L-Ascorbic Acid Metabolism in the Ascorbate-Deficient Arabidopsis Mutant vtc7

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The biosynthesis of l-ascorbic acid (vitamin C) is not well understood in plants. The ozone-sensitive Arabidopsis thaliana mutant vitamin c-1 (vtc1; formerly known as sox1) is deficient in ascorbic acid, accumulating approximately 30% of wild-type levels. This deficiency could result from elevated catabolism or decreased biosynthesis. No differences that could account for the deficiency were found in the activities of enzymes that catalyze the oxidation or reduction of ascorbic acid. The absolute rate of ascorbic acid turnover is actually less in vtc1 than in wild type; however, the turnover rate relative to the pool of ascorbic acid is not significantly different. The results from [U-14C]Glc labeling experiments suggest that the deficiency is the result of a biosynthetic defect: less 1-14Cascorbic acid as a percentage of total soluble 14C accumulates in vtc1 than in wild type. The feeding of two putative biosynthetic intermediates, d-glucosone and d-sorbose, had no positive effect on ascorbic acid levels in either genotype. The vtc1 defect does not appear to be the result of a deficiency in t-galactono-1,4-lactone dehydrogenase, an enzyme able to convert t-galactono-1,4-lactone to ascorbic acid.

As is the case with all aerobic organisms, plants have the ability to detoxify reactive oxygen species. This is accomplished in part through the synthesis of a variety of antioxidant molecules, including thylakoid-associated α-tocopherol and carotenoids, and soluble molecules such as AsA (vitamin C), GSH (and homoglutathione in some legumes), polyamines, and phenolics (Albers and Hess, 1993; Foyer et al., 1994b; Kangasjärvi et al., 1994). Enzymes that are involved in the regeneration of the reduced forms of antioxidants include mono (and dehydro-)-AsA reductase and GR (Foyer and Halliwell, 1976; Jablonski and Anderson, 1981; Hossain and Asada, 1984). In addition, enzymes with varied antioxidant activities are known, including superoxide dismutases, AsA peroxidases, guaiacol peroxidases, GSH S-transferases, and catalases (Bowler et al., 1992; Lea et al., 1992; Bartling et al., 1993; Foyer et al., 1994a).

To define the roles of these antioxidants and antioxidant enzymes in reactive oxygen stress detoxification, we have isolated Arabidopsis thaliana mutants altered in response to the air pollutant ozone, a well-documented cause of enhanced reactive oxygen species production in plants. To date, vitamin C-1 (vtc1; originally named sox1 and renamed in this manuscript to reflect the fact that it has a demonstrated defect in AsA metabolism) is the most thoroughly characterized of these mutants (Conklin et al., 1996). The ozone sensitivity of vtc1 is conferred by a semidominant monogenic trait that causes reduced accumulation of AsA with 2-week-old mutant seedlings accumulating approximately 30% of wild-type AsA. The vtc1 mutant also has altered sensitivity to other conditions that generate reactive oxygen species, such as UVB light and sulfur dioxide. The results of these studies are consistent with the hypothesis that AsA plays a role in protection from oxidative stress generated by a variety of conditions.

Many physiological effects of AsA deficiency have been described in animals (for example, in the disease scurvy), but our understanding of the specific roles for AsA is limited. In addition to the ability to directly scavenge hydroxyl radicals, superoxide, and singlet oxygen, AsA is able to reduce the oxidized form of the membrane-associated antioxidant α-tocopherol (Padh, 1990). AsA also plays an important role in preserving the activity of enzymes that contain prosthetic transition metal ions (Padh, 1990). In plants the antioxidant role of AsA has received the most attention (Smirnoff, 1996; Smirnoff and Pallanca, 1996). For example, chloroplastic AsA is thought to scavenge hydrogen peroxide produced by photosynthesis via the AsA-GSH cycle (Foyer and Halliwell, 1976; Asada and Takahashi, 1987), and apoplast-localized AsA may reduce reactive oxygen species from extracellular sources such as ozone (Polle et al., 1995). AsA in plants is also thought to play roles in other physiological processes. For example, it is utilized as a reductant in the hydroxylation of Pro residues during extensin biosynthesis (Liso et al., 1985), has a likely involvement in cell wall expansion (Smirnoff, 1996).

Abbreviations: AO, ascorbate oxidase; AP, ascorbate peroxidase; AsA, l-ascorbic acid; DHAR, dehydroascorbate reductase; GLDH, t-galactono-1,4-lactone dehydrogenase; GR, glutathione reductase; MDAR, monodehydroascorbate reductase.

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and may be involved in the transition from G1 to S during the cell cycle (Kerk and Feldman, 1995).

In plants AsA can be catabolized to a four-carbon product, tartrate, or to a two-carbon product, oxalate (for review, see Loewus, 1988). The remainder of the carbon skeleton of AsA is then recycled. In some plants tartrate or oxalate accumulate to quite high levels. For example, oxalate accounts for 35% of the dry weight of *Haloclon glomeratus*, a desert weed (Loewus, 1988).

AsA-deficient mutants provide valuable tools for probing both the metabolism and physiological roles of AsA. A loss of the ability to synthesize AsA has occurred in pri-mates, including *Homo sapiens*, guinea pigs, some passeriforme birds, and at least two species of bats (Chatterjee, 1973, 1978). However, the Arabidopsis mutant *vct1* (Conklin et al., 1996) and the scurvy prone, osteogenic disorder rat (Kawai et al., 1992) are the only AsA-deficient mutants that have isogenic wild-type counterparts for comparison. The rat mutant contains a missense mutation in l-gulono-1,4-lactone oxidase, the enzyme catalyzing the final step in animal AsA biosynthesis: conversion of l-gulono-1,4-lactone to AsA (Kawai et al., 1992).

In contrast to animals, the AsA biosynthetic pathway in plants remains to be elucidated. Two different pathways have been proposed, one of which has characteristics in common with the animal pathway, whereas the second is chemically distinct (Loewus, 1988). Animals generate AsA via the inversion pathway in which the carbon skeleton of Glc is inverted in the final product (Fig. 1A). This pathway proceeds through the substrates d-gluconate and l-gulono-1,4-lactone. A similar pathway has been demonstrated in the chrysomonad *Ochromonas danica*, with d-galacturonate and l-galactono-1,4-lactone as intermediates (Helser et al., 1982). Published biochemical evidence for such an inversion pathway in higher plants rests largely on the identification of a mitochondrial enzyme with GLDH activity. This oxidizes exogenous l-galactono-1,4-lactone to AsA (Fig. 1B).

The ability to convert l-galactono-1,4-lactone to AsA has been demonstrated in several plant species (Mapson and Breslow, 1958; Baig et al., 1970; Hausladen and Kunert, 1990; DeGara et al., 1992, 1994; Oba et al., 1994; Conklin et al., 1996). GLDH has been purified from the mitochondria of sweet potato roots as a single 56-kD polypeptide (Oba et al., 1994; Conklin et al., 1996). This oxidizes exogenous l-galactono-1,4-lactone to AsA (Fig. 1B).

The noninversion pathway involving ozone intermediates (Loewus, 1988; Saito et al., 1990). Evidence for l-sorbosone as an AsA intermediate is somewhat less clear. No difference was found in the incorporation of label into AsA from d-[14C]Glc and l-[14C]sorbosone, and unlabeled sorbosone only partially inhibited (approximately 3-fold) the conversion of d-[14C]Glc to l-[14C]AsA (Saito et al., 1990). However, an enzymatic activity that is able to convert l-sorbosone to AsA has been partially purified from both spinach and bean (Loewus et al., 1990). Although no enzymatic activities from plants have been isolated that convert d-Glc to d-glucosone or d-glucosone to l-sorbosone, there is radiotracer evidence for an epimerization at C5 that would be required for conversion of d-glucosone to l-sorbosone (Grün et al., 1982).

The biochemical evidence described above does not conclusively demonstrate the existence of either pathway acting in vivo, nor does it allow us to assess the possibility that both pathways function in plants. Analysis of a collection of Arabidopsis mutants defective in AsA biosynthesis will be an effective method to critically evaluate the pathway(s) that function in vivo. Toward that goal, we describe here the evidence that the AsA-deficient mutant *vct1* is defective in AsA biosynthesis rather than catabolism.

Figure 1. Two proposed pathways for AsA biosynthesis in plants. A, The inversion pathway that is present in rats and many other animals. *Euglena* and other protists also synthesize AsA via an inversion pathway, although the intermediate substrates differ from the classical animal pathway as shown. B, The noninversion pathway involving ozone intermediates (Loewus, 1988; Saito et al., 1990).
MATERIALS AND METHODS

Plant Culture

The Arabidopsis thaliana genotypes used in these analyses were the Columbia (Col-0) wild-type ecotype and the AsA-deficient vitamin C-1 (vtc1) mutant (Conklin et al., 1996), which was backcrossed twice to wild type. This mutant was previously described as soz1 (Conklin et al., 1996) but has been renamed. Plants used for the assay of endogenous AsA turnover in the dark and those used to determine L-galactono-γ-lactone dehydrogenase activity were grown in a constant-environment chamber on a 16-h photoperiod from 6 AM to 10 PM, with other conditions as described previously (Conklin and Last, 1995).

Tissue for enzyme assays was obtained from plants grown in a greenhouse in Exeter (UK) in April, 1996. Plants used in the 14C-feeding experiments were grown in a controlled-environment chamber with a photoperiod of 16 h (300 μmol m$^{-2}$ s$^{-1}$ PPFD). The temperature was 20°C in the light and 15°C in the dark. Rosette leaves of intermediate age (i.e. neither the youngest nor the oldest leaves) were selected for the analyses, and labeling experiments were started during the first half of the photoperiod. Plants utilized for both the enzyme assays and 14C-feeding experiments were grown in a medium containing equal volumes of coconut fiber compost (with slow-release fertilizer), vermiculite, perlite, and fine sand.

Enzyme Assays

Enzymes were extracted from rosette leaves of plants at the start of primary flower stalk elongation. For the assay of MDAR, DHAR, GR, and AO, the leaves were homogenized in the extraction medium (0.1 g fresh weight mL$^{-1}$ described by Smirnoff and Colombé (1988). Total AP activity was extracted with 50 mM potassium phosphate buffer containing 1 mM EDTA, 0.1% (v/v) Triton X-100, and 1 or 10 mM AsA. The homogenates were centrifuged at 12,000g for 1 min in a microcentrifuge. Enzymatic activities were measured as follows: AP, MDHAR, and GR activities as previously described (Smirnoff and Colombé, 1988), and DHAR activity using the method of Nakano and Asada (1980).

AO is found both in soluble form and ionically bound to the cell wall (Esaka et al., 1989). Therefore, activities were measured in the supernatants and pellets from leaf homogenates. To measure insoluble AO activity, the pellet from the centrifuged leaf homogenate was resuspended in 0.5 mL of 100 mM phosphate citrate buffer, pH 5.5. AO activity in the supernatant and resuspended pellet was measured as ascorbate-dependent oxygen consumption with a liquid-phase oxygen electrode (Hansatech, Kings Lynn, UK). The assay contained 0.8 mL of 100 mM phosphate citrate buffer, pH 5.5, 0.1 mL of sample, and 10 mM AsA in a total volume of 1 mL. Rates were corrected for non-enzymatic AsA oxidation.

Turnover of Endogenous AsA in the Dark

The largest true leaves from approximately 3-week-old plants were used to measure the turnover of endogenous AsA in an assay similar to that previously described (Smirnoff and Pallanca, 1996). Leaves were excised and sliced perpendicular to the midrib with a razor blade into approximately 5-mm-wide segments. These leaf slices were floated in Petri plates (equivalent of approximately 16 leaves/plate) containing 25 mL of water. The plates were then incubated either in the light (approximately 60-70 μmol m$^{-2}$ s$^{-1}$ PPFD) or wrapped in foil (dark) for 0, 6, 12, or 24 h. Tissue was harvested from a single plate for each time point and genotype, and samples from each plate were then divided into three aliquots and stored at −80°C until analysis. The three replicate samples for each time point were extracted and assayed for AsA as described below.

Conversion of L-Galactono-1,4-Lactone to AsA

The method described above was used with several changes. Leaf slices were incubated in 25 mL of water (control) or 25 mL of 25 mM L-galactono-1,4-lactone (Sigma) in the light. Tissue was collected from both control and experimental plates for each genotype at 0, 2, 4, 6, and 8 h.

Treatment of Leaves with D-Glucosone and L-Sorbosone

Petioles of excised leaves were placed in 40 μL of 50 mM glucosone or 25 mM sorbosone (Nippon Roche) until the solution was absorbed (6 h) in the light (80 μmol m$^{-2}$ s$^{-1}$ PPFD). The leaves were then floated on water in Petri dishes and incubated in the light (300 μmol m$^{-2}$ s$^{-1}$ PPFD) for 17 h. Neutralized 5% perchloric extracts were assayed for total AsA by the AsA oxidase assay as described below. D-Glucosone was prepared by oxidation of Glc with pyranose-2-oxidase that was partially purified from Phanerochaete chrysosporium (Liu et al., 1983) and its identity and purity confirmed by GC-MS, TLC, and HPLC (J.E. Pallanca and N. Smirnoff, unpublished data).

Labeling of Leaves with L-[1-14C]AsA or D-[U-14C]Glc

To measure turnover of L-[1-14C]AsA, leaves from 6-week-old plants were detached at the petioles using a very sharp double-edge razor blade. Each pair of leaves was placed with their petioles in 25 μL of 1 mM AsA containing 0.5 μCi L-[1-14C]AsA for 160 min in the light (80 μmol m$^{-2}$ s$^{-1}$ PPFD). Labeled leaves were then transferred to water, sealed in a transparent plastic jar along with a filter paper saturated with 20% potassium hydroxide to trap carbon dioxide, and incubated for 22 h under fluorescent lamps (80 μmol m$^{-2}$ s$^{-1}$ PPFD). Labeled tissue was then stored at 80°C until processed. Three replicate sets of leaves for each genotype were labeled, and two leaves (approximately 100 mg total) were used for each replicate. For metabolism of D-[U-14C]Glc in 5-week-old plants, three detached leaves with petioles per sample (approximately 100 mg/sample) were pooled, weighed, and their petioles placed in 12.5 μL of water containing 3 μCi D-[U-14C]Glc (specific activity 57 mCi mmol$^{-1}$) for 45 min. Each set of three leaves was then transferred to 5 mM unlabeled Glc and placed in a labeling dish.
Fractionation of 14C-Labeled Tissues

Frozen, labeled tissue was ground to a powder in liquid nitrogen and extracted with 1 mL of 5% perchloric acid. The homogenate was centrifuged for 2 min at 12,000g. The pellet was saved for later measurement of radioactivity in the insoluble fraction. The soluble fraction was neutralized with 5 mM potassium carbonate and an aliquot was used to determine radioactivity and AsA concentration. The cleared neutralized extract (0.5 mL) was loaded onto an anion-exchange column with quaternary ammonium functional groups (Techelut SAX, 0.15-mL bed volume, HPLC Technology, Macclesfield, UK). The column was first washed with 4 mL of water, and then the eluent (containing neutral and basic compounds) was collected. The column was then washed sequentially with 4 mL of 60 mM formic acid and 4 mL of 2 M formic acid to recover acidic compounds. The two acidic fractions were freeze-dried. The 2 M formic acid fraction was resuspended in a small volume of 2 M formic acid and frozen until further analysis. In the 14C-AsA-labeling experiment, the water wash from the anion-exchange step was further fractionated into neutral and basic fractions with a cation-exchange column with benzene sulfonic acid functional groups (Techelut SCX, 1-mL bed volume, HPLC Technology). The column was sequentially eluted with water and 6 M ammonium hydroxide.

Fractionation of L-[^14]C]AsA by HPLC

AsA occurs in the 60 mM formic acid fraction from the SAX anion-exchange column. The freeze-dried 60 mM formic acid fraction was redissolved in 200 μL of 60 mM formic acid and AsA separated by HPLC. One-hundred microliters was loaded onto a Rezex ROA-organic acid column (300 × 7.8 mm, Phenomenex, Macclesfield, UK) and eluted with 0.75 mM H2SO4 (0.5 mM min⁻¹). The retention time of AsA under these conditions was approximately 16 min. The AsA peak was collected in 30-× 100-μL fractions starting 14 min after injection. The AsA peak was detected at 210 nm and quantified by reference to a standard curve. Comparison of the amount of AsA detected by HPLC with the amount loaded onto the anion-exchange column (quantified by the AO assay described below) enabled a correction to be made for loss (largely a result of oxidation) during the procedure.

AsA turnover was calculated in the 14C-AsA labeling experiment from the specific activity of the leaf ascorbate at the beginning of the pulse period and the amount of label either remaining as ascorbate or recovered in nonascorbate fractions at the end of the chase period.

Measurement of Radioactivity

Aliquots of all of the fractions, including the washed and resuspended pellet, (0.6 mL total volume) were added to 4 mL of scintillation fluid (Emulsifier-Safe, Packard, Meriden, CT). The potassium hydroxide-impregnated filter paper was placed in 0.6 mL of water and 4 mL of scintillation fluid to determine radiolabel in carbon dioxide. The 14C content was determined with a liquid scintillation analyzer (Tri-Carb 2500 TR, Packard) with external standard and built-in quench curve.

AsA Assays

AsA in leaf extracts from experiments without radiolabel was determined by two different methods. The results presented below of total AsA were obtained with the AO spectrophotometric assay as previously described (Conklin et al., 1996). The average from two replicate assays on the same extract is presented. In the duplicate experiments for which data are not shown, reduced AsA was extracted and measured by HPLC with electrochemical detection using isoaascorbic (erythorbic) acid as an internal standard (Kutnik et al., 1987). In the radiolabeling experiments AsA and dehydroascorbate in the neutralized perchloric acid extracts were quantified by a spectrophotometric assay similar to that described above. Total AsA was measured by first reducing dehydroascorbate to AsA with homocysteine (Okamura, 1980).

RESULTS

The AsA deficiency in vtc1 could result from either increased AsA catabolism or decreased synthesis. To determine whether vtc1 has increased AsA turnover, we measured (a) the activities of enzymes involved in either the oxidation or reduction of AsA, (b) turnover of endogenous AsA pools in the dark, and (c) turnover of L-[1-14C]AsA fed to excised leaves. To monitor the ability of vtc1 to synthesize AsA, we conducted labeling experiments with D-[U-14C]Glc, fed putative AsA precursors, and measured the activity of a putative AsA biosynthetic enzyme in both genotypes.

Activity of Enzymes Involved in AsA Metabolism

The monodehydroascorbate radical and dehydroascorbate (the two oxidized forms of AsA) are both quite unstable in an aqueous environment. Therefore, an AsA deficiency could result from a mutation causing either an elevation in the activity of an enzyme that oxidizes AsA or a decrease in the ability to regenerate reduced AsA from one of its oxidized forms. Two enzymatic activities that each oxidize AsA, AO and AP, were assayed in leaf extracts. AO, an enzyme that uses oxygen to oxidize AsA to water and monodehydroascorbate, appears to have a role in cell expansion and cell wall metabolism (for review, see Smirnoff, 1996). Its location in the apoplastic compartment of tobacco leaves was demonstrated by assaying the activity of AO in the soluble and insoluble fractions (Table I).

In contrast, the activity of AP, which uses AsA as a reductant to catalyze the reduction of hydrogen peroxide to water, was significantly lower in vtc1 than in the wild type (Table I). The activity of a chloroplastic isoform of this
Table 1. Activity of enzymes of AsA metabolism in wild type and vtc1

Results are mean ± SD (n = 3 separate extractions).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Wild Type</th>
<th>vtc1</th>
<th>p a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n kat g^-1 fresh wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AsA oxidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td>9.2 ± 1.0</td>
<td>10.4 ± 0.4</td>
<td>n.s. b</td>
</tr>
<tr>
<td>Insoluble</td>
<td>5.4 ± 1.2</td>
<td>5.0 ± 0.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>MDHA reductase</td>
<td>22.6 ± 1.8</td>
<td>22.8 ± 3.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>DHA reductase</td>
<td>355 ± 38</td>
<td>308 ± 22</td>
<td>n.s.</td>
</tr>
<tr>
<td>AsA peroxidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM AsA</td>
<td>796 ± 41</td>
<td>632 ± 25</td>
<td>0.01</td>
</tr>
<tr>
<td>10 mM AsA</td>
<td>971 ± 70</td>
<td>557 ± 17</td>
<td>0.001</td>
</tr>
<tr>
<td>GSH reductase</td>
<td>10.8 ± 0.6</td>
<td>12.3 ± 0.9</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

a Determined by one-way ANOVA. b n.s., Not significant.

enzyme in tea leaves is very sensitive to AsA depletion, and is stabilized by extraction in buffer containing AsA (Chen and Asada, 1989).

We observed a similar stabilization of overall AP activity upon inclusion of AsA in extraction buffer for wild-type Arabidopsis plants (Table I). In contrast, total AP activity in vtc1 does not appear to depend upon the amount of AsA in the extraction buffer. Taken together, these results are consistent with the hypothesis that the decline in total AP activity in vtc1 may reflect a specific loss of a chloroplastic AP activity(s) due to the lower endogenous AsA levels in this mutant. Two lines of evidence suggest that vtc1 does not have a deficiency in cytosolic AP. First, antibodies raised against a cytosolic form of AP (GenBank accession no. X59600) revealed no difference in the level of this isoform in vtc1 and wild type (data not shown). Second, a method for assaying cytosolic AP activity in Arabidopsis (Mittler and Zilinskas, 1993) revealed similar if not elevated activity of a putative cytosolic AP in vtc1 (data not shown). It is interesting to note that Arrigoni et al. (1997a) also observed a reduction in AP activity in Lupinus albus seedlings in which AsA deficiency had been induced by lycorine treatment.

MDAR and DHAR both act to regenerate the reduced form of AsA. MDAR reduces monodehydroascorbate using NADH, whereas DHAR regenerates AsA from dehydroascorbate using GSH. The total activity of each of these reductases is unaltered in vtc1 (Table I). Antibodies raised against two different isoforms of Arabidopsis MDAR (accession nos. T43234 and T21955) revealed no change in the level of these proteins in vtc1 (data not shown).

Finally, the activity of GR was compared in wild type and vtc1. Using NADPH, GR reduces GSSG produced both nonenzymatically from the reduction of oxidized AsA, and enzymatically (as described above) by DHAR. Therefore, a deficiency in GR could result in depletion of GSH, the reducing agent necessary for activity of DHAR, and thus impair the ability of DHAR to regenerate AsA. As seen in Table I, the activity of GR is unchanged in vtc1.

AsA Turnover Is Not Elevated in vtc1

Previous work with barley demonstrated that the total AsA pool can be depleted in detached, dark-incubated leaves, presumably because turnover outpaces synthesis (Smirnoff and Pallanca, 1996; N. Smirnoff and J.E. Pallanca, unpublished data). This observation was utilized to compare the endogenous turnover of AsA in vtc1 and wild-type leaves. Leaves were sliced into sections and incubated in water either in the light or dark for different time periods, and the remaining amount of total AsA was determined (Fig. 2). As expected, no observable decline in AsA levels was seen in leaves incubated in the light. Counter to the expectation that vtc1 is AsA deficient because of elevated catabolism, the absolute rate of AsA turnover over the 24-h incubation period was lower in vtc1 than in wild type leaves (Table II). This suggests that the lower levels of AsA in vtc1 are not the result of decreased turnover, but rather an elevation in AsA biosynthetic activity.

Table 2. Comparison of AsA turnover in dark-incubated excised leaves of vtc1 and wild type

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Absolute Change</th>
<th>P-Value (Dark) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol AsA g^-1 fresh wt</td>
<td>%</td>
</tr>
<tr>
<td>0-6</td>
<td>0.028</td>
<td>n.s. b</td>
</tr>
<tr>
<td>6-12</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>12-24</td>
<td>0.005</td>
<td>n.s.</td>
</tr>
<tr>
<td>0-12</td>
<td>0.015</td>
<td>n.s.</td>
</tr>
<tr>
<td>0-24</td>
<td>0.016</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

a Two-sided t-test for difference of means; no assumption of equal variances. b n.s., Not significant.
Table III. Metabolism of [1-14C]AsA by vtc1 and wild-type Arabidopsis
Excised leaves were fed via the peptide with 14C-AsA in 1 mM AsA carrier for 160 min and then transferred to water for 22 h in the light. Values are ± SD (n = 3) of total labels in leaves.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Wild type</th>
<th>vtc1</th>
<th>% of total</th>
<th>% of soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>98.4 ± 0.5</td>
<td>97.7 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insoluble</td>
<td>1.2 ± 0.3</td>
<td>1.8 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO2</td>
<td>0.5 ± 0.3</td>
<td>0.7 ± 0.4</td>
<td></td>
<td></td>
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</tbody>
</table>

Neutral 13.2 ± 4.9 41.3 ± 6.7
60 μM Formic acid 56.4 ± 5.3 35.7 ± 4.0
2 mM Formic acid 30.2 ± 6.2 22.2 ± 10.0
AsA* 51.0 ± 5.6 34.9 ± 3.2
Basic 0.3 ± 0.1 0.5 ± 0.3

ASA poolb 3.91 ± 0.97 1.05 ± 0.34
ASA + DHA poolb 3.83 ± 1.13 1.28 ± 0.81

Absolute ASA turnover ratec 1.58 ± 0.60 0.57 ± 0.10
Relative ASA turnover ratec 0.41 0.44

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a From integration of HPLC peak and corrected for recovery.
b Measured in total neutralized extract by ASA oxidase assay.
c Measured by amount of ASA metabolized.

dark incubation period was actually greater in wild type than in vtc1.

A comparison of the absolute change in AsA per unit of time between wild type and vtc1 revealed a significant difference over all time-point intervals except for the 6- to 12-h interval (Table II). It is interesting to note that a comparison of turnover as a percent change in AsA per unit time revealed no significant difference between wild-type and vtc1 over the course of the 24-h dark incubation: both genotypes declined to approximately 40% of initial levels after 24 h in the dark (Table II). Based upon these experiments we conclude that the rate of AsA turnover in dark-treated leaves relative to the AsA pool size was similar in vtc1 and wild type.

The above experiment estimates AsA turnover under conditions in which synthesis is decreased. To extend this analysis, AsA turnover was estimated by following the metabolism of L-[1-14C]AsA by leaves incubated in the light under conditions that permit ascorbate synthesis. Excised leaves were fed with L-[1-14C]AsA for approximately 3 h and then transferred to water for 22 h in the light. The amounts of 14C present in the neutral, acidic, and basic fractions were then determined and are expressed as a percentage of 14C in the total soluble fraction (Table III). The ratio of L-[1-14C]AsA to total soluble 14C was lower in vtc1 than in wild type (35% versus 51%, respectively). However, the L-[1-14C]AsA in wild type would have been diluted more by the larger endogenous pool, causing an underestimation of turnover in wild type compared with vtc1. To account for this, the actual AsA turnover was estimated from the amount of L-[1-14C]AsA metabolized, correcting for the specific activity of the AsA pool. As seen in Table III, although the actual rate of AsA turnover was approximately 3-fold less in vtc1 than in wild type, the turnover rate relative to the pool size ("Relative AsA turnover rate" at the bottom of Table III) was not significantly different in vtc1 and wild type. These results are similar to endogenous AsA turnover in dark-incubated leaves presented in Figure 2 and Table II.

Table IV. Metabolism of [U-14C]Glc by illuminated detached vtc1 and wild-type leaves
Leaves were incubated in label for 45 min followed by a chase in cold 5 mM Glc for the indicated times. Two samples/genotype were labeled and processed for all time points. The data from both replicates are shown only for AsA. Values are percentage of total 14C.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incubation Time</th>
<th>2 h</th>
<th>6 h</th>
<th>20 h</th>
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<tr>
<td></td>
<td>wt vtc1</td>
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<td>wt vtc1</td>
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<tr>
<td>Soluble</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Insoluble</td>
<td>72.3 72.8</td>
<td>63.1 66.1</td>
<td>58.4 47.3</td>
<td></td>
</tr>
<tr>
<td>Carbon dioxide</td>
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<td>36.5 31.6</td>
<td>40.1 51.1</td>
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<tr>
<td>Neutral</td>
<td>49.0 41.9</td>
<td>3.7 3.7</td>
<td>3.0 4.8</td>
<td></td>
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<tr>
<td>60 mM Formic acid</td>
<td>4.2 11.5</td>
<td>7.0 10.6</td>
<td>6.8 4.4</td>
<td></td>
</tr>
<tr>
<td>2 mM Formic acid</td>
<td>19.3 24.8</td>
<td>26.7 25.5</td>
<td>32.6 28.7</td>
<td></td>
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<tr>
<td>AsA*</td>
<td>Replicate 1</td>
<td>1.0 0.8</td>
<td>1.8 1.0</td>
<td>2.0 0.5</td>
</tr>
<tr>
<td></td>
<td>Replicate 2</td>
<td>1.2 0.8</td>
<td>1.7 0.9</td>
<td>2.6 1.0</td>
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</tbody>
</table>

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a From integration of AsA HPLC peak, corrected for recovery.
Accumulation of l-[^14]C]AsA from d-[U-[^14]C]Glc is Reduced in vtc1

Glc can provide the carbon skeleton for either of the proposed AsA biosynthetic pathways (Fig. 1). Therefore, if vtc1 has a mutation directly affecting AsA biosynthesis, we would expect a decreased ability to convert labeled Glc into AsA, and this was observed in two independent experiments. The metabolism of d-[U-[^14]C]Glc by detached leaves was analyzed in an experiment similar to that described above for AsA. Excised leaves were fed through the transpiration stream with either water, 50 mM D-glucose, or 25 mM l-sorbosone (see Fig. 1B). After incubation in the light for 17 h, these proposed intermediates did not significantly increase the level of AsA in either genotype. In fact, the l-sorbosone treatment significantly reduced the concentration of AsA in the wild type (control, 5.84 ± 1.25 μmol/g fresh weight; l-sorbosone, 3.03 ± 1.13 μmol/g fresh weight). Similar results were seen in an independent experiment.

Feeding of l-galactono-1,4-lactone to various species of plants results in its conversion to AsA by the last enzyme in the proposed inversion pathway, GLDH (Fig. 1A). The in

Figure 4. The vtc1 mutant displays no defect in conversion of l-galactono-1,4-lactone to AsA. The method is the same as that described in Figure 2, except that leaf slices were incubated in water or 25 mM l-galactono-1,4-lactone (GalL) in the light. Tissue was collected for AsA analysis from both control and experimental samples for each genotype at the time points shown. Solid lines indicate samples incubated with GalL; dotted lines indicate control samples incubated without GalL. △, Total AsA in wild-type leaves −GalL; □, total AsA in wild-type leaves +GalL; ○, total AsA in vtc1 leaves −GalL; ◆, total AsA in vtc1 leaves +GalL. The SD (n = 3) is shown.

20-h incubation: wild-type leaves contained approximately 2- to 5-fold more l-[^14]C]AsA than the vtc1 leaves (Table IV). Figure 3 illustrates the HPLC traces for the 20-h time point samples, in which the amount of [^14]C (as a percentage of total soluble dpm) in the AsA-containing fraction is shown for two replicate samples for each genotype. No major differences were seen in the distribution of label among all of the other (non-AsA) fractions between wild type and vtc1, indicating no major disturbances in carbohydrate metabolism in vtc1 (Table IV). Therefore, vtc1 appears to be defective in conversion of Glc to AsA.

Treatment of vtc1 and Wild-Type Leaves with Putative AsA Biosynthetic Intermediates

The feeding of the two putative intermediates in the proposed noninversion pathway to vtc1 and wild-type leaves did not result in increased levels of AsA. Detached leaves were fed through the transpiration stream with either water, 50 mM D-glucose, or 25 mM l-sorbosone (see Fig. 1B). After incubation in the light for 17 h, these proposed intermediates did not significantly increase the level of AsA in either genotype. In fact, the l-sorbosone treatment significantly reduced the concentration of AsA in the wild type (control, 5.84 ± 1.25 μmol/g fresh weight; l-sorbosone, 3.03 ± 1.13 μmol/g fresh weight). Similar results were seen in an independent experiment.

Feeding of l-galactono-1,4-lactone to various species of plants results in its conversion to AsA by the last enzyme in the proposed inversion pathway, GLDH (Fig. 1A). The in
vivo activity of this putative plant AsA biosynthetic enzyme was estimated by floating leaf slices on water or 25 mM l-galactono-1,4-lactone for the indicated times (Fig. 4). AsA was then assayed in three replicate samples per time point for each genotype. Both wild type and vtc1 contain an activity that is able to convert l-galactono-1,4-lactone to AsA. At the earlier time points, the rate of formation of AsA was greater in vtc1 than in wild type. This difference in the initial rate of conversion of l-galactono-1,4-lactone to AsA was also observed in an independent experiment.

**DISCUSSION**

The data presented in this paper are most consistent with the hypothesis that the vtc1 mutant has a defect in ascorbic acid biosynthesis. First, the AsA biosynthetic rate in wild type was compared with vtc1 by following the incorporation of 14C-Glc into AsA. This type of analysis is difficult because, although the carbon skeleton of Glc can potentially be utilized by either of the two proposed pathways, only a very small proportion of exogenously supplied Glc is actually incorporated into AsA by higher plants (for example, see Saito et al., 1990). Despite this technical difficulty, after feeding excised leaves with d-[U-14C]Glc, vtc1 contained a smaller percentage of l-[14C]AsA relative to total 14C than did wild-type leaves at all time points assayed (Fig. 3). Despite being dedicated intermediates in the proposed noninversion pathway (Fig. 1B), exogenously supplied unlabeled d-glucosone and l-sorbosone had no positive effects on the AsA pool size. In contrast, both wild-type and vtc1 plants were able to oxidize l-galactono-1,4-lactone to AsA, making it unlikely that the AsA deficiency in vtc1 is caused by a defect in GLDH activity. Because elevated turnover during the chase period could also result in less l-[14C]AsA accumulation, we assayed AsA turnover in several different ways.

The two oxidized forms of AsA are relatively unstable. Therefore, it is plausible that the vtc1 AsA deficiency is caused by increased activity of an enzyme that causes its oxidation, or by a decreased ability to reduce monodehydroascorbate or dehydroascorbate back to AsA. We obtained no evidence to support this idea, since no differences could account for the deficiency in vtc1 were found in the total activities of DHAR, MDAR, AP, AO, or GR (Table I). Although we cannot rule out the possibility that vtc1 has a defect in an isozyme that was not measured in our experiments, the results of AsA turnover experiments make this unlikely. The modest reduction of AP activity, which is presumably caused by inactivation of the chloroplastic AP isoenzyme, could contribute to the sensitivity of vtc1 to reactive oxygen species, but could not account for the AsA deficiency.

Results of two different experiments that directly measured AsA turnover suggest that the AsA deficiency in vtc1 cannot be explained by elevated turnover. As previously shown in barley (Smirnoff and Pallanca, 1996), the AsA pool declines in excised leaves of Arabidopsis during dark incubation (Fig. 2). This technique was utilized to compare changes in the pool sizes of AsA in dark-incubated vtc1 and wild-type leaves. An additional experiment that measures turnover of AsA in the light, while synthesis is active and pool size is constant, was conducted by following the metabolism of l-[1-14C]AsA. Contrary to the hypothesis that vtc1 has increased AsA catabolism, both methods showed that the absolute rate of AsA turnover is in fact significantly greater in wild type than in vtc1. However, after the difference in AsA pool size is taken into account, the relative turnover rate in the two genotypes is quite similar. This result is consistent with the idea that AsA catabolism is regulated in response to the AsA pool size. To our knowledge, the specific enzymes involved in the catabolism of AsA have not been identified (Loewus, 1988), precluding a more direct test of this hypothesis.

The observation that vtc1 appears to have an elevated ability to convert l-galactono-1,4-lactone to AsA relative to wild type (Fig. 4) suggests that GLDH activity may also be regulated in response to AsA pools. Whereas wild-type leaves showed a lag before the full rate of l-galactono-1,4-lactone oxidation was seen, vtc1 leaves oxidized the substrate at a similar rate throughout the time course. Taken together, our observations suggest that regulation of both AsA biosynthesis and turnover may be sensitive to endogenous pool size.

The vtc1 mutant is not completely deficient in AsA, containing approximately 30% of wild-type levels. This partial deficiency could be explained if the vtc1 allele is leaky, allowing partial activity of the mutant gene product. Alternatively, synthesis of AsA in plants may be biosynthetically and/or genetically redundant. In this scenario, the vtc1 mutation could cause complete loss of function of the affected gene product, whereas the remaining pathway would remain intact. These pathways could be compartmentalized in different subcellular locations or as redundant pathways within the same compartment.

AsA is present in millimolar concentrations in all plants and has a broad range of functions (Smirnoff, 1996); consequently, the present lack of information about the pathway(s) of AsA biosynthesis represents a major gap in our knowledge of plant metabolism. Given the technical difficulties associated with studying this biosynthetic pathway in higher plants, biochemical approaches to identifying intermediates and enzymes of AsA synthesis have not succeeded. The isolation of a collection of mutants defective in the various steps in the biosynthetic pathway(s) should help to elucidate the pathway(s) of AsA biosynthesis.

Cloning of VTC1 and other genes of AsA biosynthesis will facilitate genetic engineering approaches to manipulating the levels of AsA in plants. This would provide the opportunity to enhance the nutritional quality and stress tolerance in agronomically important species.

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