

Identification of Ascorbic Acid-Deficient *Arabidopsis thaliana* Mutants

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ABSTRACT

Vitamin C (l-ascorbic acid) is a potent antioxidant and cellular reductant present at millimolar concentrations in plants. This small molecule has roles in the reduction of prosthetic metal ions, cell wall expansion, cell division, and in the detoxification of reactive oxygen generated by photosynthesis and adverse environmental conditions. However, unlike in animals, the biosynthesis of ascorbic acid (AsA) in plants is only beginning to be unraveled. The previously described AsA-deficient *Arabidopsis* mutant *vtc1* (vitamin *c*-1) was recently shown to have a defect in GDP-mannose pyrophosphorylase, providing strong evidence for the recently proposed role of GDP-mannose in AsA biosynthesis. To genetically define other AsA biosynthetic loci, we have used a novel AsA assay to isolate four *vtc* mutants that define three additional *VTC* loci. We have also isolated a second mutant allele of *VTC1*. The four loci represented by the *vtc* mutant collection have been genetically characterized and mapped onto the *Arabidopsis* genome. The *vtc* mutants have differing ozone sensitivities. In addition, two of the mutants, *vtc2-1* and *vtc2-2*, have unusually low levels of AsA in the leaf tissue of mature plants.

MUTANTS have been extremely valuable tools for the dissection of biosynthetic pathways ever since the pioneering work on arginine-requiring mutants of *Neurospora crassa* in the 1940s by Srb and Horowitz. In plants, mutants have greatly facilitated the elucidation of a wide variety of complex pathways, including (for example) the biosynthesis of tryptophan (Radwanski and Last 1995), the hormone abscisic acid (Koornneef *et al.* 1998), carotenoids (Buckner and Robertson 1993; Norris *et al.* 1995), and fatty acids (Miquel and Browse 1998). Although mutants have been utilized to uncover a number of plant primary and secondary metabolic pathways, many more pathways have yet to be unraveled.

The antioxidant and cellular reductant vitamin C (l-ascorbic acid; AsA) has diverse physiological roles in plants (for a review see Smirnoff 1996). Despite the importance of this abundant small molecule in plant physiology and animal health, only very recently has significant progress been made toward elucidation of the plant AsA biosynthetic pathway (Wheeler *et al.* 1998). In contrast, the AsA biosynthetic pathway in animals has been known for some time. Animals generate AsA via the intermediates d-glucose, d-glucuronic acid, l-gulonic acid, and finally l-gulono-1,4-lactone, which is then oxidized to AsA. In this pathway the stereochemistry of the carbon skeleton of the primary substrate

glucose is inverted in the final product (for a review see Burns 1967). A similar pathway was proposed for plants (Isherwood *et al.* 1954). However, it was later shown that inversion of the glucose carbon skeleton does not occur during plant AsA biosynthesis (Loewus 1963). A noninversion pathway with the intermediates d-gluco-sonone and l-sorbosone was then proposed to accommodate the observed stereochemistry (Loewus 1988) although there has been no recent evidence for this pathway. Strong biochemical evidence for a novel plant AsA biosynthetic pathway was recently published. This proposed pathway, with conversion of d-mannose to l-galactose via a little known GDP-mannose-3,5-epimerase as a key step, does not invoke inversion of the carbon skeleton of glucose (Wheeler *et al.* 1998). Furthermore, the Smirnoff/Wheeler pathway leads to conversion of l-galactose to l-galactono-1,4-lactone, the substrate for galactono-1,4-lactone dehydrogenase (GLDH) enzyme (Ōba *et al.* 1995; Østergaard *et al.* 1997), which oxidizes l-galactono-1,4-lactone to AsA.

We are using *Arabidopsis thaliana* mutants that are deficient in AsA to identify genes involved in the maintenance of AsA levels in plants. The identification of such genes will facilitate critical analysis of the plant AsA biosynthetic pathway as well as identify the pathway(s) of AsA catabolism. Screens for ozone-sensitive *A. thaliana* mutants led to the isolation of many mutants, two of which contain only ~25–30% of wild-type (wt) AsA (Conklin *et al.* 1996; P. L. Conklin and R. L. Last, unpublished data). Complementation analysis that relied upon the ozone-sensitive phenotype indicated that these lines have nonallelic recessive mutations and they were named *vtc1-1* and *vtc2-1* (*vtc*, vitamin *c*). The *vtc1-1*

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mutant has a reduced ability to convert d-glucose (the primary substrate for AsA) into AsA, suggesting that this locus encodes a biosynthetic pathway enzyme (Conklin *et al.* 1997). Indeed, the *VTC1* locus was recently cloned and shown to encode a GDP-mannose pyrophosphorylase (Conklin *et al.* 1999). This enzyme catalyzes the conversion of d-mannose-1-P to GDP-mannose, a step in the Smirnov/Wheeler AsA biosynthetic pathway (Wheeler *et al.* 1998). *VTC1* is the first locus that has been genetically identified as having a role in plant AsA biosynthesis, and our results provide strong support for the role of GDP-mannose in AsA biosynthesis.

As the recovery of AsA-deficient mutants from our screen for ozone-sensitive mutants was very low (2/100,000 M₂ plants screened or ~0.002%), we turned to a more direct screen to identify additional *VTC* loci. As detailed below, a nitroblue tetrazolium (NBT)-based assay was developed and used to screen ethyl methanesulfonate (EMS)-mutagenized Arabidopsis seedlings for an AsA-deficient phenotype. The characterization of five new *vtc* mutants isolated by this screen is described below. Together, our collection of mutants represent four different *VTC* loci.

MATERIALS AND METHODS

Plants and growth conditions: All wt and mutant *A. thaliana* lines used in this study are derived from the Columbia (Col-0) ecotype. Mutant lines used for both the quantitative measure of AsA and determination of ozone sensitivity were derived from at least one backcross to Col-0 wt. The *vtc1-1* line was derived from four backcrosses while the *vtc1-2* and *vtc4-1* lines were each backcrossed twice.

Specific growth conditions varied depending on the experiment. Seeds of mutagenized M₂ and F₂ polymorphic mapping populations, as well as F₂ seed used for segregation and linkage analyses, were plated on sterile nutrient medium without sucrose (PN; Haughn and Somerville 1986) and grown on a 24-hr photoperiod as previously described (Li *et al.* 1995). The F₂ *vtc/vtc* mapping individuals were then transplanted to soil-less Cornell mix and grown under a 24-hr photoperiod with other conditions as described (Conklin *et al.* 1999). All plant material used for the quantitative measure of AsA were grown under a 16-hr photoperiod (Conklin *et al.* 1999). Mutant and wt plants to be fumigated with ozone were grown in a constant environment chamber under a 16-hr photoperiod as described (Conklin *et al.* 1996).

L-ascorbic acid assays: A qualitative AsA assay was developed that utilizes NBT as a reagent for the visual detection of AsA. Arabidopsis leaves ~3–8 mm in length are excised and laid on a sheet of chromatography paper. Whatman 3030-6185 paper (Whatman Ltd., Kent, UK) works well for this assay while generic brands do not. Each leaf is then squashed onto the chromatography paper using a curved metal weigh spatula. Ten microliters of a 1 mg/ml aqueous solution of NBT (Sigma, St. Louis) is then pipetted directly onto each squashed leaf. Within ~5 min, a bluish-purple formazan precipitate is visualized around each wt leaf. As the formazan tends to bleed through the chromatography paper, this precipitate can often be visualized better on the back side of the paper.

Two different quantitative AsA assays were used in this study: a previously described ascorbate oxidase-based spectrophotometric assay (Conklin *et al.* 1996) and an HPLC-based assay

modified from the procedure of Kutnink *et al.* (1987) and Mitton and Trevithick (1994). For the detection of total AsA by an electrochemical (EC) assay coupled to HPLC, frozen samples were extracted using Tenbroeck tissue grinders (Lawson Mardon Wheaton Inc., Millville, NJ) in cold 5% metaphosphoric acid containing 0.54 mM Na₂EDTA and samples were microcentrifuged for 10 min to remove solids. The lysate was diluted to 0.2% metaphosphoric acid, 0.54 mM Na₂EDTA, 1 mM cysteine, and 1 mg/ml dithiothreitol (DTT); incubated for 60 min at room temperature prior to injection; and then microfuged for 5 min. Samples of the supernatant were injected onto a C-18 ODS column (BAS, W. Lafayette, IN) with a 0.02 M NaAcetate, 0.54 mM Na₂EDTA, 1.5 mM N-octylamine mobile phase, and AsA was detected with a BAS LC-4B electrochemical detector containing a glassy carbon working electrode. The applied potential was +0.5 V (oxidative) with the sensitivity set at 50 nA. The run time for each sample was 10 min with the AsA peak eluting at ~2 min and a wide DTT peak appearing at ~6 min.

Mutant screen: Col-0 wt seed was mutagenized with EMS as described (Barczak *et al.* 1995). M₂ seed was suspended in 0.1% agar and hand-plated in a grid pattern on PN medium in 100-mm-diameter petri plates at a density of 100 seeds/plate. A single true leaf from each 2-wk-old plant was assayed for AsA using the NBT-based assay. The plants that showed a reduced formazan halo were then transplanted to soil and allowed to self-pollinate. Two-week-old M₃ plants were retested for reduced AsA by the NBT-based assay as well as the ascorbate oxidase- and EC-HPLC-based assays. Mutants that contained ≤50% of wt AsA were subjected to further analysis.

Genetic analyses: To test whether the *vtc* mutation segregated as a single monogenic trait, F₁ seed was obtained by pollination of *VTC/VTC* stigmas with *vtc/vtc* pollen or vice versa. F₁ progeny were allowed to self-pollinate to obtain segregating F₂ populations. Two-week-old plants from these populations were then scored using the NBT-based assay. To test for allelism, an F₂ segregating population was obtained from a cross between two independently isolated ascorbic acid-deficient lines. Two-week-old F₂ plants were then scored for AsA using the NBT-based assay. Two independently isolated *vtc* mutants were judged as nonallelic if F₂ progeny with wt levels of AsA were obtained.

Each of the *VTC* loci was mapped onto the Arabidopsis genome by scoring genetic markers throughout the genome on *vtc/vtc* individuals (scored as Nbt⁻) from a polymorphic F₂ mapping population generated by a cross between the *VTC/VTC* (Ler ecotype) and *vtc/vtc* (Col-0 background). Both microsatellite (Bell and Ecker 1994) and cleaved amplified polymorphic sequences (Konieczny and Ausubel 1993) were used as markers. *VTC2*, *VTC3*, and *VTC4* were mapped using 50 *vtc2-1/vtc2-1*, 54 *vtc3-1/vtc3-1*, and 31 *vtc4-1/vtc4-1* F₂ individuals. Genetic map locations were calculated using the Kosambi mapping function (Koornneef and Stam 1992).

Assessment of ozone sensitivity: Ozone fumigations were performed on 2-wk-old *vtc* mutant and wt plants grown on a 16-hr photoperiod from 6:00 AM to 10:00 PM as previously described (Conklin and Last 1995). Plants were exposed to 400 ppb ozone for 8 hr from 9:00 AM to 5:00 PM. Photographs were taken 16 hr after the end of the exposure.

RESULTS

As described above, the AsA-deficient Arabidopsis mutants *vtc1-1* and *vtc2-1* were isolated by virtue of their ozone sensitivity and are part of a large collection of ozone-sensitive mutants. However as *vtc* mutants repre-

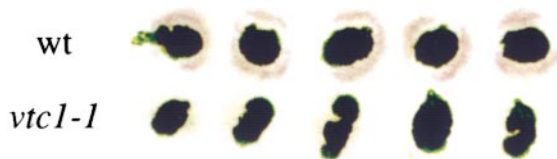


Figure 1.—Differential formation of purple formazan precipitate from reduction of NBT in wt and AsA-deficient *vtc1-1* leaves. One of the first two true rosette leaves from five 2-wk-old plants per genotype was squashed onto Whatman chromatography paper. A solution of NBT was then immediately pipetted onto each squashed leaf as described in materials and methods. After ~ 5 min, the purplish-blue formazan precipitate was scored as a “halo” around each wt leaf, indicative of the presence of wt levels of AsA (top). In contrast, the formazan precipitate was virtually absent in the treated *vtc1-1* leaves (bottom).

sented $<5\%$ of the ozone-sensitive mutant class, it was necessary to develop a direct AsA assay to obtain additional *vtc* mutants. We developed a simple and rapid screen for AsA-deficient Arabidopsis mutants based upon the ability of AsA to convert NBT to formazan. Five additional *vtc* mutants including new alleles of *VTC1* and *VTC2* as well as mutants that define two new complementation groups were identified with this technique.

A novel L-ascorbic acid detection method: Our new AsA assay utilizes the electron transfer dye NBT, which can be reduced by four electrons to yield the dark bluish-purple insoluble formazan. Purified AsA reduces NBT to the formazan, and the high AsA content in plant tissue has allowed us to take advantage of this property. In this method, Arabidopsis leaves are excised and then squashed onto chromatography paper causing the aqueous contents of the leaf to be wicked into the paper. Each squashed leaf is then treated with NBT, and the formazan precipitate is visualized around the (wt) leaf after ~ 5 min.

This simple assay was titrated to reliably differentiate between the AsA level in leaves from 2-wk-old wt and *vtc1-1* plants as shown in Figure 1. Wt Col-0 Arabidopsis at 2 wk of age contains 3–4 μmol AsA/g fresh weight (FWT) while the AsA-deficient mutant *vtc1-1* contains ~ 0.8 – 1.0 μmol AsA/g FWT (Conklin *et al.* 1996). Therefore this assay permits discrimination between the ~ 7 nmol of AsA in a single 2-mg wt leaf and the ~ 2 nmol of AsA in a single *vtc1-1* leaf.

The efficacy of the NBT-based assay was tested by demonstrating that the presence of formazan (Nbt^+) cosegregates with wt levels of AsA while the absence of formazan (Nbt^-) correlates with low AsA levels in a population segregating for the *vtc2-2* mutant allele. Individual F_2 progeny from a population segregating for the *vtc2-2* mutant allele (described below) were scored for NBT. Ten Nbt^- progeny and 20 Nbt^+ progeny were then assayed for total AsA using an EC-HPLC-based method. The Nbt^- individuals were all AsA deficient while the Nbt^+ individuals all contained higher levels of

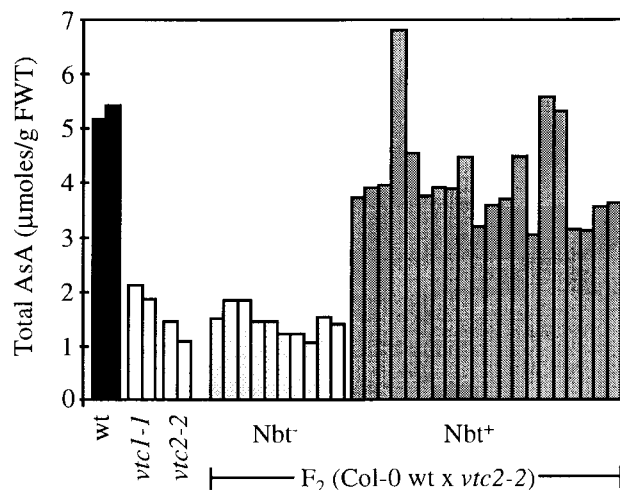


Figure 2.—The purple formazan precipitate in NBT-treated squashed leaves predicts AsA concentrations. One of the first two true leaves from 2-wk-old plants in an F_2 population segregating for a *vtc* allele (Col-0 wt \times *vtc2-2*) was scored for AsA using the NBT-based assay. The plants were then grown for an additional two days and entire rosettes from 10 Nbt^- (little or no visible formazan) and 20 Nbt^+ (readily visible formazan) individuals and control plants (wt, *vtc1-1*, *vtc2-2*) were then harvested, extracted, and subjected to a quantitative total AsA assay using EC-HPLC.

AsA (Figure 2). This demonstrates that *vtc2/vtc2* mutant individuals can be accurately detected in a background of *VTC2/VTC2*.

Isolation of *vtc* mutants: Using this NBT-based assay, ~ 6300 *M. thaliana* plants (ecotype Col-0) from an EMS-mutagenized M_2 population were screened for an AsA deficiency. Forty-eight of the M_2 individuals scored as negative in this primary screen. For each putative mutant line, M_3 generation populations were re-screened for an AsA deficiency using the NBT-based assay, an EC-HPLC-based assay (Kutnick *et al.* 1987; Mitton and Trevithick 1994), and a spectrophotometric assay (Conklin *et al.* 1996). Five of the original 48 putative mutant lines rescored as Nbt^- in the M_3 generation. These 5 lines were independently isolated from different pools of mutagenized seed, and all exhibited AsA deficiencies (based on the spectrophotometric and EC-HPLC assays). Therefore, 5/6300 M_2 plants ($\sim 0.1\%$) screened using the NBT-based assay were AsA deficient as compared to a $\sim 0.002\%$ recovery of *vtc* mutants with the ozone sensitivity-based screen. The fact that all mutants rescoring as Nbt^- in the M_3 generation were AsA deficient underscores the specificity of the NBT-based screen for AsA mutant identification.

Genetic analysis: In addition to using the NBT-based assay to identify new mutants, it was also used for analyses of genetic segregation and allelism. In both cases, individual progeny from two independent crosses per mutant line were scored for the presence (Nbt^+) or absence (Nbt^-) of wt levels of AsA.

TABLE 1

Segregation analysis of NBT phenotype of F₂ progeny from crosses between *vtc* mutant and wild-type plants

Cross ^a	Cross no.	F ₂		χ^2 (<i>P</i>)
		Nbt ⁺	Nbt ⁻	
<i>(vtc1-2</i> × Col-0)	1	178	53	0.52 (0.47)
	2	168	50	0.50 (0.48)
<i>(vtc2-1</i> × Col-0)	1	121	20	8.80 (0.003)
<i>(vtc2-1</i> × <i>Ler</i>)	1	92	27	0.40 (0.53)
<i>(vtc2-2</i> × Col-0)	1	190	76	1.62 (0.2)
	2	185	61	0.005 (0.94)
<i>(vtc2-3</i> × Col-0)	1	233	105	6.63 (0.01)
(Col-0 × <i>vtc2-3</i>)	1	175	74	3.09 (0.08)
(<i>Ler</i> × <i>vtc2-3</i>)	1	251	102	2.86 (0.09)
(Col-0 × <i>vtc3-1</i>)	1	157	46	0.59 (0.44)
	2	158	68	3.12 (0.08)
(Col-0 × <i>vtc4-1</i>)	1	133	60	3.81 (0.05)
	2	133	70	9.73 (0.001)
(<i>Ler</i> × <i>vtc4-1</i>)	1	355	101	1.98 (0.16)

^a In each cross, the pollen recipient is listed first while the pollen donor is listed second.

Our data indicate that the AsA deficiency in the mutants *vtc1-2*, *vtc2-1*, *vtc2-2*, *vtc3-1*, and *vtc4-1* is conferred by single monogenic recessive traits. As shown in Table 1, F₂ progeny from crosses between three of the *vtc* mutant lines (*vtc1-2*, *vtc2-2*, *vtc3-1*) and wt Col-0 segregate in a statistically significant 3:1 ratio of Nbt⁺:Nbt⁻ plants ($P \geq 0.2$). In contrast, the F₂ progeny from the cross between Col-0 wt and *vtc2-1* yielded an unexpectedly high number of Nbt⁺ individuals ($P = 0.003$) while the F₂ progeny of the cross between Col-0 and *vtc4-1* included a somewhat high number of Nbt⁻ individuals ($P \leq 0.05$; Table 1). These data are unlikely to result from a gene dosage effect as both *VTC2/vtc2-1* and *VTC4/vtc4-1* heterozygotes contain wt levels of AsA (data not shown). The reason(s) for the observed anomalies in these segregation ratios is not clear. However, crossing both these mutant alleles to a different wt ecotype (*Ler*) yielded F₂ progeny in the expected 3:1 ratio of Nbt⁺:Nbt⁻ suggesting that the AsA deficiencies in these mutants are indeed conferred by single monogenic recessive traits.

The phenotypes of F₂ progeny from crosses between the mutant *vtc2-3* and Col-0 were somewhat skewed toward the presence of Nbt⁻ individuals. To test the hypothesis that this is a gene dosage effect, AsA levels were quantitatively measured in two sets of pooled F₁ progeny from the cross (*vtc2-3* × Col-0). As seen in Figure 3, these F₁ heterozygotes contain levels of AsA intermediate between the two parents, suggestive of a gene dosage effect. Given the fact that the NBT-based assay is only semi-quantitative, some of the *VTC2/vtc2-3* F₂ progeny were probably scored as Nbt⁻ resulting in the observed skewed ratio of Nbt⁺:Nbt⁻ individuals.

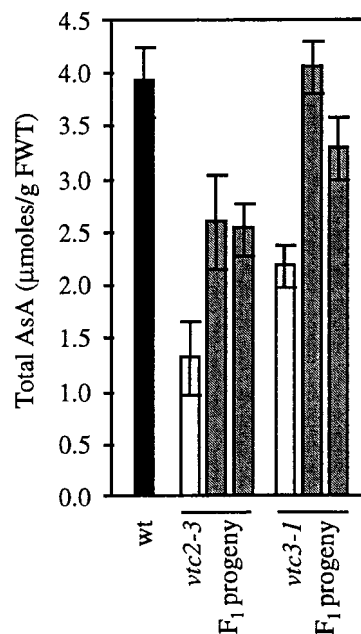


Figure 3.—The *VTC2* gene exhibits a gene dosage effect. Using a spectrophotometric assay, total AsA levels were measured in 2-wk-old wt, *vtc2-3/vtc2-3*, and *VTC2/vtc2-3*. Five individual rosettes were pooled for each extract and three independent extracts were assayed per sample with the mean and standard deviation shown. Assays were performed on F₁ progeny from two independent crosses between Col-0 (wt) and the *vtc2-3*.

We tested for allelism between the AsA-deficient Arabidopsis mutants and these results indicate that the *vtc* mutants represent four different loci: *VTC1*, *VTC2*, *VTC3*, and *VTC4*. As shown in Table 2, both wt (Nbt⁺) and mutant (Nbt⁻) individuals were found in the segregating F₂ progeny from crosses between nonallelic mutants such as *vtc1-1* and *vtc2-2*. In contrast, the F₂ segregating progeny from a cross between mutants harboring mutations at the same locus scored as mutant. A compilation of the segregation data in Table 2 shows that there are two *vtc1* mutants and three *vtc2* mutants, as well as single *vtc3* and *vtc4* alleles.

Genetic map positions: The F₂ segregation data were extended by genetically mapping *VTC2* through *VTC4* (Figure 4). The loci were mapped using polymorphic F₂ mapping populations generated from crosses between these mutants and the wt line *Ler*, which contains well-characterized microsatellite polymorphisms (Bell and Ecker 1994) and cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel 1993) compared with Col-0. The *vtc2-1* mutation was found to map to a position on chromosome 4 ~3 cM centromere distal to CAPS marker WU95, which resides at position 71.70 on the latest Arabidopsis recombinant inbred (RI) genetic map (<http://genome-www.stanford.edu/Arabidopsis/ww/Aug98RImaps/index.html>) and ~5 cM centromere proximal to CAPS marker *PRHA* (position 76.17). *vtc2-2* and *vtc2-3* map to the same region

TABLE 2
Allelism tests of *vtc* mutants

Cross ^a	Cross no.	F ₂		Allelic? ^b
		Nbt ⁺	Nbt ⁻	
<i>(vtc1-1 × vtc1-2)</i>	1	0	48	+
	2	0	50	+
<i>(vtc1-1 × vtc2-1)</i>	1	95	66	-
<i>(vtc1-1 × vtc2-2)</i>	1	175	136	-
	2	130	90	-
<i>(vtc1-1 × vtc2-3)</i>	1	139	126	-
	2	124	94	-
<i>(vtc1-1 × vtc3-1)</i>	1	107	96	-
<i>(vtc3-1 × vtc1-1)</i>	1	138	97	-
<i>(vtc1-1 × vtc4-1)</i>	1	129	75	-
	2	93	79	-
<i>(vtc2-1 × vtc2-2)</i>	1	0	193	+
	2	0	211	+
<i>(vtc2-1 × vtc2-3)</i>	1	0	128	+
	2	0	150	+
<i>(vtc2-1 × vtc3-1)</i>	1	103	75	-
<i>(vtc2-1 × vtc4-1)</i>	1	123	67	-
	2	101	57	-

^a In each cross, the pollen recipient is listed first while the pollen donor is listed second.

^b -, not allelic; +, allelic.

as *vtc2-1* (S. R. Norris and R. L. Last, unpublished results). *VTC3* was mapped to a position on chromosome 2 close to *VTC1*, ~4 cM centromere distal from microsatellite marker nga168 (position 73.01). The *VTC4* locus was mapped to the top of chromosome 4 ~2 cM centromere distal from microsatellite marker nga172 (position 6.83). *VTC1* was previously mapped on chromosome 2 to a position 0.9 cM centromere distal from CAPS marker m429 (Conklin *et al.* 1996, 1999).

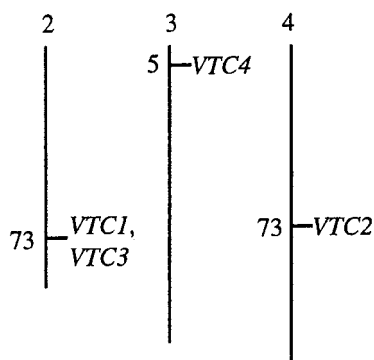


Figure 4.—Relative genetic map positions of the *VTC1*, *VTC2*, *VTC3*, *VTC4* loci. The *VTC* loci were mapped by scoring microsatellite or CAPS markers on *vtc/vtc* individuals from F₂ polymorphic mapping populations segregating for *vtc1-1*, *vtc2-1*, *vtc3-1*, or *vtc4-1*. Numbers beside loci designations refer to approximate position in centimorgans on the latest published RI map while those at the top of lines indicate chromosome numbers.

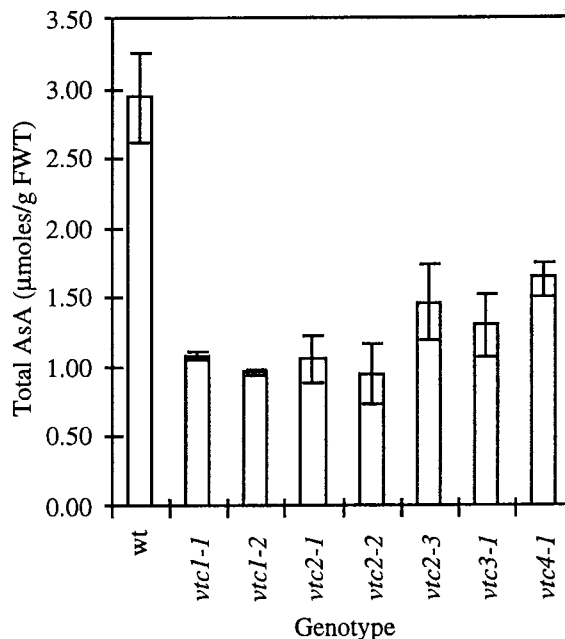


Figure 5.—Quantitative analysis of total AsA levels in 2-wk-old *vtc* mutant and wt leaves. *vtc* and wt lines were spectrophotometrically assayed for total AsA. Whole rosette leaf tissue (50 mg) from pooled 2-wk-old plants was used for each extract. Three independent extracts were assayed per genotype with the mean and standard deviation shown. Each of the *vtc* lines used in this analysis was backcrossed (BC) to wt at least once to remove unlinked mutations (*vtc1-1* was BC4; *vtc1-2* and *vtc4-1* were BC2; *vtc2-1*, *vtc2-2*, *vtc2-3*, and *vtc3-1* were BC1).

1-Ascorbic acid levels in *vtc* mutants: The new *vtc* mutants described above were identified as having a diminished ability to reduce NBT to formazan. To quantitatively measure the AsA deficiencies in these mutants, a spectrophotometric method was used to measure total AsA in 2-wk-old rosettes from each of the *vtc* mutant lines. The lines used in this analysis have all been backcrossed at least once to the wt progenitor to segregate away unlinked mutations. Our results indicate that the *vtc* mutants contain 1/3 to 1/2 the total AsA present in the wt Col-0 progenitor as shown in Figure 5.

In plants, AsA levels are known to increase upon transition from the vegetative to reproductive state (Gander 1982). To determine whether such an increase occurs in the wt and *vtc* mutants, total AsA was measured in mature rosette leaves, immature green siliques (seed pods), and the inflorescence (containing a mixture of opened and unopened flowers) of 6-wk-old plants. As shown in Figure 6, reproductive tissues from wt (green siliques and inflorescences) contain approximately twice the amount of AsA found in wt mature leaves. All the *vtc* mutants are also able to maintain higher levels of AsA in the reproductive tissues relative to that in leaves. In fact, the floral tissues of these “AsA-deficient” mutants contain $\geq 3 \mu\text{mol/g FWT}$ AsA, matching the levels found in rosette leaves of wt.

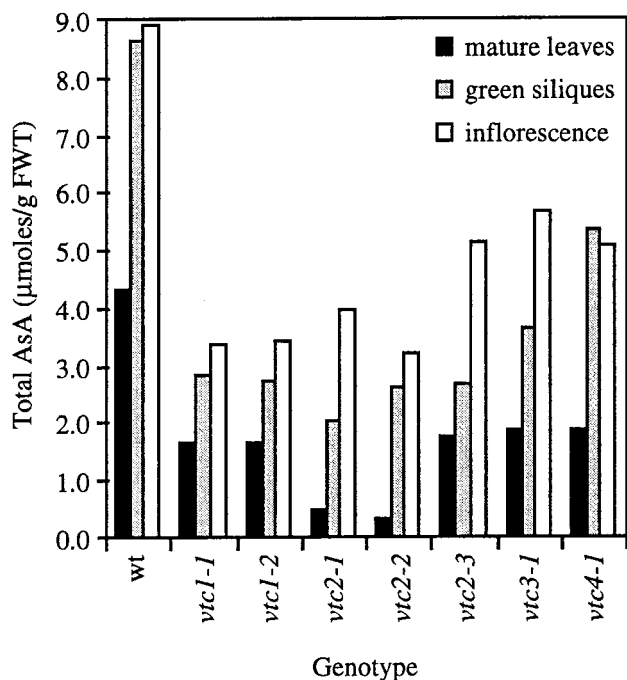


Figure 6.—Analysis of AsA in vegetative and reproductive tissues of 6-wk-old *vtc* mutants and wt. Samples of tissue (~ 100 mg) from fully expanded mature leaves, developing siliques longer than ~ 1 cm, and the inflorescence (including opened and closed buds) were pooled from several individual 6-wk-old plants of each genotype. Extracts were prepared from these samples and assayed for total AsA as in Figure 5. AsA levels in the different tissue types are indicated by the shaded boxes defined in the figure. The entire analysis was repeated with similar results (data not shown).

An interesting anomaly was uncovered upon comparison of the AsA levels in the leaves from 6-wk-old wt and *vtc* mutants. In mature (fully expanded) leaves, the majority of *vtc* mutants maintain AsA levels at $\sim 40\%$ ($\sim 1.7 \mu\text{mol/g FWT}$) of wt. *vtc2-1* and *vtc2-2* represent an exception and mature leaves from these two mutant lines have unusually low levels of AsA ($\sim 10\%$ of wt; $\sim 0.40 \mu\text{mol/g FWT}$). *vtc2-1* is also severely AsA deficient in younger leaves and cauline (stem) leaves from older plants. In summary, 6-wk-old *vtc2-1* and *vtc2-2* have a very severe AsA deficiency in leaves while siliques and inflorescences from these same plants as well as leaves from 2-wk-old plants are not as severely deficient. This suggests either that there is an underlying difference(s) in AsA metabolism in these different tissue types or that *VTC2* is a regulatory gene. As described below, the *vtc* mutants also have unexpected differences in their ozone sensitivities.

Ozone sensitivity: The anthropogenic air pollutant ozone (O_3) is a well-documented cause of oxidative stress in plants. Ozone enters the plant through open stomata and then presumably degrades into $\cdot\text{O}_2^-$, H_2O_2 , and $\text{OH}\cdot$ in the aqueous apoplastic environment (Heath 1994). The wt Col-0 ecotype of Arabidopsis is

quite tolerant to O_3 , probably because these plants mount an effective antioxidant response (Sharma and Davis 1994; Conklin and Last 1995). In contrast, the AsA-deficient mutant *vtc1-1* is extremely O_3 sensitive with visible injury including lesion formation, enhanced chlorosis, and/or tissue collapse (Conklin *et al.* 1996). Severely injured leaves do not recover after the exposure; however, immature leaves emerging during the treatment are not visually injured, presumably due to a lack of fully functioning mature stomata (Conklin *et al.* 1996).

To test the hypothesis that AsA is important for protection against O_3 injury, we examined the sensitivity of the *vtc* mutants and found a surprisingly wide range of response to this source of oxidative stress. Two-week-old *vtc* and wt plants were exposed to 400 ppb O_3 for 8 hr. Directly before this treatment, one set of plants was moved to a control chamber with very similar environmental conditions but where O_3 was depleted by activated charcoal filtration. Photographs were taken of representative treated and control plants 16 hr after the end of the O_3 exposure (Figure 7) and tissue from the control plants was assayed for total AsA (Figure 5). Surprisingly, the different AsA-deficient mutant lines have varied O_3 sensitivities, sometimes even within an allelic series. As previously reported, the *vtc1-1* mutant is very sensitive to O_3 damage (Conklin *et al.* 1996). Consistent with our observation that *vtc1-2* has the same missense mutation as *vtc1-1* (Conklin *et al.* 1999) this mutant exhibits a similar sensitivity. This injury is seen as total collapse and death of both cotyledons and fully expanded leaves. The different *vtc2* mutants have highly varied O_3 -sensitive phenotypes. *vtc2-1* is as sensitive to O_3 as the *vtc1* mutants, and *vtc2-3* appears to be somewhat sensitive as O_3 exposure of this mutant leads to partial collapse of at least one fully expanded leaf per plant. Curiously, *vtc2-2* is not visibly injured by this high dose of O_3 despite that fact that it contains a steady state level of AsA very similar to *vtc2-1* (Figure 5). As with *vtc2-3*, the mutants *vtc3-1* and *vtc4-1* are only slightly more O_3 sensitive than the wt.

DISCUSSION

A rapid and simple assay has been developed to semi-quantitatively measure AsA in leaf tissue using NBT, which is reduced by AsA to generate a purple formazan precipitate. We have defined assay conditions whereby formazan is formed when wt Arabidopsis leaves are treated with NBT, but little or no formazan is visible when assaying the AsA-deficient mutant *vtc1-1*. This assay was utilized to screen populations of EMS-mutagenized 2-wk-old seedlings. Five independent mutant lines were isolated that had a heritable Nbt⁻ phenotype. It was somewhat surprising that these five Nbt⁻ lines were all AsA deficient as other cellular constituents

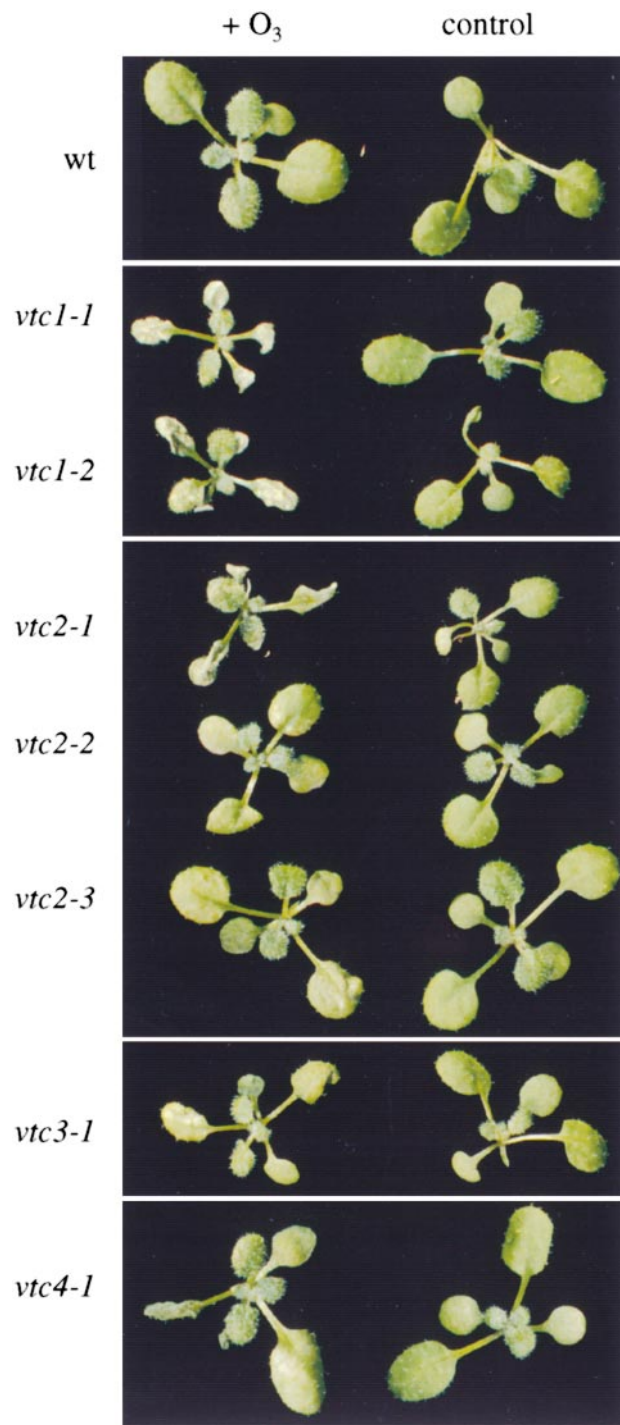


Figure 7.—Analysis of O₃ sensitivity of the *vtc* mutants. Two-week-old plants were grown under a 16-hr photoperiod in a controlled environment chamber and exposed to 400 ppb O₃ for 8 hr (+O₃). Prior to fumigation the control plants were placed in an adjacent O₃-depleted chamber set to similar environmental conditions (control). Photographs of representative plants from each genotype were taken 16 hr after the end of the O₃ exposure.

(such as superoxide) are also able to reduce NBT to formazan. However, as ascorbic acid is present in very high concentrations relative to other possible NBT-

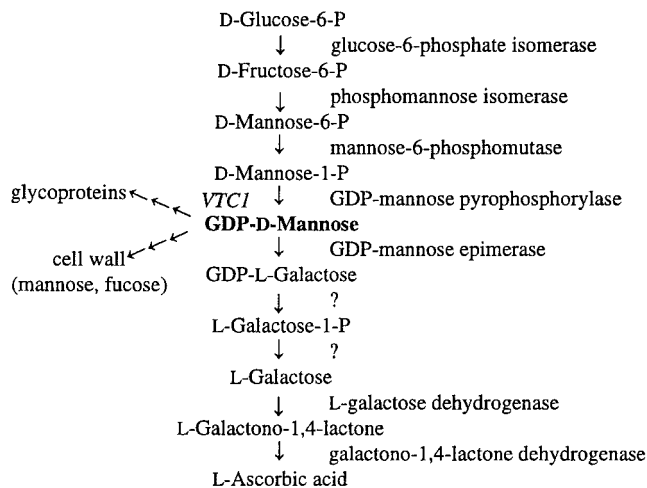


Figure 8.—Proposed pathway for AsA biosynthesis in higher plants (Wheeler *et al.* 1998). The enzymatic step catalyzed by the *VTC1* gene product is shown as well as branch-points to the synthesis of cell wall carbohydrates and glycoproteins. Enzymes: GPI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMPP, GDP-d-mannose pyrophosphorylase; GME, GDP-d-mannose-3,5-epimerase; GDH, 1-galactose dehydrogenase; GLDH, 1-galactono-1,4-lactone dehydrogenase.

reducing agents and the assay was titrated using *vtc1-1* and wt plants, the screen was most likely biased toward the isolation of additional *vtc* mutants. The five new *vtc* mutants represent four *VTC* loci. These are in addition to two *vtc* mutants previously isolated as O₃ sensitive. In total, there are two *vtc1* mutant alleles, three *vtc2* mutants, as well as *vtc3-1* and *vtc4-1*. All four loci were genetically mapped, with *VTC1* and *VTC3* found to be linked at the bottom of Chr 2, *VTC2* on Chr 4, and *VTC4* at the top of Chr 3.

The *vtc* mutants are valuable tools for studying the regulation of AsA accumulation in plants, including elucidation of the biosynthetic pathway. Identification of AsA-deficient Arabidopsis mutants that harbor mutations in AsA biosynthetic enzymes would lend strong genetic evidence for the pathway proposed by Smirnoff and colleagues (Figure 8; Wheeler *et al.* 1998) or an alternative pathway. We have recently shown that *VTC1* encodes GDP-mannose pyrophosphorylase, a predicted enzyme in the Smirnoff/Wheeler pathway (Conkl in *et al.* 1999). Preliminary labeling data suggest that the *vtc2* and *vtc3* mutants have reduced ability to convert mannose to AsA (S. R. Norris, G. L. Wheeler and N. Smirnoff, unpublished data).

Isolation and analysis of the AsA biosynthetic genes and gene products should also help to clarify the subcellular locale(s) of the proposed pathway. The enzymes responsible for the generation of fructose-6-P are well-studied glycolytic enzymes. In plants, glycolysis occurs in the cytosol and at least the first half of the glycolytic pathway is also present in the plastid. Isoenzymes en-

coded by different nuclear genes are thought to be responsible for catalysis of each of the steps in the two parallel pathways (for a review see Plaxton 1996). In yeast, phosphomannose isomerase, phosphomannomutase, and GDP-mannose pyrophosphorylase are all localized to the cytosol (Kepes and Schekman 1988; Smith *et al.* 1992; Hashimoto *et al.* 1997). Similarly, the Arabidopsis GDP-mannose pyrophosphorylase protein inferred from the *VTC1* coding sequence contains no obvious targeting sequences, suggestive of a cytosolic localization of this enzyme in plants (Conklin *et al.* 1999). In contrast, the last biosynthetic enzyme in the pathway, GLDH, is found in the mitochondria of plants (Ôba *et al.* 1995; Østergaard *et al.* 1997). It is plausible that portions of the plant AsA biosynthetic pathway could occur in at least three different subcellular compartments, the plastid, cytosol, and mitochondrion.

In addition to aiding in the elucidation of the AsA biosynthetic pathway and its cellular biology, the *vtc* mutants should help us to better understand the physiological roles of AsA. We have examined the AsA level in both mutant and wt plants at different ages and in different tissue types and have measured the O₃ sensitivity of the different *vtc* mutants. At 2 wk of age, all of the *vtc* mutants contain $\geq 33\%$ of wt AsA. As AsA has many roles, both as an antioxidant and as a cellular reductant, it is plausible that more severe decreases in AsA would be lethal. Consistent with this hypothesis, a nonsense mutation at the *VTC1* locus, which is predicted to abolish the activity of the GDP-mannose pyrophosphorylase, was identified as a cytokinesis-defective embryo lethal (*cyt1-2*; Nickle and Meinke 1998; Somerville *et al.* 1998; W. Lukowitz and C. Somerville, personal communication). While this is suggestive of an essential requirement for AsA, the GDP-mannose synthesized by GDP-mannose pyrophosphorylase (Figure 8) is used in protein glycosylation and cell wall synthesis in addition to AsA synthesis. Because of the predicted pleiotrophy of a mutant at this step in the pathway, the embryo-lethal phenotype of *cyt1-2* is presumably due to a combination of factors including defective protein glycosylation and cell wall structure as well as a lack of AsA.

As mentioned above, the *vtc* mutants are all only partially AsA deficient. These deficiencies could result from mutations causing partial inactivation of wt functions (leaky mutations) and/or functional redundancy of some of the genes involved in AsA biosynthesis. For example, we believe that the mutant *vtc1-1* is partially deficient due to a leaky mutation. *vtc1-1* contains a missense mutation that is predicted to alter a highly conserved proline residue in the GDP-mannose pyrophosphorylase amino acid sequence. As this mutant still contains $\sim 33\%$ of wt AsA and partial GDP-mannose pyrophosphorylase activity, we hypothesized that the nonlethal *vtc1-1* mutation negatively affects but does

not abolish this enzyme activity (Conklin *et al.* 1999).

In 6-wk-old wt Arabidopsis, the AsA levels in reproductive tissues (open and closed flowers and green siliques) are approximately double the amount assayed in mature leaves. It is well established that AsA levels are generally high in plant reproductive organs relative to vegetative tissue (reviewed in Gander 1982). This is likely due to a greater need for AsA in these tissues. Reproductive tissues have higher metabolic rates, which would necessitate the need for increased levels of antioxidants. In addition, such tissues have higher rates of cell expansion and division and AsA is thought to have a role in these processes (for a review see Smirnoff 1996). While the control mechanism(s) orchestrating this organ-specific up-regulation is not known, it has been shown that carbohydrate status has a role in governing AsA levels in plants (Smirnoff and Pallanca 1996). The relative higher level of AsA in flowers and siliques over that in mature leaves is maintained in the *vtc* mutants despite their overall AsA deficiency, implying that the mutants retain the control mechanism(s).

vtc2-1 and *vtc2-2* rosette and cauline leaves from 6-wk-old plants contain $\sim 10\%$ of wt amounts of AsA while the amount of AsA in the seedlings, inflorescence, and green siliques of these mutants is similar to that of *vtc1-1* and *vtc1-2* ($\sim 33\%$ of wt). Despite the very low leaf levels of AsA in the leaves of these older plants, this tissue looks quite normal, suggesting that other antioxidants are compensating in this tissue or that high levels of AsA are not necessary in the leaves of older plants. We have preliminary evidence that *vtc2-1* is defective in AsA biosynthesis (S. R. Norris, G. L. Wheeler and N. Smirnoff, unpublished data). If *VTC2* encodes a biosynthetic enzyme, one could imagine specific protein alterations that would affect enzymatic activity as flux through the pathway changes. For example, a mutation increasing the K_m of an AsA biosynthetic enzyme might cause a severe decrease in AsA only in tissues where carbohydrate is limiting. Limited carbon may be available for AsA synthesis in leaves of older plants (*i.e.*, it is getting shunted to the reproductive tissues) while carbon may not be limiting in young leaves, growing siliques, and the inflorescence. As *vtc2-1* and *vtc2-2* have low AsA in the leaves of older plants, but not in these other tissues, we hypothesize that these two mutant alleles might represent such a biochemical defect. The *vtc2-3* mutant does not follow this pattern as the AsA level in mature leaves of this mutant are not severely affected ($\sim 33\%$ of wt). An alternative hypothesis is that *VTC2* is a regulatory locus rather than encoding a biosynthetic enzyme. Given the fact that AsA levels in wt vary in different tissues, specific mutations at a site involved in this regulation could adversely affect AsA levels in a tissue-specific manner. The *VTC2* locus is currently being cloned; identification of the *VTC2* gene product and the nature of the *vtc2* mutations will hopefully shed some light on this interesting question.

In addition to the differing AsA phenotype of the *vtc* mutants, we have also found unexpected differences in O₃ sensitivity between the mutants. O₃ generates oxygen free radicals within plant tissue and the mutants *vtc1-1*, *vtc1-2*, and *vtc2-1* are all quite O₃ sensitive relative to wt. In contrast, the other *vtc* mutants are also AsA deficient, while exhibiting only mild O₃ sensitivity. Given that the mutants *vtc2-3*, *vtc3-1*, and *vtc4-1* have slightly higher levels of AsA (~50% of wt) than the more O₃-sensitive *vtc1-1*, *vtc1-2*, and *vtc2-1*, it is possible that the threshold between O₃ resistance and sensitivity is just below this amount. Alternatively, side products and/or metabolic intermediates with antioxidant activity could be accumulating in *vtc3-1* and *vtc4-1*, affording some protection against the free radicals generated by O₃. The difference in O₃ sensitivity of the mutants *vtc2-1* and *vtc2-2* cannot be explained by a threshold mechanism as they have very similar levels of AsA yet vastly different sensitivities to O₃. The physiological basis for this difference remains to be determined.

Identification of a broad range of mutants has revealed the complexity of AsA physiology and metabolism in plants. As several of the *vtc* mutant alleles have a response to O₃ similar to that of wt, they would not have been isolated in a screen for O₃-sensitive mutants. Alternatively, isolation of several *vtc* mutants by a direct screen has demonstrated the utility of the NBT-based assay as a facile method for the specific isolation of AsA-deficient mutants. Identification of *VTC1* as encoding GDP-mannose pyrophosphorylase provided strong genetic support for the proposed role of GDP-mannose in AsA biosynthesis. Likewise, cloning of the *VTC2*, *VTC3*, and *VTC4* loci should also add greatly to our understanding of how plants synthesize AsA.

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