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L-Ascorbic Acid Metabolism in the Ascorbate-Deficient Arabidopsis Mutant *vtc1*[1]

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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 *soz1*) 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-^{14}C]Glc labeling experiments suggest that the deficiency is the result of a biosynthetic defect: less L-[^{14}C]ascorbic acid as a percentage of total soluble ^{14}C accumulates in *vtc1* than in wild type. The feeding of two putative biosynthetic intermediates, D-glucosone and L-sorbosone, 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 L-galactono-1,4-lactone dehydrogenase, an enzyme able to convert L-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 (Alscher 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 *soz1* 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),

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

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 *Halogeton 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 primates, including *Homo sapiens*, guinea pigs, some passeriforme birds, and at least two species of bats (Chatterjee, 1973, 1978). However, the Arabidopsis mutant *vtc1* (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-glucuronate 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 (Helsper 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; Ôba et al., 1994; Conklin et al., 1996). GLDH has been purified from the mitochondria of sweet potato roots as a single 56-kD polypeptide (Ôba et al., 1995). Although it is clear that GLDH is present in higher plants, there is little evidence for its role in AsA synthesis. Correlations between GLDH activity (the final enzyme in the proposed inversion pathway) and endogenous AsA content have been demonstrated only in wounded potato tubers and seedlings of the parasitic plant *Cuscuta reflexa* (Tommasi et al., 1990; Ôba et al., 1994).

The correlation between GLDH activity and ascorbate biosynthesis was recently extended by the demonstration that the alkaloid lycorine, which causes reduced AsA accumulation when administered to a variety of plant species, inhibits GLDH activity in preparations of mitochondrial membrane from broad bean (*Vicia faba*) and maize (*Zea mays*) (Arrigoni et al., 1997b). Another equivocal line of evidence for this pathway is that pea (*Pisum sativum*) seedling extracts convert D-galacturonate methyl ester to L-galactono-1,4-lactone, but the affinity for the substrate is very low (Mapson and Isherwood, 1956), calling into question the physiological significance of this activity.

A biosynthetic pathway that does not invert the Glc carbon skeleton in the final AsA product has been proposed for plants (Loewus, 1988) and has been termed the noninversion pathway (Fig. 1B). The osones glucosone and sorbosone are the proposed intermediate substrates in this pathway (Loewus et al., 1990; Saito et al., 1990). Evidence that D-glucosone is an AsA intermediate include ^{14}C-tracer studies in which the label from D-[^{14}C]glucosone was incorporated more than 6-fold more efficiently into AsA than that from D-[^{14}C]Glc, and isotope dilution experiments in which unlabeled D-glucosone inhibited the conversion of D-[^{14}C]Glc to L-[^{14}C]AsA by approximately 12-fold (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-[^{14}C]Glc and L-[^{14}C]sorbosone, and unlabeled sorbosone only partially inhibited (approximately 3-fold) the conversion of D-[^{14}C]Glc to L-[^{14}C]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 Arabididopsis 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 *vtc1* is defective in AsA biosynthesis rather than catabolism.

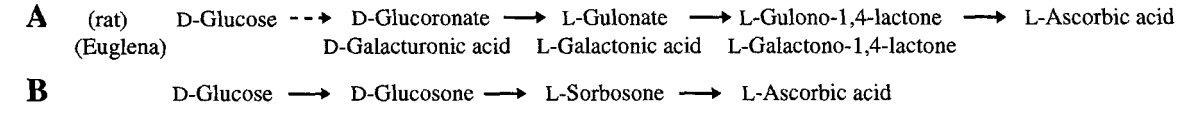

Figure 1. Two proposed pathways for AsA biosynthesis in plants. A, The inversion pathway that is present in rats and many other animals. *Euglena* and other protists also synthesize AsA via an inversion pathway, although the intermediate substrates differ from the classical animal pathway as shown. B, 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 ^{14}C-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 ^{14}C-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 12,000*g* for 2 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 nonenzymatic 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^{-1} 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-^{14}C]AsA or D-[U-^{14}C]Glc

To measure turnover of L-[1-^{14}C]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-^{14}C]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-^{14}C]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-^{14}C]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

jar as described above for 2, 6, or 20 h. Two replicate samples were labeled for each genotype.

Fractionation of ^{14}C-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 12,000*g*. The pellet was saved for later measurement of radioactivity in the insoluble fraction. The soluble fraction was neutralized with 5 M 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 ^{14}C-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 H_2SO_4 (0.5 mL 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 ^{14}C-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 papers were placed in 0.6 mL of water and 4 mL of scintillation fluid to determine radiolabel in carbon dioxide. The ^{14}C 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 spectophotometric 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 isoascorbic (erythorbic) acid as an internal standard (Kutnink 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 oxidization or reduction of AsA, (b) turnover of endogenous AsA pools in the dark, and (c) turnover of L-[1-^{14}C]AsA fed to excised leaves. To monitor the ability of *vtc1* to synthesize AsA, we conducted labeling experiments with D-[U-^{14}C]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 apoplast results in the recovery of a large fraction of AO activity in the insoluble fraction. There was no significant difference between *vtc1* and wild type in the activity of AO in either the soluble or 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 I. *Activity of enzymes of AsA metabolism in wild type and vtc1*

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

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

[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

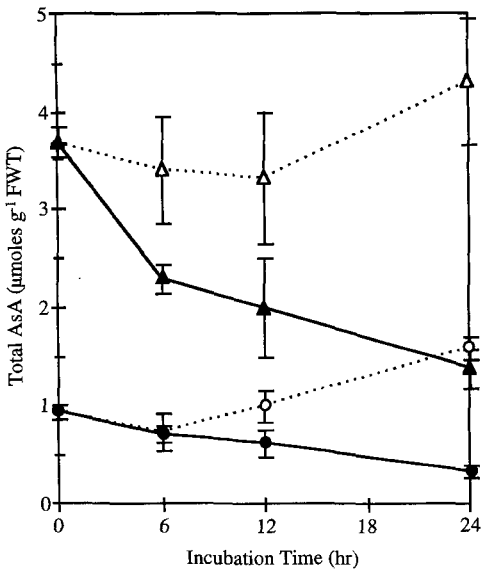

Figure 2. The endogenous pool of AsA in excised leaves of wild type declines faster than in *vtc1* during dark incubation. Leaf slices from both wild-type and *vtc1* plants were floated in water in Petri dishes and incubated in either the light or dark for 0, 6, 12, and 24 h. The concentration of total AsA was then determined. The standard deviation (n = 3) is shown. Solid lines indicate samples incubated in the dark, whereas dotted lines indicate control samples incubated in the light. ▲, Total AsA in wild-type leaves incubated in the dark; △, total AsA in wild-type leaves incubated in the light; ●, total AsA in *vtc1* leaves incubated in the dark; ○, total AsA in *vtc1* leaves incubated in the light. In a replicate experiment conducted with the same conditions, relative AsA levels in dark-incubated leaves were also not significantly different between *vtc1* and wild type (each approximately 50% of initial levels at 24 h). FWT, Fresh weight.

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

Incubation	P-Value (Dark)[a]	
	Absolute Change	Percent Change
h	*μmol AsA g^{-1} fresh wt*	%
0–6	0.028	n.s.[b]
6–12	n.s.	n.s.
12–24	0.005	n.s.
0–12	0.015	n.s.
0–24	0.016	n.s.

[a] Two-sided t-test for difference of means; no assumption of equal variances. [b] n.s., Not significant.

Table III. *Metabolism of [1-^{14}C]AsA by vtc1 and wild-type Arabidopsis*

Excised leaves were fed via the peptide with ^{14}C-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.

Fraction	Wild type	*vtc1*
	% of total	
Soluble	98.4 ± 0.5	97.7 ± 0.9
Insoluble	1.2 ± 0.3	1.8 ± 0.4
CO_2	0.5 ± 0.3	0.7 ± 0.4
	% of soluble	
Neutral	13.2 ± 4.9	41.3 ± 6.7
60 μM Formic acid	56.4 ± 5.3	35.7 ± 4.0
2 M Formic acid	30.2 ± 6.2	22.2 ± 10.0
AsA[a]	51.0 ± 5.6	34.9 ± 3.2
Basic	0.3 ± 0.1	0.5 ± 0.3
	μmol g^{-1} fresh wt	
AsA pool[b]	3.91 ± 0.97	1.05 ± 0.34
AsA + DHA pool[b]	3.83 ± 1.13	1.28 ± 0.81
	μmol 22h^{-1} μmol g^{-1} fresh wt	
Absolute AsA turnover rate[c]	1.58 ± 0.60	0.57 ± 0.10
Relative AsA turnover rate (absolute rate/pool size)	0.41	0.44

[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-^{14}C]AsA by leaves incubated in the light under conditions that permit ascorbate synthesis. Excised leaves were fed with L-[1-^{14}C]AsA for approximately 3 h and then transferred to water for 22 h in the light. The amounts of ^{14}C present in the neutral, acidic, and basic fractions were then determined and are expressed as a percentage of ^{14}C in the total soluble fraction (Table III). The ratio of L-[^{14}C]AsA to total soluble ^{14}C was lower in *vtc1* than in wild type (35% versus 51%, respectively). However, the L-[1-^{14}C]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-^{14}C]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.

There were several differences in the pattern of label incorporation into the various fractions between wild type and *vtc1*, the largest being in the neutral fraction in the experiment shown in Table III (approximately 13% in wild type versus approximately 41% in *vtc1*). However, the only difference that was consistently seen in two independent ^{1}L-[1-^{14}C]AsA labeling experiments was in the AsA-containing 60 mm formic acid fraction.

Table IV. *Metabolism of [U-^{14}C]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 ^{14}C.

Fraction	Incubation Time					
	2 h		6 h		20 h	
	wt	*vtc1*	wt	*vtc1*	wt	*vtc1*
			% of total ^{14}C			
Soluble	72.3	72.8	63.1	66.1	58.4	47.3
Insoluble	27.4	26.7	36.5	31.6	40.1	51.1
Carbon dioxide	0.3	0.5	0.4	2.3	1.5	1.6
			% of soluble			
Neutral	49.0	41.9	3.7	3.7	3.0	4.8
60 mM Formic acid	4.2	11.5	7.0	10.6	6.9	4.4
2 M Formic acid	19.3	24.8	26.7	25.5	32.6	28.7
AsA[a]						
Replicate 1	1.0	0.8	1.8	1.0	2.0	0.5
Replicate 2	1.2	0.8	1.7	0.9	2.6	1.0

[a] From integration of AsA HPLC peak, corrected for recovery.

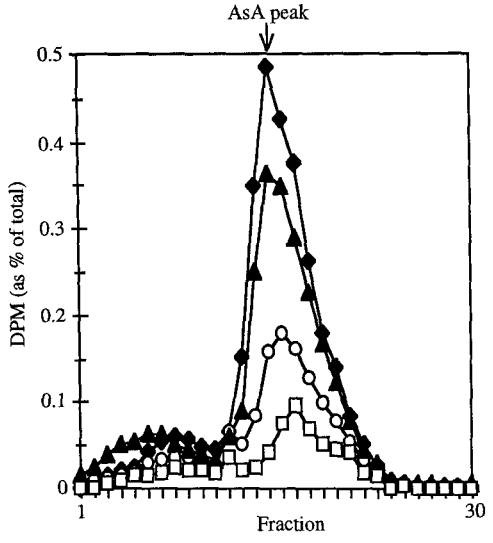

Figure 3. The percentage incorporation of ^{14}C from D-[U-^{14}C]Glc into ^{14}C-AsA is less in *vtc1* than in wild type. Excised leaves were labeled with D-[U-^{14}C]Glc for 45 min and then transferred to unlabeled Glc for 20 h. Soluble extracts were prepared from the labeled leaves. From each sample, the AsA-containing fraction (60 mM formic acid) was further fractionated by HPLC. The AsA peak was collected in 30 fractions and the collected fractions were then assayed for ^{14}C. The dpm in each of the 30 fractions/sample as a percentage of total soluble dpm is shown. The AsA peak is indicated by an arrow. Note that the dpm of the AsA-containing peak was corrected for oxidation of AsA during the fractionation. ▲, dpm in wild type, replicate 1; ◆, dpm in wild type, replicate 2; □, dpm in *vtc1* replicate 1; ○, dpm in *vtc1* replicate 2.

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 ^{14}C-AsA. Excised leaves were fed with D-[U-^{14}C]Glc for approximately 1 h, and then transferred to water for 2, 6, or 20 h in the light, and extracts from these labeled leaves were fractionated as above. The amount of ^{14}C in the fractions was then determined and expressed as a percentage of ^{14}C in the total soluble fraction (Table IV). Unlike the AsA pool, the pool size of Glc is not significantly different in *vtc1* and wild type (data not shown). Therefore, the accumulation of AsA from Glc can be directly compared in the two genotypes by analysis of the percentage of soluble ^{14}C present as L-[^{14}C]AsA by the end of the incubation period.

In every sample at each time point a greater percentage of ^{14}C was present as L-[^{14}C]AsA in wild type than in *vtc1* (Table IV). Even at the 2-h time point, leaves from wild-type plants contained more L-[^{14}C]AsA (as a percent of total ^{14}C) than did *vtc1* leaves. For example, less ^{14}C was present as ^{14}C-AsA in *vtc1* than in wild-type leaves after a 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-glucosone, 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

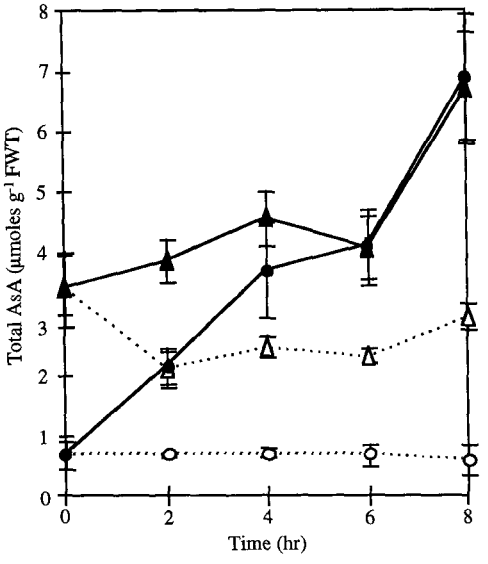

Figure 4. The *vtc1* mutant displays no defect in conversion of L-galactono-1,4-lactone to AsA. The method is the same as that described in Figure 2, except that leaf slices were incubated in water or 25 mM L-galactono-1,4-lactone (GaL) in the light. Tissue was collected for AsA analysis from both control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

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 ^{14}C-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-^{14}C]Glc, *vtc1* contained a smaller percentage of L-[^{14}C]AsA relative to total ^{14}C 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-[^{14}C]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 that 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 isoform 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-^{14}C]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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