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Dehydroascorbate and glucose are taken up into *Arabidopsis thaliana* cell cultures by two distinct mechanisms

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Abstract **The possible involvement of glucose (Glc) carriers in the uptake of vitamin C in plant cells is still a matter of debate. For the first time, it was shown here that plant cells exclusively take up the oxidised dehydroascorbate (DHA) form. DHA uptake is not affected by 6-bromo-6-deoxy-ascorbate, an ascorbate (ASC) analogue, specifically demonstrating ASC uptake in animal cells. There is no competition between Glc and DHA uptake. Moreover, DHA and Glc carriers respond in the opposite manner to different inhibitors (cytochalasin B, phloretin and genistein). In conclusion, the plant plasma membrane DHA carrier is distinct from the plant Glc transporters.**

Keywords: Vitamin C; Dehydroascorbate transport; Glucose transport; Plasma membrane; Plant cells

1. Introduction

In *Arabidopsis* seedlings, ascorbate (ASC) biosynthesis is confined to the so-called Smirnoff–Wheeler pathway starting from guanosine diphosphate-mannose [1]. The final step, converting L-galactonolactone into ASC is mediated by L-galactonolactone dehydrogenase, an enzyme exclusively present on the inner mitochondrial membrane [2]. From its site of synthesis ASC is transported throughout the plant cell. In physiological conditions with a pH above 5, ASC nor its oxidised form dehydroascorbate (DHA) will easily diffuse through a lipid bilayer supporting the necessity of specific plant transport systems for ASC and DHA [3]. A plant vitamin C transporter was first described in the chloroplast [4,5] showing a low affinity ASC transport mechanism (K_m 20 mM, 18–40 mM). High affinity transporters with K_m values ranging from 40 to 139 μM are present in the plasma membrane [6], the thylakoid membranes [7] and the inner mitochondrial membrane [8].

Although the presence of plasma membrane vitamin C transport systems seems to be a general feature of all plant tissues investigated so far, data on the redox state of the transported molecules (ASC or DHA) and on the possible involvement of hexose transporters are still inconclusive (for review see [6]). In animal tissues ASC is transported through sodium-dependent transporters [9,10], whereas DHA is transported through glucose carriers (glucose transporter (GLUT) [11]). In plants, both glucose (Glc) and DHA uptake have recently been shown to change under oxidative stress conditions [12–14]. For example, immediately after addition of the pathogenic elicitor cryptogein, Glc uptake activity decreased rapidly [12]. This initial decrease in Glc uptake could be lifted by treatment of the cells with $LaCl_3$, a Ca-channel blocker but was not affected by diphenyleniodonium (DPI), a known inhibitor of NADPH-oxidase [12]. Intriguingly, the DHA transporter of plant cells also decreased in response to oxidative stress evoked by $CdCl_2$ [14] or cryptogein (Horemans, unpublished results). The timing of the DHA transport response was strikingly slower then that of the Glc carrier responding to elicitation with cryptogein [12]; hours compared to seconds. On the other hand, similar to what is observed for the Glc carrier, the decrease of DHA uptake after a $CdCl_2$ treatment was not affected by DPI, but returned to control values in the presence of $LaCl_3$ [14]. Based on this similar response, we investigated the possible interaction between Glc and DHA uptake in *Arabidopsis* plant cell cultures.

2. Materials and methods

2.1. Plant material

Cell cultures of *Arabidopsis thaliana* (PSB-L line) were obtained from VIB (University of Ghent), and propagated in Murashige and Skoog basal salt mixture (Duchefa Biochemie, Haarlem, The Netherlands) at pH 5.7 (KOH), enriched with 30 g l^{-1} sucrose, 0.05 mg l^{-1} kinetin and 0.5 mg l^{-1} naphthaleneacetic acid at 22 °C at 100 rpm (New Brunswick Sci Co.) with a photoperiod of 16 h light (50 μE min m^2). Weekly, cells were subcultured by transferring 15–100 ml fresh medium. All experiments were performed with four to 5 d old, exponentially growing cell cultures. Cells were harvested by filtering the cell suspensions over a Büchner filter with a cellulose filter

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Abbreviations: ASC, ascorbate; BrASC, 6-bromo-6-deoxy-ASC; DHA, dehydroascorbate; DPI, diphenyleniodonium; Glc, glucose; GLUT, glucose transporter

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(grade 1) and washed once with growth medium devoid of sucrose at 22 °C. Harvested cells were resuspended in a concentration of 0.1 g FW ml^{-1} of growth medium without sucrose (pH 5.7, 22 °C).

2.2. Determination of redox status of DHA in medium

In order to manipulate the external redox state of ASC different freshly prepared redox-active compounds (concentrations see legend of Fig. 1) were mixed with ASC just prior to adding the resulting mixture to the cells. ASC was always added in a concentration of 50 μM to the cells. At different time points cells were spun down at $1000 \times g$ for 30 s. As a sample of the extra cellular medium, 100 μl aliquots of cell-free supernatant was taken, added to 200 μl of 6% (w/v) metaphosphoric acid, snap-frozen in liquid nitrogen and kept at −80 °C until HPLC analysis. Prior to HPLC analysis samples were thawed on ice and further clarified by centrifugation at $16000 \times g$ at 4 °C for 10 min.

A parallel set of experiments was run to test the effect of the changing of the external redox state of ASC on its uptake in the cells. The upset differed only in the addition of radio-labelled L-[^{14}C]ascorbic acid (Amersham, Ghent, Belgium) in stead of the non-labelled component. After 20 min, 100 μl of this mixture was diluted 50-fold with ice-cold washing medium (10 mM of non-labelled ascorbate) and further manipulated as described in the section on DHA uptake.

2.3. ASC or DHA concentration

The concentration and redox state of ASC and DHA was determined with HPLC analysis essentially as described by Horemans et al. [14]. Briefly, antioxidants were separated on a 100 mm × 4.6 mm Polaris C18-A reversed phase HPLC column (3 μm particle size; 30 °C; Varian, CA, USA) with an isocratic flow of 1 ml min^{-1} of 25 mM KPO_4-buffer (pH 3.0) and identified and quantified using a diode array detector (SPD-M10AVP, Shimadzu, Hertogenbosch, The Netherlands) on line with a home made electrochemical detector with glassy carbon electrode and a Schott pt 62 reference electrode (Mainz, Germany). The amount of oxidised DHA concentration was measured indirectly as the difference between the total concentration of ASC in a DTT reduced fraction and the concentration in the sample prior to reduction. Reduction of the sample was obtained by adding an aliquot of the extract in 400 mM of Tris and 200 mM of DTT for 15 min in the dark. The pH of this mixture was checked to be between 6 and 7. After 15 min, the pH was lowered again by four-fold dilution in elution buffer prior to HPLC analysis.

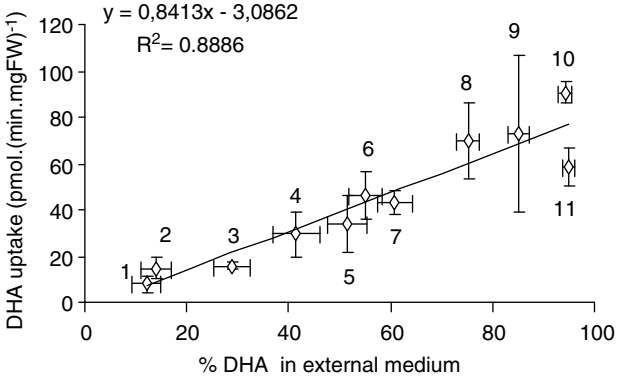

Fig. 1. Correlation between the percentage of DHA to the total vitamin C concentration in the external medium after 20 min and the uptake rate in 4 d old cells at that time point. At the start of the experiment just prior to addition to the cells 50 μM of non-radioactive or of ^{14}C-labelled ASC was mixed with redox-active compounds in following concentrations: (1) dithitreitol 50 mM, (2) dithioerythrol 10 mM, (3) dithitreitol 10 mM, (4) dithioerythrol 1 mM, (5) dithitreitol 1 mM, (6) β-mercaptoethanol 20 mM, (7) cystein 10 mM, (8) glutathione 10 mM, (9) glutathione 1 mM, (10) ascorbate oxidase 5 U and (11) control. Mean ± S.E. ($n = 3$).

2.4. DHA and Glc uptake

Uptake of DHA or Glc was measured according to Horemans et al. [14] by addition of either 50 μM of L-[^{14}C]ascorbic acid (Amersham, Ghent, Belgium) or 55 μM of [^{14}C]-Glc (Amersham, Ghent, Belgium) to 30 μl of freshly harvested cells (0.1 mg ml^{-1}) in a final volume of 100 μl of growth medium without sucrose. After 20 min cells were diluted 50-fold with ice-cold washing medium (10 mM of non-labelled ascorbate or 10 mM of non-labelled D-Glc, growth medium without sucrose), collected on a Whatman cellulose filter (grade 3M) and rinsed by the further addition of 10 ml of washing medium. The filters were dissolved in scintillation cocktail (Filter count: Packard, Brussels, Belgium). For background experiments, samples were washed immediately after addition of the radioactively labelled molecules. To determine the substrate kinetics the concentration of the radioactive Glc or DHA was varied between 0 and 300 μM.

2.5. Data analysis

Data are mean values with S.E. from three independent experiments with three replicates each (unless indicated otherwise). Significance levels were tested by a two tailed Student t-test with α of 0.05. Estimation of the K_m and V_{max} values was achieved by minimising the difference between the measured uptake values and the theoretical v values using Microsoft Excel Solver tool and the Michaelis–Menten equation $v = (V_{max} \times [S])/(K_m + [S])$ with v the initial uptake rate and $[S]$ the substrate concentration.

3. Results

Manipulation of the redox state of vitamin C in the external medium was achieved by mixing ASC with different redox-active compounds just prior to addition of the cells. As shown in Fig. 1, the ASC redox state in the external cell medium ranged from 87.9% reduced in the presence of 50 mM DTT to only 5.7% in the presence of ASC oxidase, after 20 min of treatment. Remarkably, in control conditions without any additional redox-active compound, added ASC is completely oxidised after 20 min in the presence of the cells. Samples taken at shorter time intervals (2–5 min after addition of ASC to the cells) showed that this oxidation of ASC in control conditions was instantaneous (data not shown). Moreover, this oxidation of ASC was dependent upon the presence of the cells as in cell free medium ASC remained completely reduced for at least 1 h (data not shown).

Furthermore, the capacity of the cells to take up radio-labelled ASC in the presence of different redox compounds showed a linear correlation between the amount of DHA in the external medium and the amount of radioactive DHA taken up (Fig. 1). This indicates a clear preference of the cells

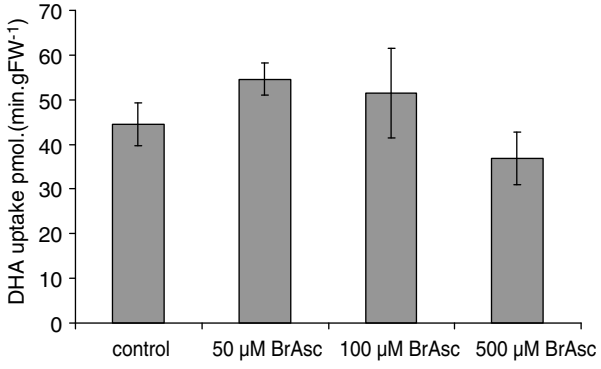

Fig. 2. Effect of different concentrations of BrASC on uptake of 50 μM ^{14}C-DHA into 4 d old cells after 20 min. Mean ± S.E. ($n = 3$).

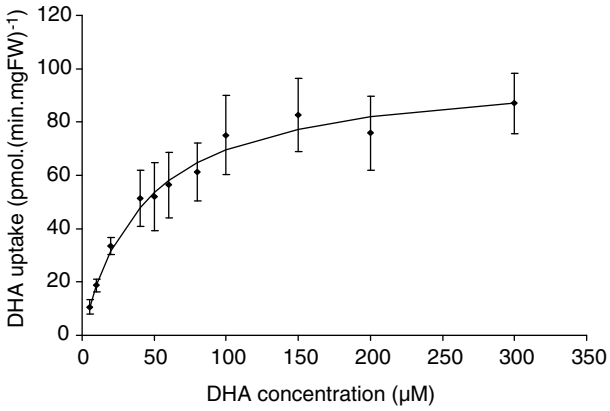

Fig. 3. Substrate kinetic of ^{14}C-DHA uptake into 4 d old cells after 20 min. Mean ± S.E. (n = 3). A curve using the estimated apparent K_m and V_{max} values was fitted onto the measured data.

for DHA. To ensure that during subsequent experiments ASC was always completely oxidised to DHA at the start of the experiment, parallel samples were run in presence and absence of an excess ASC oxidase. Even at the high DHA concentrations used in the substrate kinetic analysis, no difference was observed between the treatment with or without ASC oxidase (data not shown). From here onwards, we therefore use the term DHA uptake to describe the uptake of radio-labelled molecules added as ^{14}C-ASC to the cells.

6-Bromo-6-deoxy-ASC (BrASC) was tested as a possible competitor of DHA uptake. BrASC is an inhibitor of animal ASC transporters that has not been tested in plants before [15]. BrASC was prepared and handled as described by Corpe et al. [15]. In our cells different concentrations of BrASC could not significantly alter the rate (data not shown) nor the total amount of ^{14}C-DHA taken up into the cells after 20 min (Fig. 2).

The competition between Glc and DHA uptake was studied as follows. Firstly, it was shown that the used plant cell culture transported both compounds in a typical concentration-dependent manner showing saturation (Fig. 3 for DHA; data not shown for Glc). DHA uptake showed an apparent K_m of 42,9 μM and an apparent V_{max} of 99.5 μmol (min g FW)$^{-1}$ as determined by fitting the substrate equation onto the data (Microsoft Excel Solver tool). Uptake for both DHA and Glc was linear for at least 1 h (data not shown). In all other uptake experiments, a DHA concentration of 50 μM and a Glc of 55 μM was used as these values were close to the determined K_m value and uptake was followed for 20 min.

When Glc was added, even in concentrations up to 100-fold the ^{14}C-DHA concentration, it did not affect the amount of DHA taken up into the cells (Fig. 4A). In parallel, DHA, added in concentrations up to 10-fold the Glc concentration, did not significantly alter the amount of Glc taken up into the *Arabidopsis* cells (Fig. 4B). Furthermore, the effect of three different toxicants was tested on both Glc and DHA uptake namely; genistein (an isoflavone, known to inhibit in animals both Glc and DHA transport [16]), cytochalasin B (a mytotoxin with inhibitory effects on most Glc facilitators) and

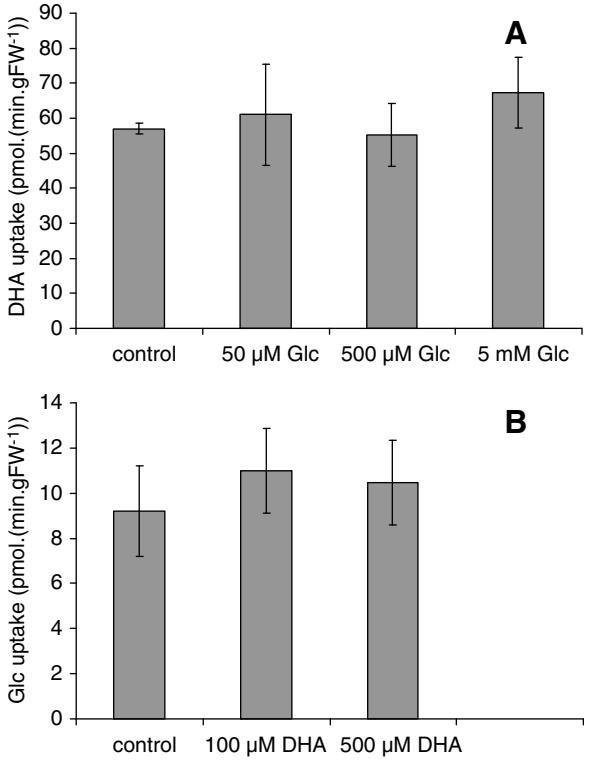

Fig. 4. Competition between Glc and DHA uptake into 4–5 d old cells after 20 min. (A) Different concentration of Glc to 50 μM of ^{14}C-DHA. (B) Different concentrations of DHA to 55 μM of ^{14}C-Glc. Mean ± S.E. (n = 3).

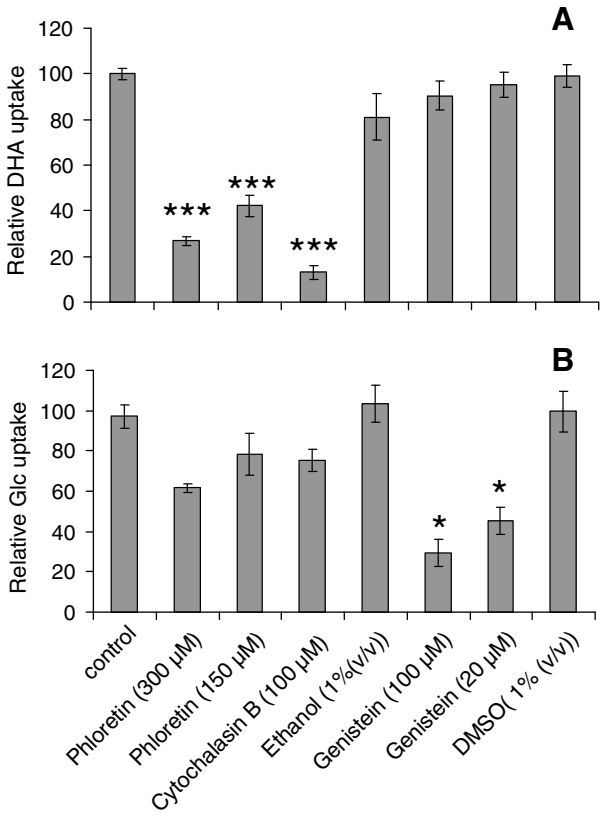

Fig. 5. Effect of different inhibitors on the uptake of (A) 50 μM of ^{14}C-DHA or (B) 55 μM of ^{14}C-Glc in 4 d old cells after 20 min. Data present mean ± S.E. of percentage uptake relative to control (n = 3 except for control: DHA uptake n = 14 and for Glc uptake n = 12).

phloretin (a flavonoid and a typical inhibitor of Glc transport in both animals and plants). Both components were added within the inhibitory concentration range [8,17]. Phloretin and cytochalasin B inhibited the uptake of Glc into the plant cells slightly but not significantly (Fig. 5A). Genistein on the other hand significantly inhibited Glc uptake in a concentration-dependent manner (Fig. 5A). However, the effect of these compounds on the uptake of DHA was completely opposite of the effect on Glc uptake (Fig. 5B). The strongest effect on DHA transport is evoked by addition of cytochalasin B (100 μM) that significantly inhibited DHA uptake with 86.8%. Phloretin (300 μM) also significantly lowered DHA transport with 73.3%. Genistein, however, did not show any inhibitory effect on DHA transport. As a control DMSO and ethanol, the solvents of these inhibitors, did not result in any significant effect on either Glc or DHA transport.

4. Discussion

Our data aimed to distinguish between ASC and DHA uptake in plants and to study to possible role of Glc carriers in uptake of vitamin C in plants. A clear correlation between the percentage of DHA in the external medium and the uptake of labelled molecules in the cells was found. Most of the redox-active compounds we used, such as DTT, DTE, mercapto-ethanol and glutathione, can interfere with thiol groups on proteins. Therefore, it is feasible that the effect on uptake activity is due to a change in the redox state of thiol-containing proteins present. However, in isolated plasma membrane vesicles sulfhydryl reagents (*p*-chloromercuribenzene sulfonic acid and *N*-ethylmaleimide) did not affect transport of DHA into the cells [18]. Moreover, under oxidative stress conditions DHA uptake decreased rather than increased as would be expected if the effect of DTT and other reducing compounds was a general effect on the redox state of the plants cells [14]. These data were therefore taken as proof that solely DHA and not ASC is taken up into the plant cell cultures. DHA uptake showed a typical saturable substrate kinetic curve with an apparent K_m and V_{max} values that are in agreement with previously obtained data in other species [6].

It is noteworthy that cells are able to quickly and completely oxidise ASC added to the cell culture medium. These data confirm earlier results using tobacco BY-2 cells [19] and bean plasma membrane vesicles, [20] indicating that the redox state of ASC in the extracellular medium of plant cell cultures is low in contrast to that of ASC in intact healthy plant tissues [3]. Although the nature of this oxidation was not further investigated, a possible source is endogenous ASC oxidase. ASC oxidase is an apoplastic enzyme shown to be actively excreted in the cell medium of pumpkin (*Cucurbita* sp.) cell suspensions and to be induced in an auxin containing MS medium (similar to the medium used to cultivate the *Arabidopsis* cells in this paper) [21].

BrASC was not able to compete with DHA uptake in our plant cells. BrASC was identified as the first analogue of ASC that is completely specific towards sodium-dependent vitamin C transporters in animal tissues and not being transported by GLUT transporters, as it can be oxidised to BrDHA but cannot cyclise into the hemiketal form that is normally transported by animal GLUT transporters [15]. In animal research it is therefore considered as a new tool to distinguish between ASC and DHA transport. The lack of effect of BrASC on the uptake of DHA is not due to instability of the oxidised BrDHA compound as both DHA and BrDHA were 100% recovered within a 20 min timespan. This was measured as the ability of extracted DHA or BrDHA to be re-reduced by DTT to ASC or BrASC prior to HPLC analysis (data not shown). This indicates that also in plants DHA is probably taken up in its hemiketal form or at least that animal-like ASC transporters are not active in plant plasma membranes.

Taken together, both the manipulation of the external redox state of ASC and the use of BrASC clearly show that no ASC but only DHA is taken up into the intact exponentially growing *Arabidopsis* cells. Earlier results performed on isolated protoplasts and plasma membrane vesicles already indicated DHA to be the preferred species [7,20]. On the other hand using pea protoplasts, Rautenkranz et al. [22] claimed that both ASC and DHA are taken up. However, they did not follow at that time the external redox state of vitamin C during their experiments and therefore the actual redox state of their transported molecules remains uncertain.

In animals, hexose transporters play a crucial role in DHA transport, as evidenced by the expression of mammalian Glc transporters (GLUT1, GLUT2 and GLUT4) in *Xenopus laevis* oocyte cells [11]. The lack of competition between BrASC and DHA uptake in our plant cells suggests that also in plant cells DHA is probably transported in its bicyclic hemiketal form. In this hemiketal form DHA shows structural similarity towards Glc [15]. In plant mitochondria competition between Glc and DHA uptake and inhibition of both Glc and DHA by genistein argued for Glc and DHA being transported by the same or at least a closely related transport system [8]. For the plant plasma membranes it was suggested in 1996 that Glc did not compete with uptake of ASC into protoplasts of pea leaf mesophyll cells [7]. However, the ASC concentrations used in this early study (4–6 mM) are much higher than the K_m values determined later, possibly hiding an effect on Glc. In purified *Phaseolus* plasma membrane vesicles, DHA uptake showed no competition with Glc or other sugars but the typical hexose transport inhibitor cytochalasin B significantly inhibited uptake [3]. However, care must be taken again when it comes to the interpretation of these experiments as both protoplasts and plasma membrane vesicles are artificial systems. This is of particular importance as the fungal cell wall degrading enzymes used in protoplast preparation cause oxidative stress with a high level of H_2O_2 production (data not shown). And as mentioned above, both Glc and DHA carriers activity responded to these type of stress [12,14]. In contrast to earlier reports, we used intact plant cells and not isolated protoplasts or membrane vesicles. Intact *Arabidopsis* cells showed no competition between DHA and Glc uptake. Moreover, these two carrier systems responded opposite to inhibitors. These data can be considered proof that in *Arabidopsis* plant cells – in strong contrast to the animal system [11] – DHA and Glc are not transported by the same carrier. A synergistic effect between DHA and Glc transport as observed in pea protoplasts [7] was not observed here. Noteworthy, for measuring Glc uptake, transport of Glc and DHA was measured in a medium not containing sugars. The average uptake rate of DHA in the *Arabidopsis* cells did not differ from that measured in the same medium containing 3% (w/v) sucrose [14], though,

indicating that sucrose does not directly influence DHA uptake either.

Interestingly, our data indicate for the first time the presence of two different types of high affinity DHA transporters being present in plant cells: a mitochondrially localised DHA transporter that is most probably similar to a Glc transporter as described in Szarka et al. [8] and one located in the plasma membrane that is distinct from a Glc carrier. An in depth study on interaction of these transport systems and ASC and sugar metabolism will contribute to understanding their physiological roles in plants.

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