Dehydroascorbate Uptake Activity Correlates with Cell Growth and Cell Division of Tobacco Bright Yellow-2 Cell Cultures

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Recently, ascorbate (ASC) concentration and the activity of a number of enzymes from the ASC metabolism have been proven to correlate with differences in growth or cell cycle progression. Here, a possible correlation between growth and the activity of a plasma membrane dehydroascorbate (DHA) transporter was investigated. Protoplasts were isolated from a tobacco (Nicotiana tabacum) Bright Yellow-2 cell culture at different intervals after inoculation and the activity of DHA transport was tested with 14C-labeled ASC. Ferricyanide (1 mM) or dithiothreitol (1 mM) was included in the test to keep the external 14C-ASC in its oxidized respectively reduced form. Differential uptake activity was observed, correlating with growth phases of the cell culture. Uptake of DHA in cells showed a peak in exponential growth phase, whereas uptake in the presence of dithiothreitol did not. The enhanced DHA uptake was not due to higher endogenous ASC levels that are normally present in exponential phase because preloading of protoplasts of different ages did not affect DHA uptake. Preloading was achieved by incubating cells before protoplastation for 4 h in a medium supplemented with 1 mM DHA. In addition to testing cells at different growth phases, uptake of DHA into the cells was also followed during the cell cycle. An increase in uptake activity was observed during M phase and the M/G1 transition. These experiments are the first to show that DHA transport activity into plant cells differs with cell growth. The relevance of the data to the action of DHA and ASC in cell growth will be discussed.

Ascorbate (ASC) is one of the best known antioxidants present in plants, protecting plant cells from oxidative challenges (Noctor and Foyer, 1998). However, ASC is more than just a defense molecule. A clear role in a number of plant physiological processes has been attributed to this molecule, for example, electron donation in different biosynthetic pathways (Arrigoni and De Tullio, 2000), regulation of cell elongation (González-Reyes et al., 1998), and cell proliferation (De Gara and Tommasi, 1999).

The last two decades of research has focused on the functioning of ASC in the regulation of plant cell growth (Arrigoni et al., 1992; Wang and Faust, 1992; De Gara et al., 1996; Tommasi et al., 1999). An ASC-deficient mutant vtcl that contains only 30% of wild-type ASC levels has also been shown to have a significant reduction in growth (Veljovic-Jovanovic et al., 2001). Recent research has indicated that ASC has effects on cell elongation as well as cell division (for review, see Horemans et al., 2000a). Moreover, ASC has been indicated as one of the possible factors important in regulating the transition of cells through the cell cycle (for review, see Potters et al., 2002).

The mechanism underlying the action of ASC in growth regulation is still a question of debate. Several studies have indicated the redox status of the ASC molecules (expressing the percentage of the total ASC pool that is in the reduced state) as an important factor in this respect. For example, if reduced ASC is added to Allium sp. or maize (Zea mays) root cells of the quiescent center, it induces these normally nondividing cells to pass form G1 into the S-phase (Liso et al., 1988; Kerk and Feldman, 1995). Moreover, as shown in the Allium sp. roots, ASC also promotes cell proliferation in the entire root meristem and the pericycle (Arrigoni et al., 1992; Paciolla et al., 2001). In strong contrast to the growth-promoting role of ASC, its oxidized form, dehydroascorbate (DHA), seems to inhibit cell proliferation (De Cabo et al., 1993; Kerk and Feldman, 1995; De Pinto et al., 1999; Potters et al., 2000). Moreover, the activity of the extracellular enzyme ASC oxidase in Bright Yellow (BY)-2 cells depends on the cell cycle phase (Kato and Esaka, 1999). More specifically, it increases during G2 and M phase. Apparently, the redox status of ASC in the apoplast is an important factor in cell cycle progression.

At a subcellular level, the concentration and redox status of ASC seems to differ between the different cell compartments (Horemans et al., 2000b). At the moment, it seems that the compartment in which the ASC redox status is most readily influenced is the apoplast. The data of Kato and Esaka (1999), as well as of De Pinto et al. (1999) indicate an intimate connection between apoplastic and cytoplasmic ASC metabolism and cell proliferation, even up to the level of the actual cell cycle. Therefore, it was postulated that...
understanding the mechanisms regulating the subcellular distribution of ASC or controlling its redox status between the apoplast and the cytoplasm will provide interesting information concerning the role and mechanism of ASC on this cell cycle control. In this respect, recent research has indicated two interesting proteins present in the plant plasma membrane. One of them is a high-potential ASC-reducible cytochrome b$_{561}$, which has been shown to mediate transmembrane electron transport, which can, at least in vitro, use monodehydroascorbate as an external electron acceptor (Horemans et al., 1994; for review, see Asard et al., 2001). In addition, the plant plasma membrane contains a high-affinity DHA transporter. This transporter has been shown to mediate the uptake of DHA into protoplasts of barley (Hordeum vulgare; Rautenkranz et al., 1994), tobacco (Nicotiana tabacum) BY-2 cell culture (Horemans et al., 1998b) or into isolated plasma membrane vesicles of Phaseolus vulgaris (Horemans et al., 1996, 1997, 1998a). Upon the discovery that the DHA uptake activity is enhanced in ASC-loaded plasma membrane vesicles, it was postulated that the carrier operates with an exchange mechanism in which uptake of DHA coincides with a simultaneous export of ASC (Horemans et al., 1998a). In this paper, it is shown that in a tobacco BY-2 cell line the activity of the DHA transporter changes throughout the growth cycle and the cell cycle. However, the physiological significance remains to be discussed.

RESULTS

DHA Uptake Activity Changes during Cell Culture

A first experiment focused on the activity of the DHA transporter throughout the growth stage of the BY-2 cell line. Therefore, uptake activity was measured in protoplasts isolated at different culture ages. Figure 1A describes the growth curve of the BY-2 cell line. It needs to be stated that a specific lag phase could not be observed. After their transfer to fresh medium, cells grow exponentially from d 1 to 5, and afterward, net growth decreases to zero and the cells enter into the stationary phase. Protoplasts were isolated at different time points and 50 μM 14C-labeled ASC was added to monitor its uptake (Fig. 1B). When no reductant or oxidant was added (control protoplasts), the uptake was seen to increase until it reached a maximum rate of 30 pmol 10$^{-6}$ cells min$^{-1}$. This maximum was reached in protoplasts isolated 4 d after culture initiation, i.e. in the middle of the exponential growth phase. As previously reported, the addition of 1 mM ferricyanide (FC) is sufficient to oxidize all externally present ASC immediately. In this test situation, all radioactive molecules are present as 14C-DHA. The curve in Figure 1B showing DHA uptake (in the presence of FC) throughout the culture of the BY-2 cells follows quite closely the uptake under control conditions: an increase of the uptake rate until d 4, and a steady activity of 25 pmol 10$^{-6}$ cells min$^{-1}$ afterward. When uptake was measured in the presence of 1 mM dithiothreitol (DTT), the uptake rate was around 10 pmol 10$^{-6}$ cells min$^{-1}$ and did not change throughout the growth cycle. DTT normally keeps 14C-ASC in the reduced form. In the presence of 1 mM DTT, the amount of radioactive molecules taken up into the cells was lower than under control conditions. At d 4, a statistically significant difference was detected between the uptake in the presence of DTT on the one hand, and the uptake into control protoplasts or in the presence of FC on the other hand ($P < 0.05$). Statistical difference could not be demonstrated at the other time points.

DHA Uptake Rates Were Not Affected by Elevated Internal ASC Levels

As shown in other reports, the endogenous ASC levels change significantly in BY-2 cells as these cells progress through the exponential growth phase and into the stationary phase (De Pinto et al., 1999; Kato and Esaka, 1999). On the other hand, it was shown that the DHA carrier possibly takes up DHA and at the same time exports ASC (Horemans et al., 1998a). Therefore, it was conceivable that an increased internal ASC level could trigger the observed increased DHA uptake throughout the growth cycle.

To test this hypothesis, a method was devised to obtain freshly isolated protoplasts containing more ASC than normal protoplasts. As described before, when culturing BY-2 cells in the presence of 1 mM ASC or 1 mM DHA for 4 h, these cells will contain 10- to 20-fold more ASC than control cells (Potters et al., 2000). Here, protoplasts were prepared from cells that had been incubated with 1 mM DHA for 4 h (Fig. 2, 4 h, treated), as well as from cells that had been subjected to a mock treatment (Fig. 2, 4 h, non-treated). ASC levels in treated protoplasts were found to be three to four times higher than non-treated protoplasts. This effect was independent of the age of the cell culture as it was observed in cells from a 4- or 7-d-old culture (Fig. 2A). Uptake into control protoplasts and ASC-loaded protoplasts did not differ significantly ($P < 0.05$) in protoplasts isolated from a 4-d-old culture (Fig. 2B) or in those from a 7-d-old culture (Fig. 2C). An elevated ASC concentration inside the protoplasts did not change uptake rates in the presence of FC or DTT (Fig. 2, B and C) as compared with protoplasts that had normal ASC levels. As expected, transport into freshly harvested cells did not differ from that into cells undergoing a mock treatment for 4 h.

DHA Uptake throughout the Cell Cycle

The observation of the differential activity of the DHA transporter throughout the growth of the BY-2 cells prompted a further analysis of this connection.
Therefore, we used the ability of the BY-2 cells to be synchronized in their cell division to study a possible connection between DHA uptake activity and the cell cycle. A BY-2 culture was synchronized with aphidicolin. Upon release of the aphidicolin block, the cells are in S phase and were permitted to fulfill their cell cycle from that point onward, until M-phase was reached (Fig. 3A). Furthermore, one extra synchronization was performed, using aphidicolin and propyzamide, to observe the cell cycle from M to S phase (Fig. 3B). Samples were taken every 2 h and were subjected to protoplastation. The time noted in the x axis of Figure 3, A and B, denotes the complete time elapsed between the release from the aphidicolin or propyzamide cell cycle block and the eventual transport test. The actual harvesting of the cells from the synchronized culture occurred 2.5 h before this point, which is the time necessary for protoplast isolation and the actual transport test. All results from these graphs will be described, with the moment of the transport test taken as a reference. Transport rates were established in cells without addition of external redox compounds, or in the presence of 1 mM FC or 1 mM DTT. Uptake in the presence of FC or into the control cells was most affected during the progression of cells through the cell cycle. A 3-fold increase (P < 0.05) was observed for the transport rate of DHA in the presence of FC around 8.5 h after aphidicolin release (Fig. 3A) as compared with the transport rates before (4.5 and 6.5 h). After this time point, transport activity decreased again. A significant difference (P < 0.025) was also found at the 8.5-h time point between uptake in the presence of FC compared with that in the presence of DTT. It is noteworthy that compared with nonsynchronized cultures, DHA uptake activity in control conditions (i.e. without FC or DTT) differed...
slightly although not significantly from the uptake measured in the presence of FC.

When cells were synchronized by the consecutive addition of aphidicolin and propyzamide, a similar 3-fold increase in transport capacity was observed 2.5 h after release from the cell cycle block (Fig. 3B). Again, it was the transport in the presence of FC or into control protoplasts that was significantly ($P < 0.05$) affected, whereas no change could be detected in the transport activity in the presence of DTT. Later time points in the experiment uptake stayed at a basal level of 50 pmol $10^{-6}$ ppl and did not show any dependence on the presence of FC or DTT.

**DISCUSSION**

In this work, evidence has been presented on the correlation between growth phase and activity of the DHA transporter, described previously (Horemans et al., 1998b). It has been established before that it is the oxidized form, DHA, that is preferentially taken up...
by plant cells (Horemans et al., 1997, 1998b). In other words, the amount of radioactive label taken up is representative of the amount of DHA that has been relocated from the external medium to the cytoplasm. In this paper, the attempt was made to investigate whether this DHA uptake activity is in some way changing throughout growth and cell cycle. Apparently, cells that exhibit a high rate of growth (cells in the middle of the exponential growth phase, Fig. 1A) also exhibit a high capacity for DHA uptake (Fig. 1B). The average size of the BY-2 protoplasts did not differ between different time points (data not shown), indicating that the observed higher DHA uptake capacity is not a consequence of cell volume.

Previous studies have shown that BY-2 protoplasts possess the capacity of oxidizing any external ASC added to their medium (Horemans et al., 1998b; Pot¬ters et al., 2000). Therefore, one may suspect that rather than a regulation of the transport mechanism, it is merely this oxidizing capacity that is up-regulated. However, uptake in the presence of FC (which causes complete oxidation of the 14C-ASC within seconds) is never different from the uptake into control cells (Fig. 1B). This indicates that the oxidation of 14C-ASC in control conditions is not a limiting factor influencing the uptake rate.

De Pinto et al. (1999) as well as Kato and Esaka (1999) demonstrated a 3- to 4-fold increase in the internal ASC concentration during the exponential growth phase. Horemans et al. (1998a) showed that the DHA carrier as measured in isolated P. vulgaris plasma membrane vesicles possibly uses an ASC/DHA exchange mechanism whereby uptake of external DHA coincides with the export of internal ASC. The same phenomenon can be demonstrated in isolated plasma membranes of BY-2 cells (N. Horemans, unpublished data). In theory, the increased uptake may thus be caused by a stimulation of the exchange mechanism due to the increased ASC concentration. However, an altered internal ASC concentration in the protoplasts (Fig. 2A), achieved by loading the cells before protoplast isolation, did not change the DHA uptake rate of the protoplasts in 4- or 7-d-old cells (Fig. 2, B and C). Therefore, the difference in the rate of DHA uptake is not dependent on the internal ASC concentration. However, this mechanism is not excluded as a possible driving force for the DHA carrier. The cytosolic concentration of ASC is in the millimolar range (Foyer and Lelandais, 1996) and it is probably high enough to provide maximal stimulation of the DHA uptake process. Therefore the amount taken up by the cells is probably only dependent on the external DHA concentration, which was evidently identical for every measurement in the different tests. Therefore, the observed differences are reflecting the overall capacity for DHA transport of the cells.

It would be interesting to support this conclusion by testing ASC-depleted protoplasts. Two treatments have been suggested in the literature: administration of lycorine, an inhibitor of ASC biosynthesis (Arrigoni et al., 1975; De Gara et al., 1994), and the addition of 4-hydroxy-(3, 3, 5, 5)-tetramethylpiperidine-N-oxyl, which oxidizes the internal ASC pool in animal cells within 30 min (Njus and Kelley, 1993; Winkler et al., 1994). However, lycorine needs several hours of incubation before the desired effect can be demonstrated, and in such a long time period, cell growth itself is affected by the slowly declining cytoplasmic ASC content. 4-Hydroxy-(3, 3, 5, 5)-tetramethylpiperidine-N-oxyl has been tested (data not shown), but in contrast to animal cells, we could not observe any effect on the ASC levels in our system. It would be interesting in this respect to test DHA uptake in the ASC biosynthesis mutant vtc1 that contains only 30% of wild-type ASC levels (Conklin et al., 1996; Veljovic-Jovanovic et al., 2001).

As mentioned above, a large increase in cellular ASC concentration with a peak during the exponential growth phase (De Pinto et al., 1999; Kato and Esaka, 1999) has been observed. In addition, several enzymes are transiently active when cells are most actively dividing: the enzyme catalyzing the last step in the ASC biosynthesis, l-galactono-γ-lactone dehydrogenase (Smirnoff et al., 2001), as well as the enzymes active in the regeneration of ASC (such as DHA reductase or MDHA reductase; Noctor and Foyer, 1998), which are significantly more active during the exponential growth phase than before or after, in the stationary phase (De Pinto et al., 2000).

Intriguingly, at least the DHA reductase and the MDHA reductase activity change throughout the cell cycle as well, and peak around G1 (Kato and Esaka, 1999). Moreover, these authors also demonstrated changes in the internal ASC redox status throughout the cell cycle. Whereas the ASC concentration did not change, the DHA concentration increased significantly, exactly at the M/G1 transition (Kato and Esaka, 1999). In this paper, we have investigated a possible relation between DHA transporter activity and the cell cycle. It was found that DHA uptake activity was up-regulated 2- to 3-fold in protoplasts made from cells collected 6 to 8 h after aphidicolin release or immediately after propyzamide release. This means that at the moment of harvesting, these cells are in M phase. The actual DHA uptake assay is 2.5 h after the harvesting in each instance (time needed for protoplastation). Therefore, the question remains whether these cells will complete their actual cytokinesis during the protoplastation process or whether they will remain in M-phase until they regenerate the cell wall. However, it is reasonably safe to propose that the increase in transport capacity occurs during M phase and the transition from M to G1.

The sudden and large increase in transporter activity around the M/G1 transition indicates for the first time that the activity of the DHA transporter on the plant plasma membrane may be highly regulated. In
animal cells, compared with plant cells, vitamin C transport has been studied more extensively. However, in animal studies, information on the regulation of the different transport systems is fragmentary. The sodium-dependent ASC carrier can be inhibited by Tyr kinase inhibitors like AG18 and genistein or ouabain (Diliberto et al., 1983; Behrman et al., 1996; Spielholz et al., 1997) or protein kinase activator phorbol 12-myristate 13-acetate (Liang et al., 2002). These observations indicate the presence of a phosphorylation site on the animal ASC carrier. On the other hand, the DHA uptake that is mediated by GLUT transporters in animal cells has been shown to be rapidly enhanced under stress conditions (Rivas et al., 1997; Laggner and Goldenberg, 2000). Apparently, an independent stress signal or an elevated DHA concentration outside were sufficient to have an increased expression of the transporter protein or to recruit additional transporter molecules to the cell surface. Which mechanisms are responsible for the fairly large and instantaneous increase in DHA transporter activity during the cell cycle that is reported here is still to be investigated. Similarly, it remains to be seen whether other defense-related phenomena are able to change DHA transport capacity as well.

MATERIALS AND METHODS

Plant Material

Tobacco (Nicotiana tabacum cv BY-2) cell suspension was propagated as described by Nagata et al. (1992) in Murashige and Skoog medium supplemented with 0.2 g L\(^{-1}\) KH\(_2\)PO\(_4\), 30 g L\(^{-1}\) Suc, 0.2 mg L\(^{-1}\) 2,4-dichlorophenoxycetic acid, 0.01 g L\(^{-1}\) thiamine-HCl, and 0.1 g L\(^{-1}\) myo-inositol, set at pH 5.8 with KOH. Cells were cultured in a rotary shaker at 130 rpm and at 27°C in the dark. Normally, 1 g of cells was diluted in 8 mL of this medium. The addition of 1 m\(\text{M} \text{KH}_2\text{PO}_4\) and 0.4 m mannitol, pH 5.5 [KOH], collected on a Buchner filter and were centrifuged at 50,000 \(g\) for 15 min at 4°C. The supernatant was collected and kept in ice and in the dark until use, or was stored at −20°C until HPLC analysis. ASC determination was carried out on reverse-phase HPLC. The chromatographic system consisted of a reverse-phase type C-18 column (3-μm particle diameter, 150 mm, internal diameter of 4.6 mm, LiChroGraph Alltech, Deerfield, IL) and an isocratic pump (LC-10ADVP; Shimadzu) coupled to a home-made amperometric detection system (glassy carbon working electrode, calomel reference electrode, reference potential 1,000 mV). The electrochemical detector was connected to a personal computer via an SS420 board (Shimadzu). Chromatogram analysis was performed with the ClassVP software package (Shimadzu). Total ASC (ASC + DHA) was determined by reducing 100 μL of each sample with 100 μL of a 200 mM DTT/400 mM Tris solution. The high Tris concentration ensures an increase in the pH of the solution to 6 (checked for every sample), which is needed for an efficient reduction of DHA to ASC. After 30 min of incubation, the reduction reaction was stopped by addition of 600 μL of the eluent, thus lowering the pH significantly to pH 3. The DHA concentration was estimated as the difference between the reduced and total ASC concentration.

ASC/DHA Transport

Uptake of ASC or DHA was measured according to Horemans et al. (1998b) by adding 50 μM l-[14C]ascorbic acid (Amersham, Gent, Belgium) to 75.10\(^{3}\) freshly prepared protoplasts (concentration generally around 2,500 protoplasts per mL). The volume was adjusted to 100 μL with protoplast medium. The addition of 1 mM FC assured that the [14C]-labeled ASC was completely oxidized to [14C]-DHA; the addition of 1 mM DTT ensures that at least a part of the [14C]-ASC is present in the reduced form. After a 20-min incubation, the cells were diluted 50-fold with ice-cold washing medium (10 mM nonlabeled ASC and 0.4 mM mannitol, pH 5.5 [KOH]), collected on a cellulose nitrate filter (0.45 μm; Sartorius, Göttingen, Germany), and rinsed by the further addition of 15 mL of washing buffer. The filters were dissolved in scintillation cocktail (Filter count; Packard, Brussels, Belgium). For blank experiments, samples were washed immediately after the addition of the radioactive labeled molecules to the protoplasts. In a typical ASC uptake experiment, three replicates were made for each condition tested together with two blanks. The number of separate experiments is added in the figure legends.

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LITERATURE CITED


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