impermeable fluorescent dyes, endocytic inhibitors, and confocal microscopy. Although effective as tools to demonstrate endocytosis, artificial fluorescent probes do not permit simultaneous tracing of the two uptake mechanisms since their transport is not carrier-mediated and since they are not likely to be perceived by cells as natural nutrients. By taking advantage of recent advances in fluorescent chemistry and microscopy technology, the aim was to demonstrate the simultaneous transport of hexoses into different cell compartments using suspension cultures of sycamore and the fluorescent glucose derivative 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG). Deoxyglucose is perceived and transported by cells indistinguishably from glucose (Oliveira *et al.*, 2002) and its non-metabolizable nature allows for its accumulation and quantification. It is demonstrated here for the first time, that the two mechanisms for solute transport, endocytosis and specific plasmalemma carriers, act concurrently in cultures of heterotrophic plant cells.

Materials and methods

Plant material

Sycamore (*Acer pseudoplatanus* L.) cells were cultured in a medium supplemented with sucrose (complete medium) as described by Frehner *et al*. (1990) in continuously agitated 250 ml flasks (200 rev min^{-1} at 28 °C). Cell samples were obtained from 6–7-d-old cultures during rapid growth phase (Bligny, 1977). Starved cells were prepared by transferring 5–6-d-old culture cells into equal volume of culture medium without sucrose (starving medium) for 24 h.

Uptake experiments

For uptake analyses into walled cells, 6-d-old cultured cells were thoroughly washed with starving medium and placed back into 250 ml of the same starving solution. After 24 h starvation, cells were transferred to fresh starving medium supplemented with 5.6 mM fluorescent deoxyglucose (2-(*N*-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)-amino)-2-deoxyglucose; 2-NBDG, Molecular Probes, Eugene, OR, USA) in the presence or absence of either 33 μM wortmannin-A (W-1628, Sigma-Aldrich, St Louis, MO, USA), 2 mM phloridzin (P-3449; Sigma-Aldrich, St Louis, MO, USA), or 10 μM gramicidin (G-5002, Sigma-Aldrich, St Louis, MO, USA). When required, cells were preincubated for 30 min with the inhibitors before the addition of 2-NBDG. Cell aliquots were taken at the indicated times, centrifuged at 2000 *g* for 5 min, the supernatant discarded, and the pellet (containing cells) washed with 1 ml starving medium. The washing and centrifugation steps were repeated three times to remove all unabsorbed solutes (2-NBDG). After the final washing, the cells were resuspended in 1 ml water, sonicated for 30 s and centrifuged at 13 000 *g* for 10 min. The amount of 2-NBDG taken up was determined fluorometrically at 520 nm in a Bio-Rad Versafluor fluorometer (Bio-Rad, Hercules, CA, USA). For some experiments, fluorescent dextran (mol.wt. 3000) tagged with Texas Red (d-TR) was used as the endocytic marker at a final concentration of 1.0 mg ml^{-1}.

Protoplast preparation

When necessary, protoplasts from cultured sycamore cells were prepared using a cell wall hydrolytic solution as described by Frehner *et al*. (1990). After 3 h incubation, protoplasts were washed three times in a solution similar to the hydrolytic medium without enzymes.

Cytoplast preparation

Cytoplasts were prepared from protoplasts according to the procedure of Komoda *et al*. (2004). Protoplasts were centrifuged for 1 h on a Percoll density gradient of 70, 40, and 30% Percoll at 40 000 *g*.

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A layer rich in cytoplasts was obtained between 30% and 40% Percoll. The collected cytoplasts were washed twice with incubation medium without Percoll and incubated at 30 °C according with the experiment described in the text.

Microscopy

Protoplasts and cytoplasts were observed with either a Nikon Eclipse TE 300 fluorescent microscope (Nikon, Tokyo, Japan) or a Leica TCS-SL confocal-fluorescent microscope (Leica, Heidelberg, Germany) depending on the experiment. Both microscopes were equipped with appropriate filters and cameras.

Results

Hexose uptake into cultured cells

Quantification of sugar uptake by live cells is often complicated by their rapid rates of metabolic utilization and conversion to other cellular components. To overcome this obstacle, and to visualize intracellular uptake more effectively, 2-NBDG, a fluorescent form of the non-metabolizable glucose derivative deoxyglucose was used. Deoxyglucose has previously been shown to serve as a substrate for hexose carriers (Buttner and Sauer, 2000; Oliveira *et al.*, 2002), but its non-metabolizable nature allows for its accumulation, and therefore, accurate measurements of uptake into cells can be made.

In sycamore cells, 2-NBDG uptake as a function of time followed a hyperbolic curve with uptake rates levelling off after approximately 12 h; achieving maximum uptake of 918±70 nmol 2-NBDG mg^{-1} biomass (Fig. 1). Using the first 6 h of incubation to approximate linearity for all treatments, a slope of 102±9.2 nmol 2-NBDG mg^{-1} biomass h^{-1} was calculated. By contrast, when incubated in the presence of phloridzin, a hexose carrier inhibitor (Stubbs *et al.*, 2004), a maximum uptake of 624 nmol 2-NBDG mg^{-1} biomass (68% of control) was achieved after 24 h of approximately linear uptake. Under the effect of phloridzin, the slope during the initial 6 h was 38±1.9 nmol 2-NBDG mg^{-1} biomass h^{-1} (37% of control). When incubated in the presence of wortmannin-A, uptake was delayed by 3–4 h and the maximum uptake during the 24 h incubation of only 96.7±2.98 nmol 2-NBDG mg^{-1} biomass equalled 10% of control. The slope calculated using the first 6 h of positive uptake data was 5.7±2.6 nmol 2-NBDG mg^{-1} biomass h^{-1} (5.6% of control). Wortmannin is an endocytic inhibitor (Emans *et al.*, 2002; Etxeberria *et al.*, 2005*a*) that exerts some non-specific inhibitory effect on plasmalemma hexose carriers (Sutherland *et al.*, 1995). Therefore, the strong inhibition of wortmannin-A on 2-NBDG uptake can be ascribed to a dual effect on both carrier-mediated and endocytic transport.

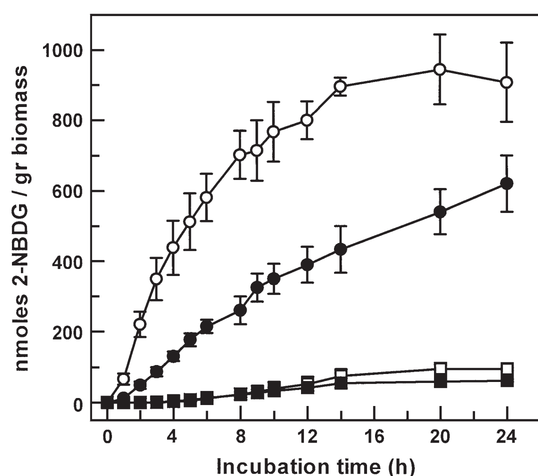

Fig. 1. 2-NBDG uptake by cultured sycamore cells. Uptake of 2-NBDG by sycamore-cultured cells incubated for 24 h. Cells were incubated in 5.6 mM 2-NBDG alone (control, open circles) and in the presence of 2 mM phloridzin (filled circles), 33 μM wortmannin-A (open squares) and both phloridzin and wortmannin-A combined (filled squares).

The decreased rates of 2-NBDG uptake as affected by both inhibitors suggests that uptake of hexoses into sycamore culture cells most likely occurs by two separate mechanisms: (i) carrier-mediated and (ii) endocytic transport.

Microscopic observations of hexose uptake

To substantiate further the above observations that imply the simultaneous presence of two hexose uptake mechanisms, uptake of 2-NBDG and d-TR into the cells was followed using fluorescent and confocal microscopy. The use of 2-NBDG offered the advantage of allowing visual examination of distinct uptake events and intracellular locations, whereas d-TR uptake would be restricted to non-carrier-mediated endocytosis. For better visualization of cell compartments, protoplasts were prepared after cells were treated according to individual experiments.

After 1 h incubation in 2-NBDG (Fig. 2A, B) most cells contained a brightly fluorescent cytosol evident against the dark translucent vacuole (Fig. 2B). The pattern of fluorescence changed gradually with time, and the majority of vacuoles began to show some fluorescence by the third hour of incubation. The result was highly fluorescent vacuoles after 24 h incubation (Fig. 2D). By this time, both vacuoles and cytosol were notably fluorescent; note that the higher concentration of fluorescence in the vacuole dims the relative fluorescence of the cytosol.

The use of transport inhibitors considerably changed fluorescence distribution. Phloridzin visibly reduced fluorescence in the cytosol making it appear almost translucent, while fluorescent cytosolic vesicles became apparent (Fig. 3). More significantly, however, phloridzin did not prevent 2-NBDG transport into the vacuole after a prolonged incubation time. When incubated with wortmannin-A, a uniform but low-level fluorescence resulted in the cytosol, whereas no fluorescence was found in the vacuole after 24 h incubation (Fig. 4).

Cells incubated for 18 h in 2-NBDG and the membrane impermeable endocytic marker d-TR showed high levels of

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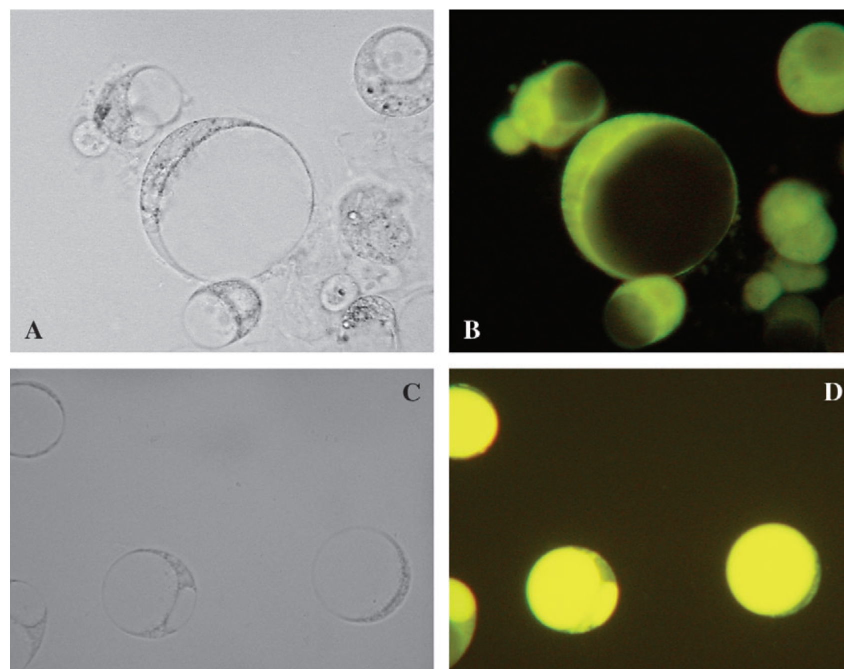

Fig. 2. Protoplasts prepared from cultured sycamore cells after incubation in 5.6 mM 2-NBDG for 1 h and 24 h. (A) light micrograph and (B) fluorescent micrograph of protoplasts after 1 h incubation. (C) light micrograph and (D) fluorescent micrograph of protoplasts after 24 h incubation. Fluorescence was observed using UV light as excitation and fluorescin filter.

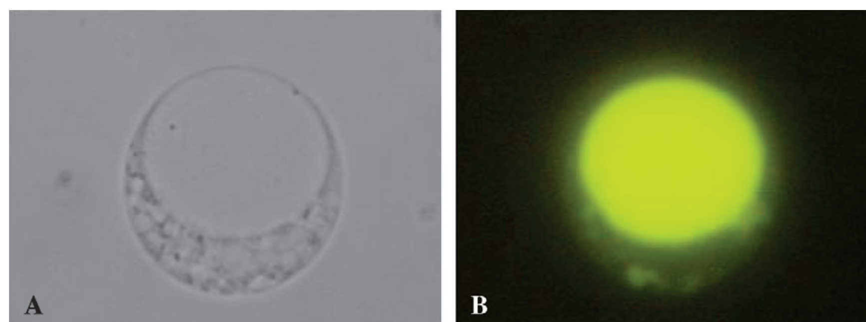

Fig. 3. Protoplasts prepared from cultured sycamore cells after incubation in 5.6 mM 2-NBDG and 2 mM phloridzin for 24 h. (A) Light micrograph; (B) fluorescent micrograph using UV light as excitation and fluorescin filter.

fluorescence in the central vacuole and in smaller cytosolic vesicular compartments (Fig. 5). These results authenticate the presence of endocytic transport into the vacuole.

Preparation and characteristics of cytoplasts

The use of large vacuolated cells was advantageous in that it allowed the visualization of 2-NBDG presence in both the cytosol and vacuoles, and made it possible to follow a time-course of events. However, the large vacuole reduces the cytosol to a relatively thin layer around the periphery of the vacuole that often hinders a detailed examination of the uptake events. To overcome this obstacle, cytoplasts were prepared. These living cells consist of all the cytoplasmic constituents except the vacuole, which was previously extracted by density centrifugation (Lörz *et al.*, 1981). A typical cytoplast is presented in Fig. 6A next to an intact protoplast. Under light Nomarsky microscopy, cytoplasts appear as heterogeneous structures with the nucleus as the only outstanding structure (Fig. 6B).

Hexose uptake into cytoplasts

Cytoplasts incubated with 2-NBDG for 24 h were highly fluorescent, but without any evidence of vesicle formation (Fig. 7). However, the lack of prominent vesicles could have been the result of the high fluorescence intensity masking smaller subcellular structures. Therefore, cytoplasts were incubated with 2-NBDG and the membrane impermeable d-TR for shorter periods. When visualized

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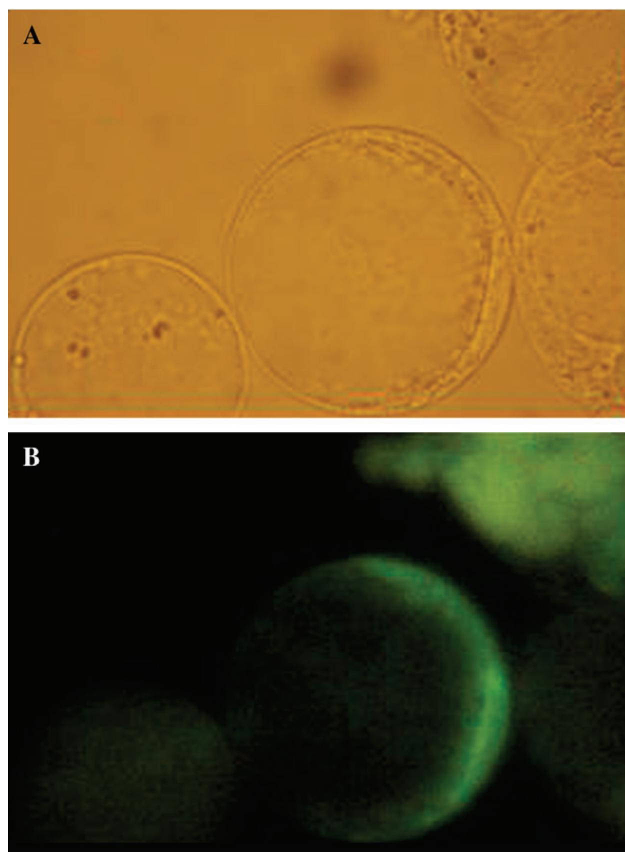

Fig. 4. Protoplasts prepared from cultured sycamore cells after incubation in 5.6 mM 2-NBDG and 33 μM wortmannin-A for 24 h. (A) Light micrograph; (B) fluorescent micrograph using UV light as excitation and fluorescin filter.

under confocal microscopy after 2 h incubation, 2-NBDG fluorescence was observed throughout the cytoplast (Fig. 8A). Several brightly fluorescent spots (presumptively vesicles) were also evident. When examined under the appropriate filter for d-TR, the brighter 2-NBDG vesicles also exhibited red fluorescence (Fig. 8B) indicating the co-localization of 2-NBDG with d-TR. This is highly significant in that d-TR was not observed in the cytosol but was confined to distinct areas co-shared with the brighter 2-NBDG spots (Fig. 8C). Preincubation in wortmannin-A prevented the formation of d-TR fluorescent vesicles (Fig. 8D), although 2-NBDG fluorescence was still observed homogeneously throughout the cytosol.

Discussion

Post-phloem transport of sugars into sink tissues is a dynamic process that can take one of two routes depending on the type of organ and developmental state (Patrick, 1997). The data of Figs 1–4 and 8, demonstrate that heterotrophic cells are adequately equipped to retrieve hexoses from the apoplast in at least two ways, substantiating an apoplastic route for these tissues.

In the present study, the use of the fluorescent, non-metabolizable glucose derivative 2-NBDG and the membrane impermeable fluorescent d-TR, combined with high definition confocal microscopy and appropriate carrier and endocytic inhibitors, allowed the existence of two parallel mechanisms for the uptake of hexoses in heterotrophic plant cells to be demonstrated for the first time. This conclusion is supported by several pieces of evidence derived from direct uptake measurements and from microscopic observations.

First, hexose uptake was significantly reduced by inhibitors of endocytosis and of hexose carriers (Fig. 1). Even after 24 h incubation, control levels of hexose uptake were not reached in the presence of either inhibitory substance.

Second, 2-NBDG transport into the vacuole was not significantly affected by inhibition of the hexose carrier although the accumulation of hexoses in the cytosol was markedly reduced (Fig. 3). Conversely, wortmannin-A strongly inhibited vacuolar accumulation of 2-NBDG (Fig. 4), but allowed some 2-NBDG transport into the cytosol.

Third, cytoplasts were capable of simultaneously taking up 2-NBDG into the cytosol and into intracellular vesicles where they co-localized with the membrane-impermeable d-TR (Fig. 8). The vesicle contents, traced by d-TR in Fig. 8, were eventually transported into the vacuole (Fig. 5; Etxeberria *et al.*, 2005*a*, *b*).

Collectively, the data demonstrate that glucose uptake into heterotrophic cells involves separate transport processes depending on the final destination of the hexose molecule (i.e. cytosol or vacuole).

The use of cytoplasts was vital in confirming the simultaneous occurrence of events leading to the transport of hexoses into different cell compartments. Heterotrophic storage cells are highly vacuolated, with the vacuole occupying as much as 95% of the cellular space (Van Praag, 1997). Cytoplasts offer the advantage of being devoid of vacuoles (Fig. 6), making it possible to follow the uptake of 2-NBDG into the cytsol and the formation of vesicles in an unobstructed manner. Figure 8 accurately summarizes these conclusions. In cytoplasts incubated with 2-NBDG, a fluorescent cytosol (Fig. 8A) is indicative of transport of hexoses across the plasmalemma, while the simultaneous inclusion of 2-NBDG and d-TR in internal vesicles demonstrate co-localization in endocytic vesicles (Fig. 8B, C). The absence of vesicles when cytoplasts were preincubated in wortmanin-A (Fig. 8D) authenticates the vesicular nature of the co-shared 2-NBDG and d-TR fluorescent structures.

Several conjectures can be made based on the data presented in this paper and on previously published results with sycamore cells (Etxeberria *et al.*, 2005*a*). The initial appearance of 2-NBDG in the cytosol (Fig. 2) suggests that transport across the plasmalemma occurs as the first phase of hexose uptake. Hexose carriers are known to be present

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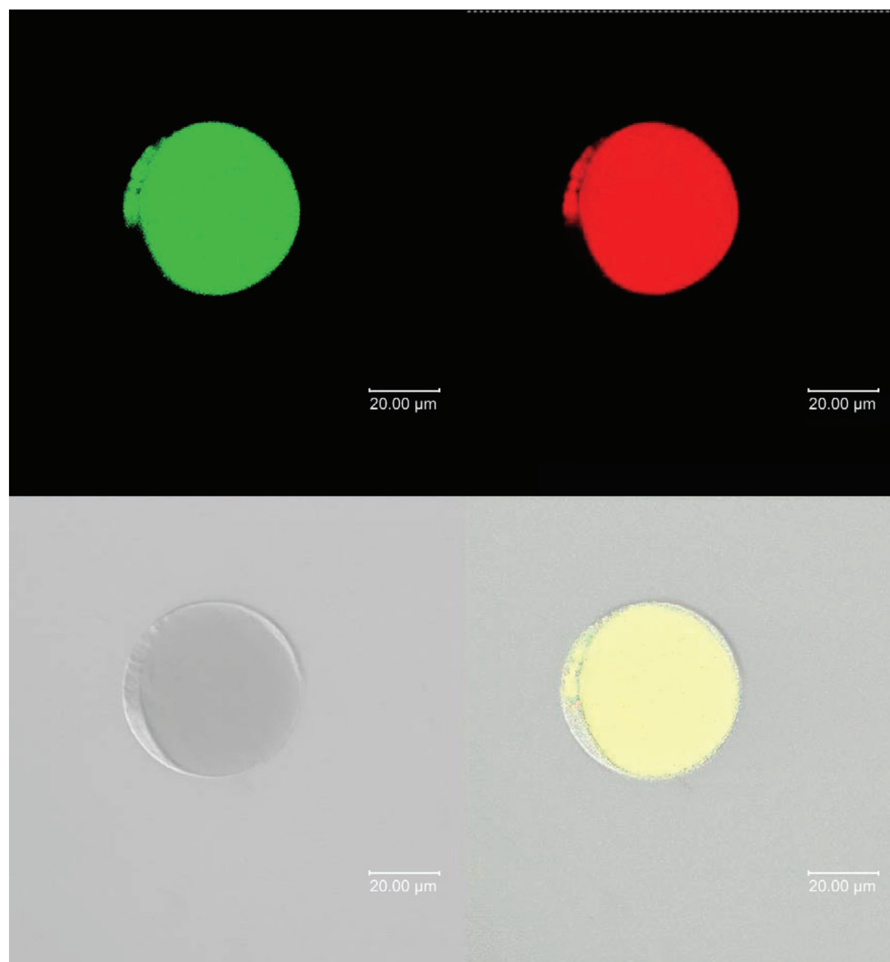

Fig. 5. Scanning fluorescent micrographs of a sycamore protoplast after 24 h incubation in 5.6 mM 2-NBDG and 1.0 mg l^{-1} d-TR. (A) Cell visualized under the fluorescin filter; (B) cell visualized under the appropriate filter for Texas Red; (C) light micrograph; and (D) under both filters for fluorescin and Texas Red.

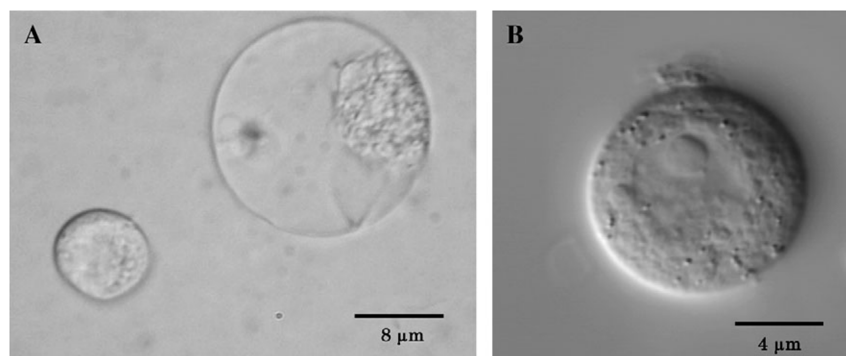

Fig. 6. Cytoplasts from cultured sycamore cells observed under (A) light micrograph and (B) Nomarski microscopy. A protoplast is shown in (A, arrow) for comparative purposes. Cells were starved for 24 h prior protoplast production. Cytoplasts were prepared from protoplasts in a density gradient.

in heterotrophic cells, and the sudden appearance of extracellular glucose triggers the further synthesis of the hexose transporters with which to catalyse additional uptake (Sherson *et al.*, 2003). The fact that 2-NBDG transport was substantially reduced by the hexose carrier inhibitor phloridzin (Figs 1, 3) confirms that 2-NBDG transport was partially mediated via a plasmalemma-bound hexose carrier as previously described (Stubbs *et al.*, 2004). The subsequent and gradual accumulation of 2-NBDG in the vacuole is, at least in part, the result of endocytosis. This conclusion is largely supported by the accumulation of 2-NBDG and the membrane impermeable d-TR into

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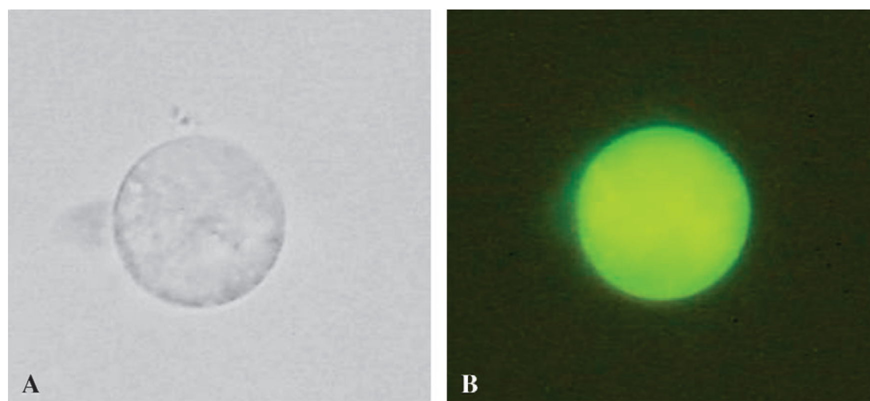

Fig. 7. Light (A) and fluorescent (B) micrographs of a cytoplast incubated in 5.6 mM 2-NBDG for 24 h. Cytoplasts were observed using a confocal microscope with appropriate filters.

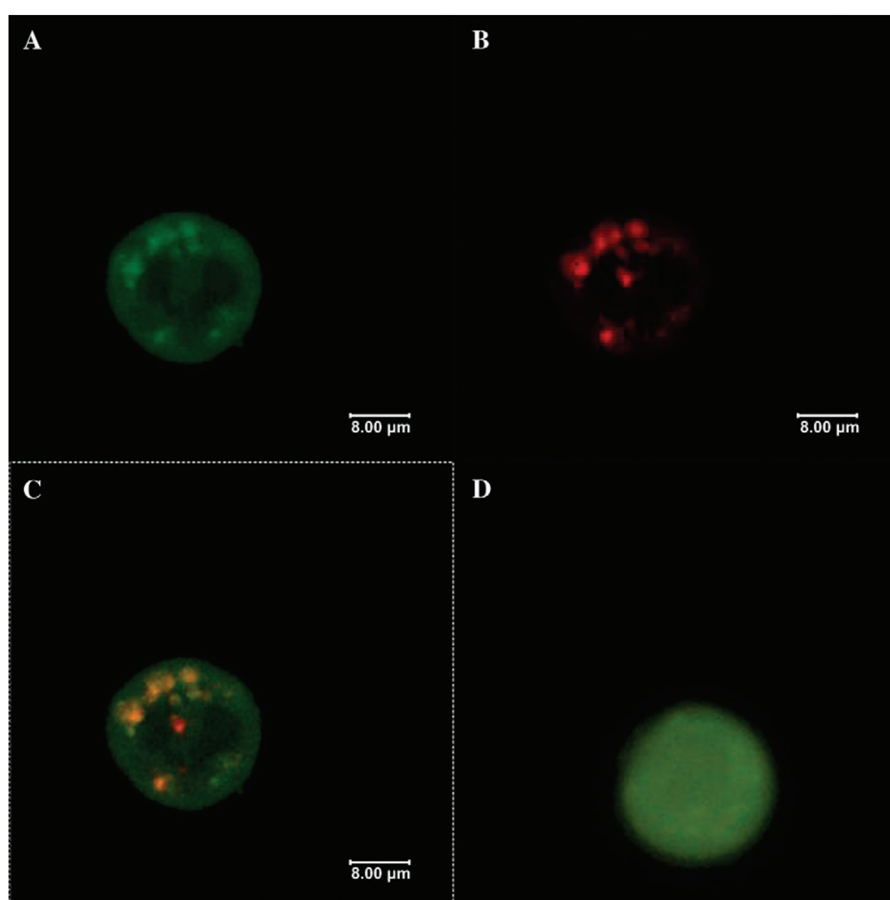

Fig. 8. High definition confocal microscopy of cytoplasts incubated for 2 h in 5.6 mM 2-NBDG and 1.0 mg ml^{-1} d-TR. Cytoplasts were observed under filters for (A) fluorescin, (B) Texas Red, and (C) a combined fluorescin/Texas Red filter. (D) Cytoplasts preincubated for 30 min in 33 μM of the endocytic inhibitor wortmannin-A prior to the addition of 5.6 mM 2-NBDG and 1.0 mg ml^{-1} d-TR for 2 h, and observed under a combined fluorescin/Texas Red filter.

the vacuole (Fig. 5), and by the prominent appearance of intracellular vesicles containing 2-NBDG and d-TR within cytoplasts after 2 h incubation (Fig. 8). These results are in accordance with a previous study (Etxeberria *et al.*, 2005*a*) in which an initial wortmanin-A-insensitive sucrose uptake corresponded to carrier-mediated transport, whereas the subsequent wortmanin-A-sensitive uptake shared characteristics of endocytosis.

It is important to note that low levels of 2-NBDG were commonly observed in the vacuole of cultured cells after prolonged incubation (i.e. 24 h) in the presence of wortmannin-A. It is very possible that wortmannin-A did

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not entirely block the endocytic process after 24 h, or that inhibition of endocytosis may enhance existing or alternative pathways on a long-term basis. Partial transport of hexoses from the cytosol into the vacuole may occur by means of tonoplast-bound hexose carriers, inasmuch as these transporters have been identified in the vacuole of plant cells (Lalonde *et al.*, 1999; Martinoia *et al.*, 2000). However, it is clear that the bulk of hexose to be accumulated into the vacuole is transported in mass by a vesicle-mediated endocytic system and that tonoplast-bound hexose carriers may well be a likely mechanism for the fine regulation of cytosolic hexose concentration.

Finally, although the experiments described in this work were conducted exclusively with cultured cells, it is believed that the conclusions on the existence of two parallel uptake systems for glucose to be a general characteristic of intact heterotrophic tissues. This contention is based on the widespread presence of plasmalemma sugar carriers (as indicated in the Introduction) and the fact that endocytosis and movement of external solutes has already been demonstrated in a wide variety of tissues including intact *Citrus* juice cells (Etxeberria *et al.*, 2005*b*), in turnip roots (E Etxeberria, unpublished results), maize root cortex (Baluška *et al.*, 2004), tobacco cells (Yano *et al.*, 2004), and many others (see Etxeberria *et al.*, 2005*a*, for references).

Acknowledgement

This research was supported by the Florida Agricultural Experiment Station and approved for publication as Journal Series No. R-10672.

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