Dehydroascorbate Influences the Plant Cell Cycle through a Glutathione-Independent Reduction Mechanism

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Glutathione is generally accepted as the principal electron donor for dehydroascorbate (DHA) reduction. Moreover, both glutathione and DHA affect cell cycle progression in plant cells. But other mechanisms for DHA reduction have been proposed. To investigate the connection between DHA and glutathione, we have evaluated cellular ascorbate and glutathione concentrations and their redox status after addition of dehydroascorbate to medium of tobacco (Nicotiana tabacum) L. cv Bright Yellow-2 (BY-2) cells. Addition of 1 mM DHA did not change the endogenous glutathione concentration. Total glutathione depletion of BY-2 cells was achieved after 24-h incubation with 1 mM of the glutathione biosynthesis inhibitor L-buthionine sulfoximine. Even in these cells devoid of glutathione, complete uptake and internal reduction of 1 mM DHA was observed within 6 h, although the initial reduction rate was slower. Addition of DHA to a synchronized BY-2 culture, or depleting its glutathione content, had a synergistic effect on cell cycle progression. Moreover, increased intracellular glutathione concentrations did not prevent exogenous DHA from inducing a cell cycle shift. It is therefore concluded that, together with a glutathione-driven DHA reduction, a glutathione-independent pathway for DHA reduction exists in vivo, and that both compounds act independently in growth control.

Ascorbate (ASC) and glutathione (GSH) are well known antioxidants, participating in the cellular defense of plants against oxidative stress. Biotic and abiotic stress conditions therefore generally affect cellular ASC levels and/or the ASC redox status (defined here as the percentage of reduced ASC upon the total pool of ASC and the oxidized dehydroascorbate [DHA]). For example, the ASC content and redox status of shoots and roots of Phaseolus vulgaris is sensitive to zinc stress (Cuypers et al., 2001), and ASC is oxidized in tomato (Lycopersicon esculentum) plants infected with Botrytis cinerea (Kuźniak and Sklodowska, 1999, 2001). Especially in the apoplast, the ASC redox status is quite sensitive to stress conditions. Different types of environmental stress result in an increased oxidation of the apoplastic ASC pool (Castillo and Greppin, 1988; Luwe et al., 1993; Takahama, 1993; Gossett et al., 1994; Plöchl et al., 2000). In turn, GSH has been implicated in herbicide and heavy metal defense (Kömives et al., 1998). For example, GSH is oxidized after copper treatment in Lemna minor (Teisserre and Vernet, 2000) or under ozone fumigation in Spinacia oleracea (Luwe et al., 1993), and GSH metabolism is enhanced under different forms of stress (May and Leaver, 1993; Xiang and Olivier, 1998).

In any case, ASC and GSH are thought to be intimately connected. In the chloroplast, GSH functions as an electron donor for DHA reductase, regenerating ASC through DHA reduction (the so-called ascorbate-glutathione cycle, Foyer and Halliwell, 1976; Noctor and Foyer, 1998). The oxidized form of GSH (designated GSSG) that results from this reaction is subsequently reduced by a NADPH-dependent GSSG reductase. The components of this ASC regenerating pathway have also been demonstrated in the cytoplasm (Borracino et al., 1986; Noctor and Foyer, 1998). Moreover, transformed plants overexpressing GSSG reductase show higher foliar ASC levels and improved tolerance to oxidative stress. Conversely, reduced GSSG reductase activity resulted in increased stress sensitivity (Noctor and Foyer, 1998). Furthermore, the cellular GSH pool is drained upon addition of exogenous DHA, at least in roots of white lupin (Lupinus albus) and onion (Allium cepa; Paciolla et al., 2001). Last, recent experiments from Chen et al. (2003) show that enhanced expression of a GSH-dependent DHA reductase increases tissue concentrations of ASC.

In addition to their functioning as antioxidants, ASC and GSH are essential in different physiological phenomena in plant cells (May et al., 1998; Noctor et al., 1998; Arrigoni and De Tullio, 2000). For example, an ever-increasing body of evidence demonstrates the role of ASC and DHA in the regulation of cell growth and division (Potters et al., 2002). GSH has also been implicated in the regulation of plant and animal cell }
growth and division. GSH metabolism is intimately linked to the control of root hair development and root cell growth (Sánchez-Fernández et al., 1997). Normal cell cycle progression in a tobacco (Nicotiana tabacum) cell suspension was blocked after addition of l-buthionine sulfoximine (BSO). This compound is a nontoxic, highly specific inhibitor of γ-glutamylcysteine synthase, which is the first step in GSH biogenesis (Griffith and Meister, 1979; May and Leaver, 1993; May et al., 1998; Vernoux et al., 2000). Similar findings have been reported in animal systems (Shaw and Chou, 1986; Atzori et al., 1994; Poot et al., 1995). Moreover, in exemplary cases ASC and GSH even are involved in cell cycle and growth control in similar ways; the size of the ASC pool in the Zea mays quiescent center is markedly decreased, compared to the pool in the meristematic cells close by (Kerk and Feldman, 1995), but also the level of GSH in the Arabidopsis quiescent center is markedly lower, again compared to the surrounding tissues (May et al., 1998). This suggests that ASC and GSH may be closely linked when it comes to growth control.

We have previously demonstrated that exogenous DHA, but not ASC, when added during the G1 phase of the tobacco L. cv Bright Yellow-2 (BY-2) cell cycle, is able to delay normal cell cycle progression (Potters et al., 2000). However, internal DHA concentrations were not affected by this treatment, whereas ASC levels increased, suggesting a rapid reduction of DHA to ASC. The DHA-mediated increase of cellular ASC and effect on cell cycle progression shows an interesting specificity. A mere increase of cellular ASC may result in faster cell proliferation rates (Liso et al., 1988; Arrigoni et al., 1989; Innocenti et al., 1990; Kerk and Feldman, 1995; Davey et al., 1999; De Pinto et al., 1999) but has never been reported to delay the cell cycle. We therefore suggested that the fact that the cell’s need to invest in DHA reduction, rather than the increased levels of ASC, may influence cell cycle progression.

This system opens a way to assess the role of the GSH/GSSG redox pair in the DHA-mediated halt in cell cycle progression in BY-2 suspension cells. Hopefully, it provides information on the extent of the intimate entanglement of ASC and GSH, in terms of DHA reduction and in terms of growth control. The aim of this work, therefore, is to challenge the hypothesis that GSH is the first and foremost reductant for DHA, and that the action of either ASC or GSH on a given physiological phenomenon should always involve changes in the other component’s concentration or redox status.

RESULTS

Effect of DHA Addition on GSH Redox Status in BY-2 Cells

Addition of 1 mM DHA to the medium of an exponentially growing BY-2 culture led to a strong increase in intracellular ASC levels. Although only the oxidized form was taken up, no increase in intracellular DHA concentration could be observed, suggesting a rapid uptake and internal reduction (Potters et al., 2000). To study the contribution of GSH in this DHA reduction, changes in intracellular ASC, DHA, GSH, and GSSG levels were followed after addition of 1 mM DHA to an exponentially growing BY-2 culture. Samples were collected with 1-h intervals, with the “0-h” sample taken immediately before DHA addition. A control culture, where extra medium was added instead of DHA, was sampled as well. Although the internal ASC concentration increased 20-fold (data not shown for this set of experiments; see Fig. 4), no significant change could be observed in the intracellular GSH pool of control cells and DHA-treated cells up to 6 h after treatment (P < 0.05; Fig. 1). In addition, no changes were observed in the total amount of glutathione (GSH plus GSSG), or in its redox status (data not shown). The high variability of our data is surprising. However, this variation is for the most part due to variation between different cultures, since sampling the same culture flask produced fairly comparable data. Since the GSH content in BY-2 cells is drastically decreased during this period, as is GSSG reductase (de Pinto et al., 2000), this may cause the huge differences between cell cultures.

Nitrosourea Fails to Inhibit the Plant GSSG Reductase But Causes Cell Death

The above-presented results possibly indicate that GSH may not be involved in the rapid reduction of DHA taken up by the cells. An alternative explanation is that GSSG may itself have been rapidly re-reduced to GSH masking changes in its redox status, even after the first hour of sampling. To evaluate this possibility, we applied 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a known inhibitor of the animal GSSG

Figure 1. DHA addition has no effect on the intracellular GSH concentration. Intracellular GSH content in control conditions (black diamonds) or after addition of 1 mM DHA (white squares). Average of seven independent culture flasks (except for time [t] = 3 h, where n = 3). Bars indicate se; t0 = immediately after DHA addition.
reductase (for example, Kehrer, 1983; Harlan et al., 1984; Kaneko et al., 2002) and its plant counterpart (Gullner and Dodge, 2000; Piquery et al., 2002). Concentrations ranging from 50 to 500 μM of BCNU were added to the BY-2 culture medium in the absence or presence of 1 mM DHA, and samples were collected every hour after addition. In animal cells, concentrations of 500 μM BCNU were effective in GSSG reductase inhibition (Riley, 1984; Ueda-Kawamitsu et al., 2002). In our BY-2 culture, even after addition of 500 μM BU, no changes were observed in the cellular GSH or GSSG content, as compared to the control cells, even after addition of 500 μM BCNU (data not shown). Unfortunately, the cells started to die after 6 h of exposure to the compound (Fig. 2), which is just after the time interval during which the culture was sampled. The rate at which the cells died was concentration dependent (percentages of viable cells after addition of both 500 and 400 μM BCNU are shown in Fig. 2). Cell viability was indistinguishable from the control culture even after 24 h of incubation with 250 μM BCNU (or all concentrations below). As an aside, addition of 1 mM DHA seemed to alleviate the cell death rates after application of 500 μM BCNU. However, this trend could not be confirmed statistically. Furthermore, DHA has no effect on cell death upon application together with lower BCNU concentrations (400 μM). As a control experiment, we have run a native gel confirming that BCNU does inhibit GSSG reductase, but only slightly, even at the 500-μM concentration (data not shown).

Uptake and Internal Reduction of DHA Proceeds Even in GSH-Depleted Cells

As an alternative approach to investigate the role of GSH in the rapid reduction of intracellular DHA, we attempted to decrease the cellular GSH content. Diethylmaleate (DEM) covalently binds GSH, thereby lowering the physiologically active intracellular levels (Mulder and Ouwerkerk-Mahadevan, 1997; Nagai et al., 2002). Pretreatment of BY-2 cells for 2 h with 1 mM DEM decreases the intracellular GSH content in BY-2 cells to one tenth of the control value (Fig. 3; 0 h meaning 2 h after addition of DEM, to permit comparison with Fig. 4). Cell viability was not affected, neither by DEM nor by BSO treatments, up to 36 h and 14 h after the addition, respectively (all values in treated and control cultures between 90% and 95%).

The effect of both compounds on ASC metabolism was checked as a control experiment. Apparently, endogenous ASC concentrations in BY-2 cells were not affected by DEM or BSO addition only (Fig. 4). The effect of DHA addition was tested on DEM- and BSO-treated cells. DEM and BSO were kept in the culture medium after DHA addition to ensure continuously low or zero levels of GSH in the cells (compared with Fig. 3). Addition of DHA to control cells led to a 20-fold increase of the intracellular ASC concentration within 3 h (Fig. 4). No changes were observed in the internal DHA concentration, confirming the intracellular capacity to efficiently reduce DHA (as described before, Potters et al., 2000). The accumulation of intracellular ASC was significantly lower 1 h after DHA treatment of cells pretreated with DEM and BSO.
However, over the course of the experiment, all DHA was effectively taken up into the cells and reduced to ASC. Six hours after DHA treatment the ASC content of the DEM- or BSO-treated cells was not significantly different from the ASC content in the control cells (again, $P < 0.05$). These results demonstrated that cells effectively depleted from GSH levels by DEM or BSO treatment were still capable of reducing high intracellular DHA levels, although at different initial rates when compared to control cells. Measurements of the DHA uptake (transport) rates were performed, showing that neither BSO nor DEM did affect the DHA uptake capacity of the cells (data not shown).

**DISCUSSION**

**Without GSH, BY-2 Cells Still Possess DHA Reduction Capacity**

GSH is a well-known antioxidant, implicated in the enzymatic regeneration of ASC through the reduction of DHA (Foyer and Halliwell, 1976, 1977; Jiménez et al., 1997; Noctor and Foyer, 1998). This role is, for example, illustrated by the decrease of GSH levels in onion and white lupin roots upon DHA treatment (Paciolla et al., 2001), and by the identification of different GSH-consuming DHA reductases (for review, see Potters et al., 2002). On the other hand, different proteins have been proposed to carry out DHA reduction without using GSH in animals (for review, see Potters et al., 2002), and plant thioredoxins apparently show DHA reducing activity in a native gel assay (Morell et al., 1997; Foyer and Mullineaux, 1998). In general, it is supposed that thiol-containing proteins, and most likely those containing a dicyteinyl motif, are capable of fulfilling this function. (Paciolla et al., 2001; De Tullio et al., 2002; Wilson, 2002; Banhegyi et al., 2003). This raised the questions about the identity of the possible other reductant(s) of DHA and about the extent of the contribution of GSH in this set of DHA reducing agents.

The first part of this work involved the verification of GSH as an electron donor for DHA. In previous work, we have demonstrated that upon addition of a large amount of DHA (100 μmol in 100 mL BY-2
Glutathione-Independent Dehydroascorbate Reduction

Figure 6. Effect of DHA and GSH on the cell cycle. Cell cycle progression in synchronized cells, under control circumstances (diamonds) or after addition of 1 mM DHA (squares), 1 mM GSH (triangles) or 1 mM DHA plus 1 mM GSH (crosses). Abscissa represents the time after release from the propyzamide-induced cell cycle block (at which time point cells are restrained in M phase). Values are the average of three independent cultures; bars indicate s.

suspension), all DHA is taken up and internally reduced. Remarkably (and contrary to the results of Paciolla et al., 2001), this treatment did not affect the GSH pool in our BY-2 system (Fig. 1). Several possibilities could explain this discrepancy: a rapid turnover of cellular GSH, either by reduction of GSSG or by an increased biosynthesis, or because GSH has a limited role in DHA reduction in these cells.

To assess the role of GSH in DHA reduction, we have tried to influence cellular GSH levels by "scavenging" GSH with DEM, by inhibiting GSH biosynthesis with BSO, and by blocking GSSG reductase activity by BCNU. BCNU is a good inhibitor for the animal GSSG reductase and has even some "scavenging" GSH with DEM, by inhibiting GSH biosynthesis with BSO, and by blocking GSSG reductase activity by BCNU. BCNU is a good inhibitor for the animal GSSG reductase and has even some effect on the plant enzyme, but, unfortunately, prolonged exposure of BY-2 cells caused the cells to die (Fig. 2), and a concentration of 500 μM, which is quite effective in animal cells, does not affect GSH levels in BY-2 cells. It should therefore be questioned whether this compound is as useful in plants as in animal systems. Perhaps BCNU should only be used for long-term events (as performed by Piquery et al., 2002), which was impossible in our system. The cell death we encountered seems a rather unspecific event.

Scavenging of cellular GSH levels by DEM was effective in the BY-2 cells and reduced free GSH concentrations to 10% of that in control cells. Since only redox active molecules are detected in our HPLC analysis, our measurements indicate the effective reduction of the reducing capacity of GSH in the cells. Although the reduction rate of DHA to ASC was lowered by the DEM treatment, similar levels of reduction were reached as in the untreated cells at the end of the experiment (Fig. 4). The possibility that even small GSH concentrations might have been enough to reduce DHA due to a fast re-reduction of GSSG cannot be ruled out completely.

Experiments using the GSH biosynthesis inhibitor, BSO, however, provided further evidence that GSH is not the primary reductant for the observed DHA reduction. After a 24-h BSO treatment, cells were completely devoid of GSH (Fig. 3). Similar to the DEM-treated cells, BSO treatment did not affect the uptake rates but resulted in slower reduction of intracellular DHA to ASC (Fig. 4). The observations that addition of DHA did not change intracellular GSH levels and that depletion of intracellular GSH did not affect the reduction of DHA to ASC strongly support the idea that GSH is not the sole, and maybe even not the primary, reductant for DHA in BY-2 cells.

For example, the observation that the appearance of ASC was slower in GSH-depleted BY-2 cells (Fig. 4) can be explained in two ways (which are not mutually exclusive). It is possible that the observed DHA reduction involves different pathways, including one which is GSH dependent. Both in the case of DEM- and BSO-treated cells, DHA reduction proceeds significantly slower than under control conditions (Fig. 4). It might be argued that this difference is due to the lack of GSH in these cells. In that case we estimate that, based on the comparison of reduction rates, around 50% of the incoming DHA is being reduced with the aid of GSH. One may even speculate that the GSH-independent reduction of DHA is proportional to the area under the BSO-plus-DHA curve in Fig. 4, and the area between the control curve (1 mM DHA) and the BSO-plus-DHA curve proportional to the GSH-dependent DHA reduction. The fact that there seems to be a difference (not statistically proven) between the DEM-plus-DHA and the BSO-plus-DHA curve might then be attributed to the fact that DEM also impacts on other thiol groups.

On the other hand, it is also possible that depleting the GSH pool forces the reductants involved in the reduction of DHA to be used in other physiological processes slowing down the DHA reduction. Although our data do not allow us to distinguish between these two possibilities, this does not affect the major conclusion of the involvement of a GSH-independent pathway in cellular DHA reduction. Any distinction between both explanations requires a definitive identification of the components of the DHA reduction pathways.

Similar experiments performed on different animal cell types before have demonstrated the existence of different mechanisms for DHA reduction. For example, DHA uptake and internal reduction were severely hampered in GSH-depleted human (HepG2) and rat (H4IIE) liver cells (Li et al., 2001). DHA reduction was also shown to be impaired in GSH-depleted erythrocytes (May et al., 2001b) or endothelial cells (May et al., 2001a), suggesting the involvement of GSH in DHA reduction. However, GSH did apparently not play any role in ASC recycling in human HaCaT keratinocytes (Savini et al., 2000), or in HL-60 cells (Guaiquil et al., 1997). It should therefore be interesting to check the involvement of GSH in DHA reduction in different plant cell types. Indicative for
the presence of different mechanisms in different tissues are the results on onion and white lupin root tips, demonstrating that GSH is involved in DHA reduction (Paciolla et al., 2001). It must be noted, however, that in BY-2 cells, this effect on thiol groups could not be duplicated (G. Potters and N. Horemans, unpublished results).

ASC and GSH Are Not Linked for Cell Cycle Control

Using the tools to affect cellular GSH levels, we also explored the connection between the effect of DHA on cell cycle progression and the possible connection to GSH. GSH depletion as a result of a BSO treatment blocks the cell cycle in BY-2 cells (Potters et al., 2000). Therefore, we wanted to check whether the observed DHA-mediated delay of the cell cycle was in fact mediated by a change in GSH content (or redox status). However, an increase in internal GSH did not prevent the observed DHA-mediated cell cycle delay (Fig. 6). Moreover, GSH depletion and DHA addition were shown to have an additive effect, strongly suggesting that both compounds act in a different pathway, each ultimately influencing the cell cycle in its own fashion. Therefore, previous observations demonstrating the involvement of ASC and GSH in cell cycle progression (Liso et al., 1988; De Gara and Tommasi, 1999; De Pinto et al., 1999, 2000; Kato and Esaka, 1999; Potters et al., 2000; Vernoux et al., 2000; Paciolla et al., 2001) might probably also be the result of independent pathways. Also, the cells are completely devoid of GSH—there is no GSH left to be influenced by the exogenous DHA. That DHA still affects the cell cycle progression under these conditions is an additional indication for the general independence of both compounds.

Surprisingly, we also noted that the mere addition of GSH did influence the cell cycle slightly, although in an inhibitory way. This is in apparent contradiction to the results of Vernoux et al. (2000) or Xiang et al. (2001)—where in both instances a lower GSH content in the cell is linked to an inhibition of cell division—as well as to our own results (Fig. 5). This indicates that, more than the mere presence of a compound, its proper concentration is crucial for optimal functioning of the cell’s physiology. If this equilibrium is shifted in either direction, the cell loses its balance and normal functioning is disturbed or even disrupted. In the actual case of GSH, Creissen et al. (1999) already demonstrated that plants overproducing GSH are surprisingly more sensitive to oxidative stress.

Possible Alternative Mechanisms for DHA Reduction and Growth Control?

Our hypothesis is—albeit indirectly—also supported by other results; plants with 20% of the control GSH levels did not show altered tolerance to oxidative stress (May et al., 1998). Apparently, defense against stress situations sometimes occurs irrespective of the GSH concentration. In addition, plants with only 5% of the wild-type GSH level grew normally (Xiang et al., 2001). On the other hand, in the Arabidopsis vt-1 mutant (which contains only 30% of a wild-type plant’s ASC level), both stress resistance and growth are affected (Veljovic-Jovanovic et al., 2001). This also suggests that GSH and ASC are not necessarily linked to one another and that they rather have distinct functions to fulfill.

Of course, the question remains as to which compound(s) will assist in GSSG-independent DHA reduction. Both in animal and plant cells, the thioredoxin/thioredoxin reductase system, glutaredoxin, protein disulfide isomerase, and even less-known proteins all have been suggested to possess DHA-reduction activity (Wells et al., 1990; Del Bello et al., 1994; Trümper et al., 1994; Hou and Lin, 1997; May et al., 1997; Morell et al., 1997; Hou et al., 1999; Potters et al., 2002). Therefore different sources of electrons for DHA reduction should be considered in future work concerning the effects of ASC and DHA on cell physiology.

In any case, the involvement of the thioredoxin/thioredoxin reductase system may be a suitable working hypothesis, explaining the inhibition of cell cycle progression by DHA. Apparently, DHA is only capable of slowing down cell cycle progression when added in G1 phase. Addition of 1 mM DHA in G2 phase does not affect the cell cycle at all (G. Potters and N. Horemans, unpublished results). This suggests that DHA influences the cell cycle through processes which are specific for either G1 or S phase. Interestingly, the thioredoxin/thioredoxin reductase system is involved in the delivery of deoxyribonucleotides (obviously necessary for the passage through S phase). A competition for reducing equivalents between DHA and ribonucleotides, which both need to be reduced, might slow down the supply for deoxyribonucleotides, and therefore effectively halts S phase progression. Unfortunately, at noon no known inhibitor of the animal thioredoxin/thioredoxin reductase system has been proven to inhibit DHA reduction in our BY-2 cells, and this hypothesis remains highly uncertain.

On the other hand, the thioredoxin/thioredoxin reductase system has even in plants been shown to interact directly or indirectly with DHA, both on a protein level and on an mRNA level. For example, both DHA and GSSG deactivated thioredoxin f-activated enzymes (Nishizawa and Buchanan, 1981), and ASC influences the expression of genes, coding for thioredoxin-activated or -repressed proteins (Kiddle et al., 2003). Further research is thus needed to look for the proper integration of ASC/DHA metabolism in thiol-mediated redox signaling and regulation.

CONCLUSION

Our results demonstrate that, similar to animal cells, different pathways for DHA reduction exist in plant cells, the nature of which remains unknown. It is true
that one cannot ignore the many examples where a functional link between GSH and ASC metabolism has been crucial in understanding what happens (see the many examples listed in the introduction). Indeed, GSH is probably a team player in the whole network of DHA reducing reactions. Nevertheless, we feel that the question about DHA reduction should be broadened; whereas GSH and DHA are most likely connected, for example, in stress resistance, their influence on growth regulation may be mediated by other, distinct pathways. As an aside, we also suggest that GSH and ASC have apparently a distinct function in the regulation of cell cycle progression and may each be part of a different oxidative stress-sensitive pathway (Reichheld et al., 1999). Further research will have to identify and specify the different components on which each molecule acts.

**MATERIALS AND METHODS**

**Plant Material**

A tobacco (*Nicotiana tabacum*) L. cv Bright Yellow-2 (BY-2) cell suspension was propagated as described by Nagata et al. (1992), in Murashige and Skoog (1962) medium, supplemented with 0.2 g L⁻¹ KH₂PO₄, 30 g L⁻¹ sucrose, 0.2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid, 0.01 g L⁻¹ thiamine-HCl, and 0.1 g L⁻¹ myoinositol, set at pH 5.8 with KOH. Cells were cultured in a rotary shaker at 130 rpm and at 27°C, in the dark. Weekly subculturing was initiated by transferring 4 mL of a 7-d-old stationary culture to 100 mL of fresh medium. DHA uptake experiments were performed with cells in full exponential growth (4 d after inoculation) to ensure a valid comparison with a synchronized (dividing) culture, as used in the cell cycle experiments. Treatments were either added exactly at the beginning of the measurements (1 mM DHA, 1 mM ASC, BCNU) or at the time specified in the “Results” section (1 mM BSO, 1 mM DEM).

**HPLC Measurement of ASC and GSH**

Intracellular and extracellular ASC and GSH content was determined by reversed phase HPLC separation, followed by amperometric detection. Cells were collected on a Buchner filter (Haldenwanger, Berlin) at intervals of 1 h. Aliquots of around 0.3 g fresh weight were resuspended in 1 mL of extraction medium, consisting of 6% n-phosphoric acid and 1 mM EDTA. Insoluble polyvinylpyrridoline (1%) was added, and the cells were subsequently snap-frozen in liquid nitrogen. ASC, DHA, GSH, and GSSG were subsequently extracted through three cycles of freezing and thawing; the homogenate was centrifuged at 50,000g for 15 min at 4°C. ASC determination in the supernatant was carried out by reverse phase HPLC (RP type C-18 column, LiChroSpher, Alltech, Deerfield, IL; isocratic pump, 0.8 mL min⁻¹ potential 1,000 mV; supplied by Prof. Dr. L. Nagels, University of Antwerp, Belgium). Chromatogram analysis was performed with the Class VP software (1 mM BSO, 1 mM DEM).

**Native PAGE GSSG Reductase Assay**

Soluble proteins were extracted from 2 g of a 3-d-old BY-2 culture with 20 mL of the buffer described in De Gara et al. (1997). Native gel electrophoresis and subsequent staining for GSSG reductase activity was performed according to Foyer et al. (1991). Every gel was cut in three pieces; before the actual activity stain, each gel strip was soaked for 15 min in buffer, buffer plus 250 μM BCNU, or buffer plus 500 μM BCNU. These concentrations of BCNU were also added to the staining mixture afterwards.

**LITERATURE CITED**

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**Glutathione-Independent Dehydroascorbate Reduction**

Every hour after synchronization, a 500-μL aliquot of the cell suspension was taken from the culture and put on ice to allow the cells to sediment. The remaining medium was removed 10 min after harvesting. The cells were then fixed in a 3:1 ethanol:acetic acid mixture and stored at 4°C until further analysis. Mitotic indices were determined by staining the chromatin with orcein (2% w/v in a 1:1 lactic acid:propionic acid mixture) and determining the percentage of cells displaying one of the mitotic phases under a bright field microscope. At least 500 cells were counted in every sample.

To assess the viability of the cells after different treatments, Evans blue was added (in a final concentration of 0.5%) to 500-μL aliquots of cell suspension. Stained cells were considered dead; 500 cells were counted in each sample.

**Uptake of DHA**

Uptake of radiolabeled [¹⁴C]ASC and [¹⁴C]DHA into protoplasts of BY-2 cells was performed as described by Homemans et al. (1998).

**Synchronization of BY-2 Cells**

BY-2 cell cultures were blocked in their cell cycle with aphidicolin and propyzamide (Nagata et al., 1992; Samuels et al., 1998) and released when synchronized at the beginning of M phase. Two hours after propyzamide release, during G1 phase, 1 mM DHA, 1 mM GSH and/or 1 mM BSO were added to the cell culture. The concentrations of these compounds were chosen in accordance to existing literature (Potters et al., 2000; Vernoux et al., 2000).

**Determination of Mitotic Index and Viability**

Reichheld et al. (1999). Further research will have to identify and specify the different components on which each molecule acts.

**Synthesis of reduced and total ASC concentration.**

**Distribution and subsequent staining for GSSG reductase activity was performed according to Foyer et al. (1991). Every gel was cut in three pieces; before the actual activity stain, each gel strip was soaked for 15 min in buffer, buffer plus 250 μM BCNU, or buffer plus 500 μM BCNU. These concentrations of BCNU were also added to the staining mixture afterwards.

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