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Environmental stress sensitivity of an ascorbic acid-deficient Arabidopsis mutant

(ozone/ultraviolet B/sulfur dioxide/reactive oxygen detoxification/vitamin C)

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ABSTRACT **L-ascorbic acid (vitamin C) is a powerful reducing agent found in millimolar concentrations in plants, and is proposed to play an important role in scavenging free radicals in plants and animals. However, surprisingly little is known about the role of this antioxidant in plant environmental stress adaptation or ascorbate biosynthesis. We report the isolation of *soz1*, a semi-dominant ozone-sensitive mutant that accumulates only 30% of the normal ascorbate concentration. The results of genetic approaches and feeding studies show that the ascorbate concentration affects foliar resistance to the oxidizing gas ozone. Consistent with the proposed role for ascorbate in reactive oxygen species detoxification, lipid peroxides are elevated in *soz1*, but not in wild type following ozone fumigation. We show that the *soz1* mutant is hypersensitive to both sulfur dioxide and ultraviolet B irradiation, thus implicating ascorbate in defense against varied environmental stresses. In addition to defining the first ascorbate deficient mutant in plants, these results indicate that screening for ozone-sensitive mutants is a powerful method for identifying physiologically important antioxidant mechanisms and signal transduction pathways. Analysis of *soz1* should lead to more information about the physiological roles and metabolism of ascorbate.**

Free radicals can damage macromolecules by oxidative processes, leading to cancer and other diseases associated with aging (1). Antioxidants act to detoxify reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($\cdot OH^-$), and organic hydroperoxides. Enzymes active in ROS removal include superoxide dismutases, catalases, peroxidases, and glutathione *S*-transferases (1). Plants also synthesize abundant small molecule antioxidants including AsA (L-ascorbic acid or Vitamin C), glutathione, α-tocopherol (Vitamin E), and carotenoids (2). Although there is increasing evidence that these plant-derived antioxidants are important components of the human diet, relatively little is known about their specific functions in plants.

Microbial and animal mutants altered in antioxidant enzymes and signal transduction processes played a pivotal role in testing the importance of specific antioxidant detoxification pathways, evaluating the proposed role of ROS in cellular processes and dissecting ROS signal transduction. For example, bacterial mutants altered in response to growth under enhanced active oxygen conditions identified key antioxidant enzymes and proteins that regulate their synthesis (3). Yeast mutants deficient in glutathione are hypersensitive to H_2O_2, implicating glutathione in ROS detoxification (4). A mammalian cell line expressing a mutant $p21^{ras}$ was recently used to demonstrate a key role for this G protein in ROS signal transduction (5).

A collection of oxidative stress-sensitive plant mutants would permit a critical assessment of the roles of antioxidant systems and elucidation of ROS signal transduction pathways. With this goal in mind, *Arabidopsis thaliana* mutants with altered sensitivity to the anthropogenic oxidizing air pollutant O_3 (ozone) (6) are being identified. We describe here a semi-dominant monogenic O_3 sensitive mutant (*soz1*, sensitive to ozone), which is deficient in ascorbic acid (AsA). This deficiency also causes sensitivity to other ROS-generating compounds such as SO_2 (sulfur dioxide) and UV-B (ultraviolet-B) light.

MATERIALS AND METHODS

Plant Culture. *A. thaliana* (ecotype Col-0) used in this study were grown in "Cornell Mix" soil (7) in a constant-environment chamber, except where noted. Conditions in this chamber were as described (8). All biochemical analyses used plants grown under a 16-hr photoperiod.

Mutant Screen. Wild-type Columbia (Col-0) Arabidopsis seed was mutagenized with ethyl methanesulfonate as described (9). Twenty-thousand M_2 seeds were stratified for 4 days at 4°C in 0.1% agar and sown at a density of 40/cm^2. Plants were grown under a 24-hr photoperiod for 14 days and then exposed to 250 ppb (parts per billion) O_3 for 8 hr (8). The O_3-treated plants were screened visually for individuals with enhanced damage after 24 hr. Putative mutants were transplanted several days later and allowed to self-pollinate to obtain M_3 progeny for further studies.

Genetic Analysis. To determine dominance, F_1 seed (*SOZ1/soz1*) was obtained by pollination of *SOZ1/SOZ1* with *soz1/soz1* (M_4) pollen, and F_2 seeds were obtained by self-pollination of F_1. F_2 progeny from this cross were grown under a 16-hr photoperiod for 2 weeks, exposed to 250 ppb O_3 for 8 hr, and scored for O_3 sensitivity 16 hr after the exposure.

The *SOZ1* locus was mapped onto the Arabidopsis genome with 117 individuals from a polymorphic F_2 mapping population generated by self-pollination of F_1 seed from a cross between the *SOZ1/SOZ1* (*Ler* ecotype) and *soz1/soz1* (isolated in the Col-0 background) with the use of both cleaved amplified polymorphic sequencer (10) and microsatellite (11) markers. The Kosambi mapping function was used to calculate the map distances (12).

Biochemical Assays. For both AsA and glutathione assays, rosette tissue was pooled and acidic extracts were produced as described (13). Glutathione (total and oxidized) was measured in a neutralized extract with the 5,5′-dithiobis(2-nitrobenzoic acid) assay as described (14). AsA was measured in a neutralized extract with the AsA oxidase assay, with the oxidized form (dehydroascorbate) estimated as (AsA_{total} − $ASA_{reduced}$) (13).

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Abbreviations: AsA, L-ascorbic acid; ppb, parts per billion; ROS, reactive oxygen species; UV-B, ultraviolet B; $UV\text{-}B_{BE}$, biologically effective ultraviolet B light.

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A standard curve for AsA_{total} was generated with purified AsA (Sigma), with the addition of dithiothreitol to a final concentration of 4 mM. Dithiothreitol was also added to the samples for the standard curve because the oxidized dithiothreitol formed from reduction of dehydroascorbate absorbs UV light (15). Relative AsA concentrations for *soz1* and wild type, obtained by the spectrophotometric method, were confirmed by HPLC analysis of the bis(dinitrophenyl)hydrazone derivative of dehydroascorbate (16).

Malondialdehyde lipid peroxidation products were assayed as 2-thiobarbituric acid-reactive substances (7). Malondialdehyde levels are expressed on a dry weight basis, because O_3 causes severe dehydration of *soz1*. Average freshweight:dryweight ratios were derived from 2–3 replicate samples unless noted otherwise in the legend of Table 1.

SO_2 and UV-B Treatments. Five-week-old plants grown on a 16-hr photoperiod were exposed to 800 ppb SO_2 for 6 hr and photographed 24 hr later. A Model 43 Pulsed Fluorescent SO_2 Analyzer (Thermo Environmental, Franklin, MA) was used to measure SO_2. UV-B experiments were performed on soil grown plants under a 24-hr photoperiod as described (7). Seeds were allowed to germinate under Mylar filtered light, and 4 days after planting were exposed to continuous 0.9 kJ biologically effective UV-B ($UV\text{-}B_{BE}$) $m^{-2}\cdot h^{-1}$ (17) for 17 days by filtration through Pyrex glass, or kept under mylar for, UV-B controls.

RESULTS

Mutant Screen. Two-week-old wild-type *A. thaliana* plants (ecotype Col-0) are resistant to O_3 fumigation. For example, the wild type exhibits little sign of O_3 pathology following exposure to 400 ppb for 8 hr (Fig. 1*A*). This intrinsic tolerance facilitated the search for O_3-sensitive mutants, which were identified by exposure to 250 ppb O_3 for 8 hr. One day after treatment, fumigated plants were screened visually for those with enhanced chlorosis, lesions, or tissue collapse. Only 4 putative mutants out of 20,000 M_2 plants screened appeared unusually debilitated by fumigation, and 3 of these exhibited heritable O_3 sensitivity. One of these mutants (*soz1*) was chosen for intensive analysis because it showed dramatic leaf damage (Fig. 1*A*). Exposure of *soz1* to ≥200 ppb O_3 for 8 hr causes tissue collapse and death of all fully expanded rosette leaves. Although the affected leaves do not recover from O_3 treatment, the toxicity is not systemic and newly emerging leaves are not noticeably harmed. The relative insensitivity of immature organs presumably results from lack of access of the gas to tissues that lack functional stomata. The O_3-sensitive phenotypes of the two other mutants isolated in this screen are distinct from that of *soz1*. Upon O_3 exposure, the second mutant (which is not allelic to *soz1*) becomes chlorotic, whereas the third develops large well-defined lesions on all fully expanded leaves.

Table 1. Effect of O_3 on lipid peroxide accumulation

Genotype	O_3, hr	FWT/DWT*	MDA†
SOZ1	0	12.4 ± 0.4	82.8 ± 12.8
	4	9.9 ± 0.6	87.7 ± 8.1
soz1	0	10.7 ± 1.1	67.9 ± 16.1
	4	5.2 ± 0.5	135.8 ± 15.6

Fifteen-day-old plants were exposed to 400 ppb O_3 for 4 hr. Tissue was collected for analysis prior to (0 hr) and immediately after (4 hr) exposure. Average fresh weight:dryweight (FWT/DWT) ratios and malondialdehyde (MDA) values are shown for three independent experiments with SE.

*For the *soz1* 4-hr sample, a single DWT value was used to obtain FWT/DWT in one of the three O_3 treatments.

†MDA, μmol of 2-thiobarbituric acid-reactive substances/g DWT.

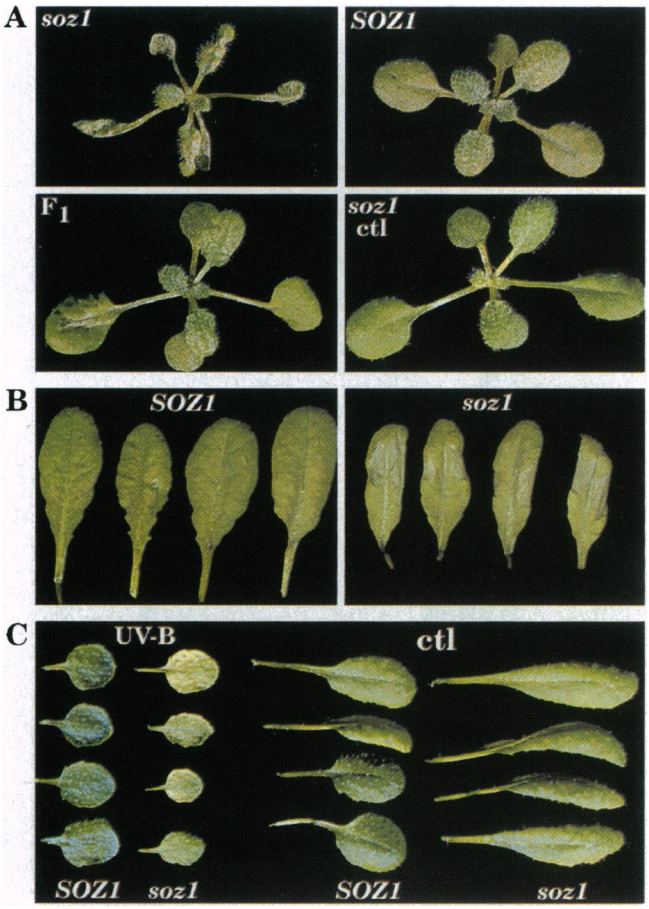


FIG. 1. Response of *soz1* to O_3, SO_2, and UV-B. Plants exposed to O_3 and SO_2 were photographed 24 hr after the exposure ended. (*A*) Representative 15-day-old *soz1/soz1*, *SOZ1/SOZ1*, and *SOZ1/soz1* (F_1) individuals exposed to 400 ppb O_3 for 8 hr. Also shown is an unexposed *soz1/soz1* homozygous mutant plant (*soz1* ctl). (*B*) Wild-type and *soz1* mutant exposed to 800 ppb SO_2 for 6 hr. Individual rosette leaves from four different 5-week-old individuals are shown for each genotype. (*C*) Wild type and *soz1* exposed for 17 days to 0.9 $UV\text{-}B_{BE}$ $m^{-2}\cdot h^{-1}$ (UV-B) or UV-B deficient Mylar-filtered light (ctl). Single rosette leaves from four different individuals are shown for each genotype and treatment.

Genetic Analysis of *soz1*. The O_3 sensitivity of *soz1* is conferred by a semi-dominant monogenic mutation, as seen when heterozygous *soz1/SOZ1* F_1 progeny were challenged with 400 ppb of O_3 for 8 hr. The F_1 exhibited an O_3 sensitivity intermediate between that of *SOZ1/SOZ1* wild-type and *soz1/soz1* mutant plants, producing O_3 induced lesions without the massive tissue collapse characteristic of the homozygous mutant (Fig. 1*A*). Unlike wild type, the F_1 plants were somewhat wilted immediately after O_3 exposure. As further evidence of this semi-dominance, three phenotypic classes (O_3 sensitive, O_3 semi-resistant, O_3 resistant) were identified in segregating F_2 progeny from a self-cross of the heterozygote. In this F_2 population, the O_3-sensitivity segregated in an approximately 3:1 O_3-resistant plus semi-resistant:O_3-sensitive ratio (358:101, $P > 0.10$), consistent with a monogenic trait. As shown in Fig. 2*A*, the *SOZ1* locus maps to the bottom of chromosome 2, 15.9 centimorgans below cleaved amplified polymorphic sequence marker m429 (10) and 16.8 centimorgans centromere distal to microsatellite marker nga168 (11).

***soz1* Is Impaired in ROS Detoxification.** Based upon the severe O_3 damage to *soz1* plants, we hypothesized that this mutant is defective in ROS detoxification. This view was strengthened by the observation that, when detached leaves were floated in increasing concentrations of H_2O_2 in an

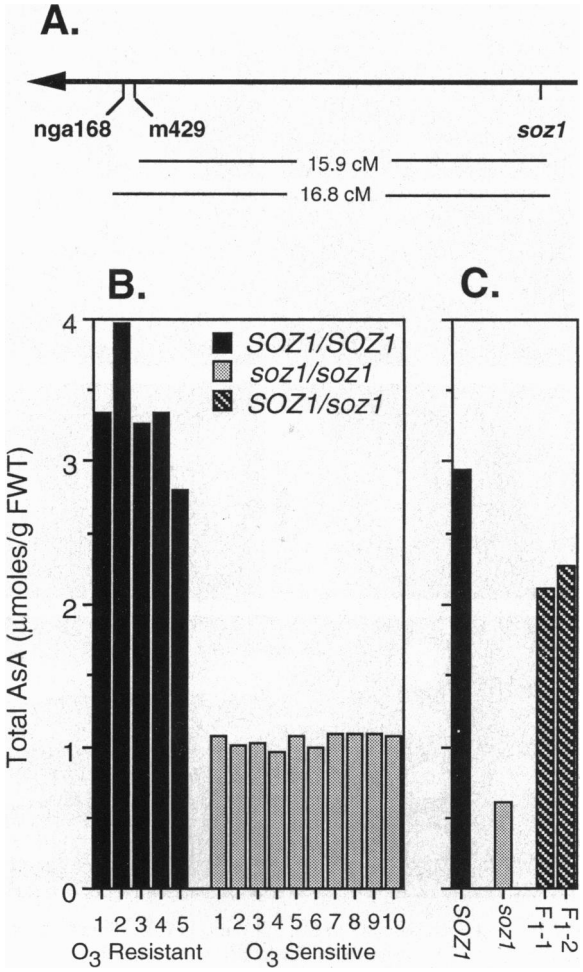


FIG. 2. Map location of *soz1* and cosegregation of both O_3 sensitivity and ascorbate deficiency with the mutation. (*A*) Map position of *soz1* relative to selected physical markers on chromosome 2. Arrowhead points toward the centromere. (*B*) Total AsA was determined in 14-day-old self-pollinated progeny from either true-breeding O_3-resistant (*SOZ1/SOZ1*, solid bars) or -sensitive (*soz1/soz1*, shaded bars) F_2 plants from the cross (*SOZ1/SOZ1* × *soz1/soz1*). (*C*) Total AsA was determined in 14-day-old *SOZ1/SOZ1*, *soz1/soz1*, and two independently generated pools of F_1 plants (*SOZ1/soz1*, crosshatched bars)

aqueous solution, *soz1* leaves became bleached at a lower concentration of H_2O_2 than did wild type (data not shown). An impaired ability to adapt to elevated ROS would result in increased oxidative damage to cellular components including lipids. To test this proposal, the accumulation of lipid peroxides in both *soz1* and wild type was measured using a malondialdehyde assay (18, 19). Lipid peroxide levels in *soz1* were increased ≈2 times after 4 hr exposure to 400 ppb O_3, and in contrast remained virtually unchanged in *SOZ1* (Table 1). This suggests that, whereas wild-type plants are able to successfully adapt to the enhanced ROS following O_3 fumigation, free radical detoxification is compromised in *soz1*. It is interesting to note that *soz1* plants do not experience enhanced lipid peroxide accumulation when grown in a growth chamber with charcoal-filtered air (Table 1).

***soz1* Is Ascorbate Deficient.** Plants contain millimolar concentrations of the soluble antioxidants ascorbic acid and glutathione. Consistent with a role for AsA in O_3 adaptation, we found that 2-week-old *soz1* plants contain only ≈30% of the wild-type level of total AsA (Table 2). As seen in Table 2, the absolute level of oxidized AsA (in the form of dehydroascorbate) is similar in the two genotypes. However, a greater

Table 2. Total and oxidized ascorbate and glutathione in *soz1*

Genotype	Total AsA	DHA	DHA % of total	Glutathione	GSSG*
SOZ1	42.7 ± 1.7	5.5 ± 2.8	≈13%	5.3 ± 0.8	0.16 ± 0.02
soz1	11.9 ± 0.6	2.8 ± 0.5	≈24%	5.0 ± 0.3	0.10 ± 0.05

Two-week-old plants were harvested for analysis. Average values (μmol/g dryweight) from five (for glutathione) or six (for AsA) independent extractions of each genotype with SD shown. DHA, dehydroascorbate. GSSG, oxidized glutathione.
*GSSG is expressed as μmol of glutathione equivalents/g dry weight.

percentage of the AsA pool is oxidized in *soz1* than in *SOZ1*. This altered ratio of reduced to oxidized AsA is presumably the result of the smaller AsA pool in *soz1*. Despite the proposed role of glutathione as a reductant for oxidized AsA (2), the presence of a diminished AsA pool in *soz1* does not affect the accumulation of reduced or oxidized glutathione (Table 2).

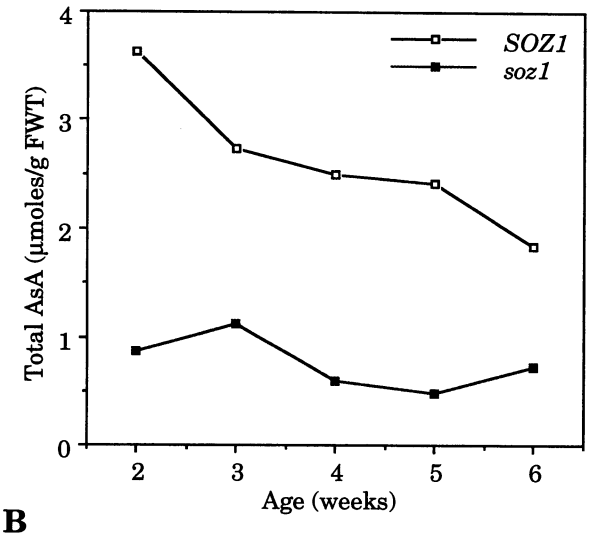
Ascorbate Deficiency Correlates with O_3 Sensitivity. A number of approaches were taken to test whether the O_3 sensitivity of *soz1* is caused by AsA deficiency. First, we asked whether the *soz1* AsA deficiency and O_3 sensitivity are the result of the same mutation by monitoring both phenotypes in a segregating population from a genetic cross (Fig. 2*B*). True-breeding F_3 families (derived from self-pollination of F_2 individuals from the cross *SOZ1/SOZ1* × *soz1*/*soz1*) were assayed for total AsA and scored for O_3 sensitivity. As would be expected if one genetic locus causes both defects, a perfect correlation was observed between O_3 sensitivity and AsA deficiency in 15 F_3 families.

If AsA plays an important role in mediating O_3 tolerance in Arabidopsis, there should be a correlation between the amount of AsA that accumulates and the degree of stress resistance. This idea was tested in two ways. First, it was found that F_1 *SOZ1/soz1* heterozygous plants, which display an intermediate O_3 sensitivity (Fig. 1*A*), accumulate a concentration of total AsA intermediate to that of *SOZ1/SOZ1* and *soz1/soz1* homozygotes (Fig. 2*C*). The heterozygote reproducibly accumulates ≈75% of the wild-type concentration, which is 250% that in *soz1*. The assertion that AsA plays an important role in O_3 tolerance is strengthened by the observation that AsA levels correlate with O_3 resistance during Col-0 wild-type (*SOZ1/SOZ1*) development. AsA levels fall between the ages of 2 and 6 weeks, whereas O_3 injury, measured as lesions of ≥1 mm in diameter, increases during this developmental time course, as shown in Fig. 3.

To further test the hypothesis that O_3 sensitivity in Arabidopsis is influenced by AsA concentration, this antioxidant was artificially elevated in *soz1*. Both wild-type and mutant plants were watered with H_2O (control), AsA, or L-galactono-1,4-lactone (a proposed AsA precursor). Forty-eight hours after this treatment plants were either harvested for AsA analysis or exposed to O_3. Pretreatment with AsA or L-galactono-1,4-lactone caused elevation of AsA in both *soz1* and wild type (Fig. 4*A*) and this restored O_3 resistance to *soz1* (Fig. 4*B*). This result further strengthens the hypothesis that the *soz1* O_3 sensitivity is caused by an AsA deficit.

Sensitivity of *soz1* to Other Abiotic Stresses. The AsA deficient *soz1* mutant was used to assess the importance of AsA in adaptation to SO_2 and UV-B, which both generate ROS (7, 20). To assess SO_2 sensitivity, 5-week-old plants were exposed to 800 ppb SO_2 for 6 hr. Under these conditions *soz1* developed large spreading bifacial necrotic lesions typical of SO_2 injury within 24 hr of the treatment, whereas *SOZ1* plants were nearly uninjured (Fig. 1*B*). Exposure to a chronic high dose of UV-B (0.9 kJ UV-B_{BE} m^{-2}·h^{-1}) for 17 days caused severe growth retardation in both wild-type and mutant plants. However, unlike wild type, *soz1* leaves became chlorotic (Fig. 1*C*), a phenotype that was consistently seen in three independent

A



B

Age (weeks)	2	3	4	5	6
SOZ1 (% injury)	4	36	53	27	47

FIG. 3. AsA and O_3 resistance both decline during development in wild type. (*A*) Total AsA was determined in *SOZ1* rosette tissue at 1-week intervals during development. (*B*) Plants were exposed to 400 ppb of O_3 for 8 hr and scored for injury 16 hr after the exposure. O_3 injury was measured as the percentage of plants (n = 25) with O_3-induced lesions of ≥1 mm in diameter.

UV-B treatments. Collectively, these results show that AsA is important in the detoxification of ROS generated by a variety of diverse sources.

DISCUSSION

The *A. thaliana soz1* mutant described above is hypersensitive to the air pollutant O_3, and has enhanced lipid peroxidation products following fumigation. Several lines of evidence have been presented that suggest the failure to adapt to oxidative stress is caused by a deficiency in L-ascorbic acid (vitamin C). First, the *SOZ1* gene dosage correlates with both AsA accumulation and O_3 tolerance in 2-week-old plants (Figs. 1*A* and 2*C*), and the two phenotypes cosegregate in a genetic cross (Fig. 2*B*). Second, developmental studies of wild-type Arabidopsis indicate a correlation between enhanced O_3 sensitivity and reduced AsA concentration as the plants age (Fig. 3). Finally, both the *soz1* O_3 sensitivity and AsA deficiency are reversed by feeding with L-galactono-1,4-lactone or AsA (Fig. 4).

Several other aspects of our results are worthy of note. The *soz1* mutation is pleiotropic, showing that AsA also plays a role in adaptation to the ROS generated by SO_2 and UV-B (Fig. 1 *B* and *C*). It is also surprising that this AsA deficient mutant has a phenotype very similar to wild type when propagated in a growth chamber under standard Arabidopsis culture conditions with a 16-hr photoperiod and charcoal-filtered air (Fig. 1*A*). This suggests that the residual AsA in *soz1* is sufficient to fulfill its roles as a scavenger of ROS and a reducing agent under controlled environment growth conditions. However, the rosette leaves of *soz1* are elongated when grown under the same conditions with constant illumination (Fig. 1*C*; "ctl" plants). Finally, the screen for O_3-sensitive mutants is influenced by the fumigation conditions and is rather subjective. For example, a greater percentage of sensitive mutants were recovered in a more recent screen using a higher dose of O_3 and progeny testing those M_2 plants with less visible injury.

A

Genotype	+ H_2O I	+ H_2O II	+ GaL I	+ GaL II	+ AsA I	+ AsA II
SOZ1	3.4	3.3	17.3	17.8	19.6	20.3
soz1	0.9	1.3	19.6	21.3	20.8	23.8

B

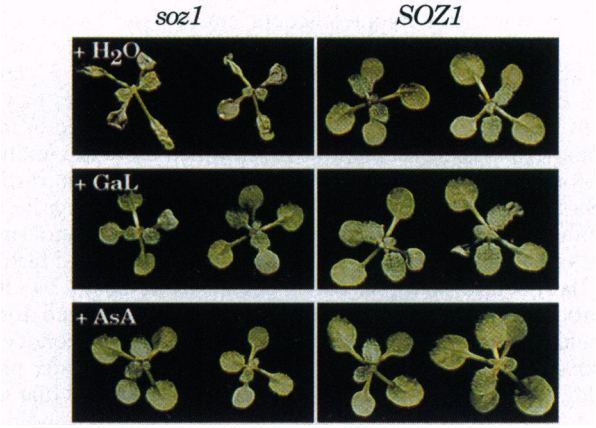


FIG. 4. Treatment of *soz1* with an AsA precursor or AsA results in elevation of total AsA and O_3 tolerance. Duplicate pots of 12-day-old plants (≈100 seeds per pot) were watered with 50 ml of H_2O, 0.25 M L-galactono-1,4-lactone (GaL), or 0.25 M AsA and allowed to grow for 48 hr. (*A*) AsA levels of leaf tissue. Total AsA (μmol/g fresh weight) in the samples from each pot (I and II) are shown. (*B*) The remainder of the treated plants in each pot described in *A* were exposed to 330 ppb O_3 for 8 hr and photographed 24 hr after the O_3 exposure. A representative plant from each of the duplicate treatments is shown. This experiment was performed on three separate occasions, and in all experiments pretreatment of *soz1* with AsA or l-galactono-1,4-lactone caused increased O_3 resistance and elevation of *in planta* AsA to greater than untreated wild-type levels.

AsA is thought to play a variety of critical roles in plants and animals. As an abundant reducing agent it is able to directly detoxify active oxygen species and is thought to maintain the reduced state of the chloroplastic antioxidant α-tocopherol. It is also a substrate for AsA peroxidase, a component of the proposed superoxide and hydrogen peroxide detoxifying ascorbate–glutathione cycle. AsA in plants may be involved in the synthesis of zeaxanthin, which dissipates excess light energy in the thylakoid membranes, preventing oxidative damage. In addition, it acts as a reductant in a number of other enzymatic reactions. AsA is also responsible for keeping prosthetic metal ions in the reduced form, thereby maintaining the activity of various enzymes. Thus, AsA acts both as an abundant antioxidant free radical scavenger and a reductant in enzymatic reactions.

A small pool of AsA located outside the plant cell wall in the apoplastic space is thought to have a major role in the detoxification of ROS generated by O_3 and was found to become highly oxidized during exposure to O_3. This was in contrast to the redox state of the total AsA pool, which was not significantly altered (21). We have also found that the redox state of total AsA does not change significantly in either wild type or *soz1* upon O_3 exposure (data not shown). Cell fractionation experiments will be needed to reveal the subcellular location of the residual AsA and dehydroascorbate in *soz1*.

Analysis of AsA-deficient mutants such as *soz1* should provide needed information on AsA metabolism. It is plausible that this mutant is defective in AsA synthesis. However, the biosynthesis of this abundant antioxidant is not well understood in plants (22). In animals and several algae it is synthesized via a pathway in which the carbon skeleton of the precursor D-glucose is inverted. Although there is no evidence for the existence of the first three steps of this pathway (22),

flowering plants do contain an activity for the terminal step, conversion of L-galactono-1,4-lactone to AsA (23). Our feeding experiments suggest that *soz1* can make this conversion (Fig. 4*A*). Data from several plant species are consistent with the existence of a direct biosynthetic pathway in which the order of the carbons in D-glucose is preserved (22). In this proposed pathway, D-glucose is converted to AsA by means of the intermediates D-glucosone and L-sorbosone.

Why is *soz1* not completely deficient in AsA? One possible explanation would be that there are biochemically and/or genetically redundant AsA biosynthetic pathways in plants. For example, both the direct and inversion pathways may be active in Arabidopsis, with only one of these affected in *soz1*. Another plausible scenario is one in which there are multiple isoforms of an AsA biosynthetic enzyme (perhaps in distinct subcellular compartments), and only one is defective in the mutant. Alternatively, *soz1* may have enhanced catabolism of AsA to shorter chain compounds such as oxalate and tartrate (22). It is also plausible that a defect in the ability to enzymatically regenerate reduced AsA from its oxidized forms could lead to decreased total AsA, since both monodehydroascorbate and dehydroascorbate are unstable under physiological conditions (24). A variation on this theme is that *soz1* may have a defect in the active transport system proposed for shuttling oxidized AsA from the apoplast into the cell (25), thus preventing this oxidized AsA pool from being reduced within the cell. Future studies will clarify the nature of the *soz1* defect as well as provide insights into the origin and biological consequences of ROS in varied stress conditions including pathogen infection, photooxidative stress, drought, and chilling or in the aging process.

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