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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.

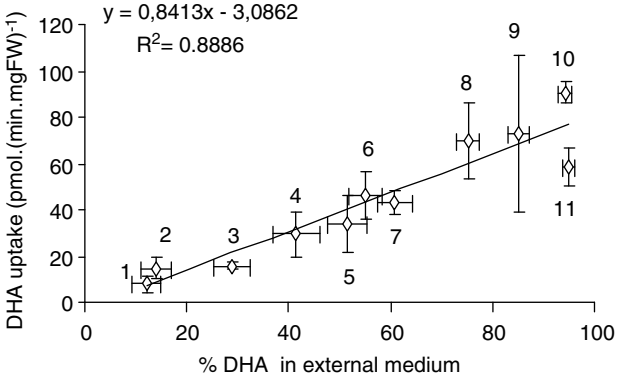

Fig. 1. Correlation between the percentage of DHA to the total vitamin C concentration in the external medium after 20 min and the uptake rate in 4 d old cells at that time point. At the start of the experiment just prior to addition to the cells 50 μM of non-radioactive or of ^{14}C-labelled ASC was 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

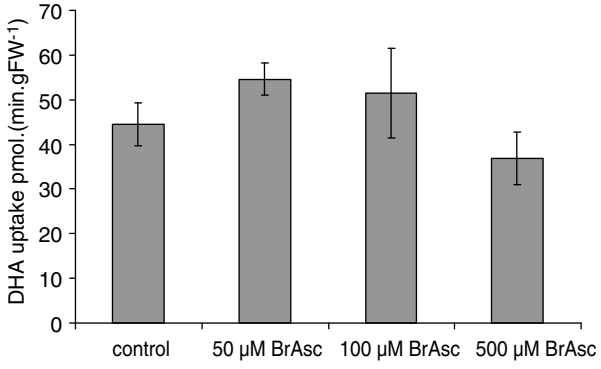

Fig. 2. Effect of different concentrations of BrASC on uptake of 50 μM ^{14}C-DHA into 4 d old cells after 20 min. Mean ± S.E. ($n = 3$).