An Important Pool of Sucrose Linked to Starch Biosynthesis is Taken up by Endocytosis in Heterotrophic Cells

Edurne Baroja-Fernández 1, Ed Etxeberria 2, Francisco José Muñoz 1, María Teresa Morán-Zorzano 1, Nora Alonso-Casajús 1, Pedro Gonzalez 2 and Javier Pozueta-Romero 1, *

1 Agrobiotecnologia institutu, Nafarroako Unibertsitate Publikoa, Gobioio de Navarra and Consejo Superior de Investigaciones Científicas, Mutxitoako etorbidea zenbait gabe, 31192 Mutxitoa, Nafarroa, Spain
2 University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850, USA

We have recently shown the occurrence of endocytic sucrose uptake in heterotrophic cells. Whether this mechanism is involved in the sucrose–starch conversion process was investigated by comparing the rates of starch accumulation in sycamore cells cultured in the presence or absence of the endocytic inhibitors wortmannin and 2-(4-morpholynyl)-8-phenyl-4H-1 benzopyran-4-1 (LY294002). These analyses revealed a two-phase process involving an initial 120 min wortmannin- and LY294002-insensitive starch accumulation period, followed by a prolonged phase that was arrested by the endocytic inhibitors. Both wortmannin and LY294002 led to a strong reduction of the intracellular levels of both sucrose and the starch precursor molecule, ADPglucose. No changes in maximum catalytic activities of enzymes closely linked to starch and sucrose metabolism occurred in cells cultured with endocytic inhibitors. In addition, starch accumulation was unaffected by endocytic inhibitors when cells were cultured with glucose. These results provide a first indication that an important pool of sucrose incorporated into the cell is taken up by endocytosis prior to its subsequent conversion into starch in heterotrophic cells. This conclusion was substantiated further by experiments showing that sucrose–starch conversion was strongly prevented by both wortmannin and LY294002 in both potato tuber discs and developing barley endosperms.

Keywords: Acer pseudoplatanus — Hordeum vulgare — Sink strength — Solanum tuberosum, Sucrose–starch conversion — Sucrose synthase.

Abbreviations: LatB, latrunculin B; LY, Lucifer yellow-CH; LY294002, 2-(4-morpholynyl)-8-phenyl-4H-1 benzopyran-4-1; SuSy, sucrose synthase.

Introduction

The arrival of sucrose at terminal sink organs is a dynamic process that can take a symplastic and/or an apoplastic route depending on the type of organ and developmental state (Patrick 1997). In either case, the overall ability of the heterotrophic organ to attract photoassimilates is strongly dependent on the capacity of individual cells to import, metabolize and store sucrose, ultimately determining plant productivity and crop yield (Ho 1988).

In organs where apoplastic sucrose unloading occurs, different routes can be envisaged for the subsequent uptake into storage cells: (i) cleavage of sucrose by an apoplastic invertase and subsequent uptake of glucose and fructose (Shannon 1972, Lalonde et al. 1999, Fernie et al. 2000, Oliveira et al. 2002, Weschke et al. 2003); and/or (ii) import of sucrose by plasma-lemma-bound carriers (Tegeder et al. 1999, Barker et al. 2000, Noiraud et al. 2000, Weshcke et al. 2000, Manning et al. 2001, Aoki et al. 2002, Kühn et al. 2003) (Fig. 1 and Supplementary Fig. 1). In addition, and as evidenced by recent studies using citrus juice cells and suspension-cultured cells of sycamore (Acer pseudoplatanus L.), sucrose can also be taken up by endocytosis and transported to the central vacuole (Etxeberria et al. 2005a, Etxeberria et al. 2005b). Subsequent conversion of internalized sucrose to starch involves a series of enzymatic reactions wherein sucrose synthase (SuSy) (EC 2.4.1.13) predominantly controls sucrose breakdown and production of a C-6 starch precursor molecule entering the amyloplast (Keeling et al. 1988, Appeldoorn et al. 1997, Hatzfeld and Stitt 1990, Etxeberria-Fernández et al. 2003). Because of its importance in the sucrose–starch conversion process, SuSy is considered to be a major determinant of sink strength in heterotrophic organs (Geigenberger and Stitt 1993, Martin et al. 1993, Fu and Park 1995, Zrenner et al. 1995, Ruan et al. 2003)

In addition to its role as the major transported assimilate, there is now compelling evidence that sucrose is an important mobile signal that regulates a variety of different genes and processes in various plant organs (Chiou and Bush 1998, Sokolov et al. 1998, Lalonde et al. 1999, Barker et al. 2000, Smeekens 2000, Yang et al. 2004). In heterotrophic cells, for instance, numerous genes and metabolic pathways involved in starch biosynthesis are induced or stimulated by sucrose (Fu et al. 1995, Geiger et al. 1998, Müller-Röber et al. 1990, Fernie et al. 2001), as is the endocytic system for sucrose uptake (Etxeberria et al. 2005a).

The existence of two entry points for sucrose into storage cells may represent the channeling of sucrose to different meta-
Endocytic sucrose uptake and starch biosynthesis

Fig. 1  Suggested metabolic pathways for the conversion of apoplastic sucrose to starch in heterotrophic cells involving plasmalemma-bound carriers as well as vesicle-mediated sucrose transport between the apoplast and the vacuole (Etxeberria et al. 2005a). Apoplastic sucrose can be converted into glucose and fructose by means of apoplastic invertase. Hexoses enter the cell by plasmalemma-bound carriers and/or diffusion (Stubbbs et al. 2004), and are converted into sucrose (Shannon 1972, Geiger et al. 1998, Fernie et al. 2000, Fernie et al. 2002). Alternatively, sucrose can be taken up from the apoplast by means of plasmalemma-bound sucrose carriers and/or endocytosis to be transported to the cytosol or to the vacuole, respectively. Vacuolar sucrose can be exported to the cytosol by means of a tonoplast-bound sucrose transporter (Getz 1991, Keller 1992). Cytosolic sucrose is then converted into ADPglucose and fructose by means of soluble SuSy or membrane-bound SuSy (Amor et al. 1995, Carlson and Chourey 1996, Baroja-Fernández et al. 2003, Etxeberria and Gonzalez 2004, Muñoz et al. 2005). According to this suggested model, SuSy catalyzes the production of cytosolic ADPglucose entering the amyloplast by means of an ADPglucose translocator (Pozueta-Romero et al. 1991, Shannon et al. 1996, Shannon et al. 1998, Patron et al. 2004), whereas ADPglucose pyrophosphorylase is involved in the scavenging of starch breakdown products (Pozueta-Romero et al. 1999, Baroja-Fernández et al. 2004, Baroja-Fernández et al. 2005, Muñoz et al. 2005). Membrane recycling takes place essentially as described by Echeverria (2000). A model involving the production of UDPglucose from sucrose by SuSy and its stepwise conversion into hexose phosphates and ADPglucose is illustrated in Supplementary Fig. 1. Whether sucrose occurring in vesicles can be transported to the cytosol by means of hypothetical membrane-bound sucrose carriers is being currently investigated.

bolic events, with both mechanisms contributing separately to the overall sink strength. As a first step to investigate to what extent endocytic sucrose uptake is involved in the control of both sink strength and starch biosynthesis in heterotrophic cells, we employed the well characterized sycamore cultured cells (Rébeillé et al. 1985, Frehner et al. 1990, Pozueta-Romero et al. 1991, Etxeberria et al. 2005a), potato (Solanum tuberosum L.) tuber slices (Viola and Davies 1991, Geiger et al. 1998, Fernie et al. 2001) and developing barley (Hordeum vulgare) endosperms. In addition, we employed the potent endocytic inhibitors wortmannin (Emans et al. 2002) and 2-(4-morpholynyl)-8-phenyl-4H-1 benzopyran-4-1 (LY294002) (Vlahos et al. 1994), which do not exert any inhibitory effect on electrogenic sucrose transport (Etxeberria et al. 2005a), and latrunculin B (LatB). The results presented in this work provide a first indication that an important pool of apoplastic sucrose must be taken up by endocytosis prior to its subsequent conversion into starch in heterotrophic organs of both mono- and dicotyledonous plants.
Results and Discussion

Starch accumulation is strongly reduced by endocytic inhibitors in sycamore cells cultured with sucrose

By employing both sycamore cultured cells and citrus juice cells, as well as the potent endocytic inhibitors wortmannin and LY294002, we recently provided strong evidence that endocytosis is an important mechanism for sucrose uptake in heterotrophic cells (Etxeberria et al. 2005a). Time course analyses of sucrose accumulation in walled cells revealed a two-phase process involving an initial 120 min period of carrier-mediated sucrose uptake, followed by a prolonged phase of rapid sucrose accumulation that was greatly inhibited by the endocytic inhibitors wortmannin and LY294002 (cf. Fig. 1, Etxeberria et al. 2005a).

To investigate whether sucrose taken up by endocytosis is involved in the starch biosynthetic process, a time course analysis of starch accumulation was performed using 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. The rationale behind this experimental approach was that, if endocytic uptake of sucrose is unimportant in the overall sucrose–starch conversion process, the rate of starch accumulation in cells cultured in the presence of endocytic inhibitors would not be affected when compared with control cells. Conversely, if sucrose linked to starch biosynthesis enters via endocytosis, cells cultured in the absence of endocytic inhibitors will accumulate starch more rapidly than cells cultured in the presence of endocytic inhibitors.

As illustrated in Fig. 2, the rate of starch accumulation in cells cultured with 50 mM sucrose was approximately 3 µmol of glucose transferred to starch g⁻¹ FW during the initial 90 min of culture, followed by an approximately 30 min asymptotic phase of low starch accumulation. Similar to the time course pattern of sucrose incorporation in sycamore cells (cf. Fig. 1, Etxeberria et al. 2005a), starch accumulation during this initial 120 min culture period was insensitive to endocytic inhibitors, indicating that sucrose uptake linked to the initial stages of starch biosynthesis in starved cells does not take place by endocytosis but presumably by plasmalemma-bound carriers. Then starch accumulation entered a second prolonged phase where starch accumulated at a rate of 1.5 µmol glucose transferred to starch g⁻¹ FW h⁻¹. This phase was strongly repressed by endocytic inhibitors, the rate of starch accumulation declining to 250 nmol glucose transferred to starch g⁻¹ FW h⁻¹ in the presence of either 500 nM wortmannin or LY294002. The overall data thus suggest that, after a 2 h culture period, an important pool of sucrose linked to starch biosynthesis enters the cell via endocytosis in starved cells. Strongly substantiating these observations was the sharp reduction in the rate of starch accumulation when wortmannin was added after 90 min of culture, a time when sucrose starts entering the cell by endocytosis (cf. Fig. 1, Etxeberria et al. 2005a).

Endocytic inhibitors lead to reduced intracellular levels of ADPglucose, but not of hexose phosphates

We measured the intracellular levels of metabolites involved in the metabolism of both starch and sucrose in cells cultured with sucrose both in the presence and in the absence of 500 nM LY294002. As shown in Table 1, analyses of the intracellular levels of sugars after 5 h of culture revealed no significant effect of LY294002 on the levels of hexoses and hexose phosphates. Subsequent time course analysis confirmed that LY294002 does not exert any significant effect on the intracellular levels of hexoses and hexose phosphates during 6 h of culture (not shown). In clear contrast, however, intracellular levels of sucrose, starch, UDPglucose and the starch precursor molecule ADPglucose in cells cultured with LY294002 were significantly lower than those of control cells.

We then performed a time course analysis of sucrose accumulated and intracellular ADPglucose content in cells previously starved for 24 h prior to re-introduction of sucrose both in the presence and in the absence of LY294002. As illustrated in Fig. 3, this analysis revealed that, similarly to sucrose, ADPglucose accumulation follows a two-phase process involving an initial 120 min LY294002-insensitive period, followed by a prolonged phase of ADPglucose accumulation which was inhibited by LY294002. This result strongly indicates that ADPglucose biosynthesis is intimately linked to the metabolism of sucrose taken up by endocytosis. Essentially similar
results were obtained when investigating the effect of LY294002 on the intracellular levels of UDPglucose. However, as illustrated in Supplementary Fig. 2, reduction of UDPglucose levels by LY294002 was less pronounced than that of ADPglucose intracellular levels.

The fact that hexose phosphate levels are normal in cells cultured in the presence of LY294002, whereas sucrose, ADPglucose, UDPglucose and starch levels are significantly lower in cells cultured in the presence of LY294002 than in control cells indicates that (i) metabolism of sucrose taken up by endocytosis is closely connected to that of both ADPglucose and UDPglucose, most probably by means of SuSy; (ii) the hexose phosphate pool is not directly connected to that of sucrose taken up by endocytosis, but most probably it is connected to the pool of sucrose and/or hexoses taken up by plasmalemma-bound carriers; and (iii) hexose phosphates are not intermediates in the sucrose–starch conversion process (Baroja-Fernández et al. 2003). In addition, the fact that the effect of LY294002 on the UDPglucose levels was less pronounced than on ADPglucose levels suggests that the UDPglucose pool is not solely connected to sucrose taken up by endocytosis, but also to hexose phosphates, presumably by means of UDPglucose pyrophosphorylase (EC 2.7.7.9).

Table 1  Metabolite levels in sycamore cells cultured with 50 mM sucrose in the presence and in the absence of 500 nM LY294002

<table>
<thead>
<tr>
<th>Metabolite (nmol g⁻¹ FW)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +LY294002</td>
</tr>
<tr>
<td>ADPglucose</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>UDPglucose</td>
<td>481 ± 20.9</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>343 ± 20.5</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>126 ± 20.0</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>120 ± 18.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>3,880 ± 410</td>
</tr>
<tr>
<td>Fructose</td>
<td>5,300 ± 1,170</td>
</tr>
<tr>
<td>Accumulated sucrose</td>
<td>19,600 ± 940</td>
</tr>
<tr>
<td>Starch</td>
<td>22,500 ± 1,600</td>
</tr>
<tr>
<td>Accumulated starch</td>
<td>8,600 ± 530</td>
</tr>
</tbody>
</table>

Cells were harvested after 5 h of culture. The results are given as the mean ± SE of extracts from four cultures. Metabolite levels are given as nmol g⁻¹ FW. Starch content and starch accumulated after 5 h of culture are given as nmol glucose g⁻¹ FW. ‘Accumulated starch’ is the difference between starch content after 5 h of culture and the starch content at zero time. Starch content at zero time (upon addition of sucrose to the culture medium) was 13,900 ± 1,300 nmol glucose g⁻¹ FW. ‘Accumulated sucrose’ is the difference between sucrose content after 5 h of culture and the sucrose content at zero time. Sucrose content at zero time was 9,000 nmol g⁻¹ FW. Values significantly different from those of control cells are indicated in bold.

Fig. 3  Time course of (A) accumulated sucrose and (B) intracellular ADPglucose content in sycamore cells cultured with 50 mM sucrose in the absence (filled squares) or in the presence (open circles) of 500 nM LY294002. ‘Accumulated sucrose’ is the difference between sucrose content at the indicated culture time and the sucrose content at zero time of culture. Sucrose content at zero time was 9 µmol g⁻¹ FW. The time course of UDPglucose is shown in Supplementary Fig. 2. Experimental conditions are the same as those of Fig. 2. Results are given as the mean ± SE of four analyses per time point.

Reduction of both ADPglucose levels and rate of starch accumulation by endocytic inhibitors is not accompanied by changes in activities of enzymes directly connected to starch and sucrose metabolism

Whether reduction of both ADPglucose and starch levels by inhibitors of endocytic sucrose uptake was a secondary effect of arrested enzyme activities was investigated by measuring maximum catalytic activities of a broad range of enzymes closely connected to starch and sucrose metabolism. As shown in Table 2, these analyses revealed no significant changes in ADPglucose pyrophosphorylase (EC 2.7.7.27), alkaline pyrophosphatase (EC 3.6.1.1), UDPglucose pyrophosphorylase, phosphoglucomutase (EC 2.7.5.1), SuSy, sucrose phosphate synthase (EC 2.3.1.14), acid invertase (EC 3.2.1.26), total starch synthase (EC 2.4.1.21), starch phosphorylase (EC 2.4.1.1) and total amylolytic activity between cells cultured in the presence or absence of wortmannin and
LY294002. Therefore, the reduction of both ADPglucose and starch levels in cells cultured with sucrose in the presence of wortmannin and LY294002 is ascribable to the inhibition of the endocytic uptake of sucrose and not to a reduction in the activities of enzymes of sucrose–starch conversion.

Starch accumulation from glucose is unaffected by endocytic inhibitors

Glucose enters the heterotrophic cell by either carrier-mediated transport, diffusion or endocytosis (Stubbs et al. 2004, Etxeberria et al. 2005c). Subsequent conversion of the internalized glucose to starch involves a series of enzymatic reactions in the cytosol and the amyloplast which are common to those involved in the sucrose–starch conversion (Geiger et al. 1998, Sokolov et al. 1998, Fernie et al. 2001). To investigate further whether reduction of both ADPglucose and starch levels by inhibitors of endocytic sucrose uptake was a secondary effect of arrested gluconeogenic machinery, and to investigate whether an important pool of glucose destined to starch production is also taken up by endocytosis, we compared rates of starch accumulation in cells cultured with glucose both in the presence and in the absence of either 500 nM wortmannin or LY294002.

As illustrated in Fig. 4, sycamore cells cultured with glucose accumulated starch at a rate comparable with cells cultured with sucrose (Fig. 2). In addition, the glucose–starch conversion process was unaffected by the endocytic inhibitors, indicating that, in contrast to sucrose, the bulk of glucose destined to starch biosynthesis enters the cell by plasmalemma-bound carriers and/or diffusion (Stubbs et al. 2004). Furthermore, the data confirm that endocytic inhibitors do not negatively affect the enzymatic machinery involved in starch biosynthesis.

Rate of starch accumulation is highly reduced by endocytic inhibitors in potato tuber discs and developing barley endosperms

Having shown that a large proportion of apoplastic sucrose linked to starch biosynthesis is taken up by endocytosis in sycamore cells, we proceeded to investigate whether this process also occurs in other species. Towards this end, we performed a time course analysis of starch production in both potato tuber discs and developing barley endosperms cultured with radiolabeled sucrose in the presence and in the absence of endocytic inhibitors as indicated in Materials and Methods.
Endocytic sucrose uptake and starch biosynthesis

As illustrated in Fig. 5, control rates of starch accumulation in developing barley endosperms and potato tuber discs were 65 and 5 nmol glucose transferred to starch g\(^{-1}\) FW h\(^{-1}\), respectively. Remarkably, and essentially identically to the case of cultured cells of sycamore (Fig. 2), rates of starch accumulation were markedly reduced when endocytic inhibitors were included in the culture medium. The overall data are thus a first indication that a sizable pool of sucrose linked to starch biosynthesis is taken up by endocytosis in different heterotrophic organs of both mono- and dicotyledonous species.

Additional remarks

Whereas much has been written about the enzymatic pathways involved in both sink strength and the sucrose–starch conversion process in heterotrophic organs, the possible involvement of different mechanisms of apoplastic sucrose transport into the cell has not been considered as part of the overall regulatory system. Our data offer the first direct evidence indicating that, essentially as illustrated in Fig. 1, and as inferred from the results of Table 1 and Fig. 2 and 5 showing that endocytic inhibitors prevent the sucrose–starch conversion process, apoplastic sucrose linked to starch biosynthesis enters the cell through two entirely different routes: by means of plasmalemma-bound carriers and by endocytosis. That both wortmannin and LY294002 strongly inhibit the sucrose–starch conversion process (Fig. 2) is consistent with previous reports showing that, although apoplastic glucose can be converted into starch (Geiger et al. 1998) (Fig. 4), cleavage of sucrose by apoplastic invertase and subsequent import of glucose into the cell plays a minor role in the overall starch biosynthetic process of some heterotrophic organs (Ross et al. 1994, Appeldoorn et al. 1997, Hajirezaei et al. 2000).

The results presented in this work are consistent with the view that, at least in some heterotrophic organs, SuSy predominantly controls sucrose breakdown and production of precursor(s) for starch biosynthesis (Zrenner et al. 1995), whereas apoplastic invertase catalyzes the hydrolytic breakdown of sucrose necessary for processes such as phloem unloading, cell growth and differentiation, osmoregulation, defense response, etc. (Miller and Chourey 1992, Sonnewald et al. 1997, Tauberger et al. 1999, for a review see Sturm and Tang 1999).

In addition, and in agreement with the idea that apoplastic sucrose may act as a signal that regulates carbohydrate metabolism (Lalonde et al. 1999, Smeekens 2000, Fernie et al. 2001), combined data of Fig. 2 and 4 strongly indicate that conversion of apoplastic glucose into starch is prevented by sucrose.

It is important to point out that, as discussed by Etxeberria et al. (2005a), endocytic uptake of apoplastic sucrose and its subsequent conversion to starch does not conflict with transport through membrane-bound carriers. Our data using starved sycamore cells indicate that carrier-mediated sucrose import may play a crucial role in the overall sucrose–starch conversion process during the initial period of sucrose unloading (i.e. during the initial part of the day), whereas endocytic uptake may play an important role in transporting the bulk of sucrose to the vacuole after a prolonged period of sucrose unloading.
Endocytic sucrose uptake and starch biosynthesis

453

Tonoplast-bound sucrose carriers (Keller 1992) and SuSy (Etcheberria and Gonzalez 2004) would subsequently mediate export of vacuolar sucrose and conversion to starch intermediates. Whether endocytic vesicles are equipped to export sucrose to the cytosol is still a matter of conjecture, which is presently under investigation.

Repression of starch biosynthesis by inhibitors of endocytic sucrose uptake was not limited to compounds interfering with phosphatidylinositol 3-kinase such as wortmannin and LY294002 (Li et al. 1995, Brunskill et al. 1998). The F-actin-depolymerizing drug LatB, which blocks the internalization of fluid-filled endocytic vesicles (Baluska et al. 2004), markedly reduced the endocytic uptake of both Lucifer yellow-CH (LY) (Fig. 6A) and sucrose (Fig. 6B) in starved sycamore cells. Most importantly, and as illustrated in Fig. 6C, sucrose–starch conversion was repressed after 2 h of culture with 5 µM LatB. The overall data thus further strengthen the view that a sizable pool of sucrose linked to starch biosynthesis is taken up by endocytosis.

The fact that endocytic inhibitors lead to reduced levels of UDPglucose (Table 1 and Supplementary Fig. 2) appears to indicate that, in addition to its involvement in the sucrose–starch conversion process, endocytic sucrose uptake may be an important determinant of UDPglucose-dependent glucono-geic processes such as synthesis of cell wall polysaccharides, glycoproteins, etc. (Amor et al. 1995, Muñoz et al. 1996, Buckeridge et al. 1999, Delmer 1999, Ruan et al. 2003, Konishi et al. 2004). Further studies will be necessary to confirm this idea.

Materials and Methods

Plant material

Sycamore (A. pseudoplatanus L) cells were cultured in a medium supplemented with 50 mM sucrose as described by Frehner et al. (1990) in continuously agitated 250 ml flasks (200 rpm at 28°C). The 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 either 50 mM sucrose or 50 mM glucose in the presence or absence of 0.5 µM wortmannin (W-1628, Sigma-Aldrich, St Louis, MO, USA) or 0.5 µM LY294002 (L-9908, Sigma-Aldrich). Cell aliquots were taken at the indicated times, washed with starving medium, filtered through Miracloth and ground to a fine powder in liquid nitrogen. Barley (cv. Scarlett) plants were grown in the fields of the Public University of Nafarroa. Potato (cv. Desirée) plants were cultivated in growth chambers in individual pots under a 16 h light (21°C)/8 h dark (16°C) regime.

Enzyme assays

All enzymatic reactions were performed at 37°C. Cultured cells of sycamore were harvested, washed with starving medium, filtered through Miracloth and immediately freeze-clamped and ground to a fine powder in liquid nitrogen with a pestle and mortar. To assay enzyme activity, 1 g of the frozen powder was resuspended at 4°C in 5 ml of 100 mM HEPES (pH 7.5), 2 mM EDTA and 5 mM dithiothreitol (DTT). The suspension was then desalted and assayed for...
enzymatic activities. The following enzymes were assayed as described by Baroja-Fernández et al. (2004): ADPglucose pyrophosphorylase, ADPglucose producing SuSy, acid invertase, UDPglucose pyrophosphorylase, alkaline pyrophosphatase, total starch synthase, starch phosphorylase, total amylolytic activity, phosphoglucomutase and sucrose phosphate synthase. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the production of 1 µmol of product min⁻¹.

Determination of sugars

Cultured cells of sycamore were harvested and immediately ground to a fine powder in liquid nitrogen with a pestle and mortar. A 0.5 g aliquot of the frozen powder was resuspended in 0.4 ml of 1.4 M HClO₄, left at 4°C for 2 h and centrifuged at 10,000 g for 5 min. The supernatant was neutralized with K₂CO₃ and centrifuged at 10,000 g. ADPglucose content in the supernatant was determined by using either one of the following methods: (i) assay A by HPLC on a system obtained from P.E. Waters and Associates fitted with a Partisil-10-SAX column as described by Sweetlove et al. (1996); or (ii) assay B by HPLC with pulsed amperometric detection on a DX-500 system (Dionex) fitted to a CarboPac PA10 column.

To confirm further that measurements of ADPglucose were correct, ADPglucose eluted from either the Partisil-10-SAX or CarboPac PA10 columns was enzymatically hydrolyzed with purified Escherichia coli ADP-sugar pyrophosphatase (Moreno-Bruna et al. 2001) and assayed for conversion into AMP and glucose-1-phosphate as described by Muñoz et al. (2005).

Glucose, sucrose, fructose, glucose-1-phosphate, fructose-6-phosphate and glucose-6-phosphate were determined by HPLC with pulsed amperometric detection on a DX-500 system as described by Muñoz et al. (2005). Starch was extracted and measured by using an amyloglucosidase-based test kit as described by Baroja-Fernández et al. (2004).

Labeling experiments

Tuber discs (diameter of 8 mm, thickness of 2 mm) were cut directly from growing tubers attached to the mother plant and washed three times in a 20-fold excess of medium containing 10 mM HEPES-KOH buffer (pH 7.0) and 100 mM sucrose (Viola and Davies 1991). Both tuber discs and barley endosperms at nearly 15 d after pollination (eight discs and 30 endosperms in a volume of 5 ml in a 100 ml Erlenmeyer flask) were incubated in medium including [U-¹³C]sucrose (5×10⁵ dpm/µl) (Amersham, UK), shaken at 90 rpm, harvested at the indicated incubation periods, washed three times in abundant medium to remove unabsorbed sucrose and debris, frozen in liquid nitrogen and homogenized in a mortar. The powder thus obtained was washed thoroughly with abundant 75% methanol/1% KCl, and ¹⁴C incorporation into starch was determined as described by Viola and Davies (1991). In all cases, >90% of the radiolabel released after digesting starch with amyloglucosidase was recovered as glucose following HPLC separation.

Uptake of LY

Four-day-old walled sycamore cells were starved for 24 h. At this time, the culture medium was replaced with fresh medium containing 50 mM sucrose and 1 mg/ml LY (Molecular Probes, Eugene, OR, USA) both in the presence and in the absence of 5 µM LatB (Calbiochem, Germany). Aliquots were taken at the indicated times and protoplasts prepared as described by Frehner et al. (1990). From the protoplast preparation, aliquots were taken and LY was analyzed fluorometrically in a Fluoroskan Neonat (Labystems, Hampshire, UK) with an excitation/emission of 425/550 nm, respectively.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.ccp.oupjournals.org.

Acknowledgments

This research was partially supported by the grant BIO2004-01922 from the Comisión Interministerial de Ciencia y Tecnología and Fondo Europeo de Desarrollo Regional (Spain). M.T.M.-Z. acknowledges the Spanish Ministry of Culture and Education for a pre-doctoral fellowship. E.E. expresses his gratitude to the Spanish Ministry of Culture and Education, to the Consejo Superior de Investigaciones Científicas and to the Public University of Nafarroa for their support. We express our gratitude to Beatriz Zugasti for technical assistance and help.

References


(Received December 5, 2005; Accepted January 17, 2006)