

# Effects of ozone on apoplast/cytoplasm partitioning of ascorbic acid in snap bean

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Apoplast/cytoplasm partitioning of ascorbic acid (AA) was examined in four genotypes of snap bean (*Phaseolus vulgaris* L.) known to differ in ozone sensitivity. Plants were grown in pots under field conditions using open-top chambers to establish charcoal-filtered (CF) air (36 nmol mol<sup>-1</sup> ozone) or elevated ozone (77 nmol mol<sup>-1</sup> ozone) treatments. AA in fully expanded leaves of 36-day-old plants was separated into apoplast and cytoplasm fractions by vacuum infiltration methods using glucose 6-phosphate as a marker for cytoplasm contamination. Apoplast ascorbate levels ranged from 30 to 150 nmol

g<sup>-1</sup> fresh weight. Ozone-sensitive genotypes partitioned 1–2% of total AA into the apoplast under CF conditions and up to 7% following a 7-day ozone exposure. In contrast, an ozone-tolerant genotype partitioned 3–4% of total leaf AA into the leaf apoplast in both CF and ozone-treated plants. The results suggest that genetic background and ozone stress are factors that affect AA levels in the extracellular space. For all genotypes, the fraction of AA in the oxidized form was higher in the apoplast compared to the cytoplasm, indicative of a more oxidizing environment within the cell wall.

## Introduction

Tropospheric ozone is a major air pollutant that has adverse effects on the growth of agricultural crops and natural vegetation (Heagle 1989, Runeckles and Chevone 1992, Sandermann 1996). Ozone enters the plant primarily through open stomata and then rapidly decomposes (Laisk et al. 1989) to form free radicals (Grimes et al. 1983, Mehlhorn et al. 1990) presumably through reactions with cell wall constituents. To limit plasma membrane damage and subsequent injury responses, ozone and the associated activated oxygen intermediates formed during ozone decomposition must be neutralized in the apoplast space. Ascorbic acid (AA) in the apoplast space could minimize ozone injury through two types of reactions. A direct ozonolysis reaction between AA and ozone is possible (Chameides 1989), although a recent report suggests that this reaction is not a major pathway for ozone detoxification in the apoplast (Jakob and Heber 1998). AA in the apoplast can also act as an antioxidant in enzymatic reactions that deactivate peroxides generated during ozone decomposition (Castillo and Greppin 1988). A number of studies have identified AA as a

constituent of apoplastic fluid (Castillo and Greppin 1988, Takahama and Oniki 1992, Luwe et al. 1993, Luwe and Heber 1995). In addition, exposure of plants to ozone has been shown to affect apoplast ascorbate content and redox status (Castillo and Greppin 1988, Luwe et al. 1993, Luwe and Heber 1995). The objective of the present study was to determine if genetic variation exists in apoplast AA content and the effect of such differences on the response of plants to ozone stress.

## Materials and methods

### Plant material and treatments

Based on preliminary assessment of ozone-induced visual leaf injury, two ozone-tolerant cultivars (Strike and Tenderette), one ozone-sensitive cultivar (Oregon 91) and one ozone-sensitive experimental line (S248) (R. Reinert, unpublished data) of snap bean (*Phaseolus vulgaris* L.) were selected for this study. Seeds were planted in cups of

*Abbreviations* – AA, ascorbic acid; CF, charcoal-filtered; DAP, days after planting; DHA, dehydroascorbic acid; G6P, glucose 6-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; IWF, intercellular wash fluid.

MetroMix-220 (Scotts-Sierra Horticultural Products, Marysville, OH, USA) in a charcoal-filtered (CF) greenhouse on June 2, 1997. Fourteen days after planting (DAP), uniform plants of each genotype were selected, transplanted into 15-l pots of MetroMix-220 and placed in open-top field chambers (Heagle et al. 1973) under CF conditions. Plants were watered daily, fertilized weekly with Peter's 20-10-20 (Scotts-Sierra Horticultural Products) water-soluble nutrient solution and fertilized once at 28 DAP with Peter's standard trace element mixture (Scotts-Sierra Horticultural Products). The third main stem trifoliate leaf was tagged at 26 DAP and expansion of the tagged leaf was completed by 32 DAP for all genotypes.

Beginning at 36 DAP, plants were either maintained under CF conditions ( $36 \pm 3$  nmol mol<sup>-1</sup> ozone) or exposed to elevated ozone by adding ozone to CF air for 12 h each day (08:00–20:00 h eastern standard time). An ozone generator (Griffin Technics Corporation, Lodi, NJ, USA) was used to produce ozone by electrostatic discharge in dry oxygen. Ozone was monitored using a UV photometric ozone analyzer (Thermo Environmental Instruments, Franklin, MA, USA). Ozone was dispensed in proportion to a daily profile that followed the average hourly concentration of ambient ozone measured at the experimental site from 1993 to 1996 during the months of June, July and August (F. Booker, unpublished data). The targeted 12-h mean was 80 nmol mol<sup>-1</sup> ozone and the actual exposure during the experimental period was  $77 \pm 1$  nmol mol<sup>-1</sup> ozone. The final experimental design consisted of three blocks, each containing one elevated ozone and one CF chamber.

Leaf tissue was analyzed between 12:00 and 17:00 h each day following 1, 3 and 7 days of ozone exposure. For each sample, two leaflets from the third main stem trifoliate were excised and transported to an adjacent laboratory, where processing of the tissue was begun within 5 min of excision. One leaflet was used to prepare intercellular wash fluid (IWF) and to determine leaf AA. The second leaflet was used for chlorophyll analysis.

### Isolation and assessment of IWF

The IWF isolation protocol was based on the vacuum infiltration procedure described by Luwe and Heber (1995). Each sample consisted of one leaflet from the third main stem trifoliate of a single plant. The mid-vein was removed and the initial fresh weight was determined. The leaf tissue was suspended in 50 ml of 100 mM KCl inside a 60-ml polypropylene syringe and the leaf air spaces were infiltrated with KCl solution by manipulation of the plunger to apply alternating vacuum and pressure. Leaves were then blotted and the infiltrated leaf was weighed. The infiltrated leaves were rolled and inserted into a 5-ml syringe barrel that was then placed in a centrifuge tube containing a small collection tube to receive the IWF. The collection tube contained 50 µl of cold 2% (w/v) *meta*-phosphoric acid and 2 mM EDTA to stabilize the AA in the IWF. The IWF (200–300 µl g<sup>-1</sup> fresh weight) was

recovered by centrifugation at 556 g for 5 min at room temperature and then stored on ice for AA analysis. In recovery experiments not shown,  $92 \pm 5\%$  (n = 5) of exogenous AA added to freshly prepared IWF was recovered after storage on ice for 5 h, with no significant effect on the AA redox status. Following centrifugation to recover the IWF, leaf tissue was re-weighed, frozen with liquid nitrogen and stored at  $-80^\circ\text{C}$  for later analysis of leaf AA content.

The IWF isolation conditions were established in preliminary experiments using leaves from snap bean plants grown in the greenhouse or open-top chambers. The presence of the metabolite glucose 6-phosphate (G6P) in the IWF was used as an indicator of cytosol contamination (H. Winter, personal communication). For these tests, IWF was prepared as above, without stabilizing solution, then quickly frozen with liquid nitrogen and stored at  $-80^\circ\text{C}$ . G6P was detected using commercial glucose 6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) from yeast. The reduction of NADP was measured at 334 nm ( $\epsilon_{334\text{ nm}} = 6.18$  mM<sup>-1</sup> cm<sup>-1</sup>) in a kinetic assay, where the IWF was the source of G6P substrate. The assay contained 50 mM HEPES-KOH, pH 6.9, 1.5 mM MgCl<sub>2</sub>, 0.2 mM NADP, with an aliquot of IWF added quickly after thawing. Monitoring of the absorbance at 334 nm was begun immediately to obtain a baseline. The reaction was initiated by the direct addition of 5 µl of G6PDH to the cuvette on a plastic stir rod used to rapidly mix the enzyme with the cuvette contents. G6PDH was dissolved in 50 mM HEPES-KOH, pH 6.9, 50% (w/v) glycerol. Sufficient G6PDH was used to observe the inflection point between stir rod removal from the cuvette and the initial increase in 334 nm absorbance from the enzyme reaction. The rise to a final maximum absorbance at 334 nm was complete within 2 min when G6P was present. Centrifugation speed was found to be the most critical parameter in the preliminary studies. No G6P was observed using 556 g to recover IWF, but G6P was occasionally detected in the IWF when infiltrated leaf tissue was subjected to a higher centrifugation setting of 844 g. As a control, IWF was isolated from each genotype after 6 days of ozone exposure and analyzed for G6P. No G6P was found (data not shown), indicating that the ozone treatment did not cause changes in leaf tissue that resulted in cytosol contamination during isolation of the IWF.

### AA extraction and assay

Frozen leaf tissue was quickly transferred to a cooled mortar containing cold extraction solution (2% [w/v] *meta*-phosphoric acid, 2 mM EDTA) and homogenized. The extraction solution was prepared fresh each day and used in a ratio of 5 ml g<sup>-1</sup> fresh weight. The homogenate was subjected to centrifugation at 10500 g for 10 min at 4°C.

TCA was added to the leaf extract supernatant or isolated IWF to a final concentration of 5% (w/v). After a 15-min incubation on ice, the TCA-precipitated proteins were removed by centrifugation in a micro-centrifuge. The TCA-treated extracts were assayed for total ascorbate

(AA + dehydroascorbic acid [DHA]) and reduced AA according to Okamura (1980).

### Chlorophyll extraction and assay

Samples consisting of four 2.5-cm<sup>2</sup> leaf disks were frozen in liquid nitrogen at harvest and stored at -80°C. Chlorophyll was extracted from leaf tissue in dimethylformamide in the dark for 48 h at 4°C. The extract was then assayed for chlorophyll using the spectrophotometric procedure of Moran (1982).

### Statistical analysis

The treatment design was a 2 × 4 factorial with two ozone levels (CF or elevated ozone) and four snap bean genotypes. The experimental design was a split plot, with open-top chambers serving as main plots and arranged in a randomized complete block design with three blocks. The four snap bean genotypes were the subplot treatments, with four plants per genotype randomly placed within each chamber. The magnitude of change in a variable caused by ozone exposure was examined by calculating the ozone/CF ratio for each genotype within each block. Data for each dependent variable were grouped by number of days of exposure and analyzed separately using general linear models. Because of heterogeneity of variances for the different exposure periods, the data were not combined. Residual plots for each variable were examined to identify variables that required transformation. Box-Cox tests (Rawlings 1988) were used to select the most appropriate transformation. For the 7-day exposure data, two variables (leaf AA and apoplast AA) were transformed using  $Y^{-1}$ . Orthogonal contrasts were used to compare genotypes and genotype × treatment interactions. Differences were considered to be significant at  $P \leq 0.05$ .

## Results

### Leaf injury

Ozone injury of leaf tissue was assessed by measuring the reduction in leaf chlorophyll during the 7-day ozone treatment. Because the four genotypes exhibited a range of leaf chlorophyll contents under CF conditions (1.3–1.8 mg g<sup>-1</sup> fresh weight), ozone/CF ratios were calculated so that a decrease in the ratio would be indicative of ozone-induced chlorophyll loss. A 40% loss of leaf chlorophyll was observed in S248 after the 7-day ozone treatment (Fig. 1). Chlorophyll losses in the sensitive genotype Oregon 91 and the two tolerant genotypes were not significantly different within the 7 days of ozone exposure. Based on these results, S248 was the most ozone sensitive. The inability to distinguish ozone sensitivity in the other genotypes suggested that leaf chlorophyll is not an effective indicator of injury in studies that utilize short-term exposures to moderate ozone concentrations.

### AA content and partitioning

Genotype differences in the leaf total ascorbate (AA + DHA) content were observed (Fig. 2). Total leaf ascorbate in Oregon 91 was significantly higher ( $P < 0.02$ ) than in S248 on all harvest dates. Intermediate levels of leaf ascorbate were found in Strike and Tenderette throughout the experiment. No significant differences were observed when genotype × ozone treatment interactions were analyzed. Ozone/CF ratios of leaf AA content were approximately one for each genotype and harvest date (data not shown).

Genotype and treatment were important factors in the ascorbate content (AA + DHA) of the apoplast compartment. Under CF conditions, Oregon 91 and S248 contained relatively low amounts of apoplast ascorbate (30–45 nmol g<sup>-1</sup> fresh weight) on day 1 (Fig. 3), representing approximately 1% of total leaf ascorbate (Fig. 4). Apoplast ascor-

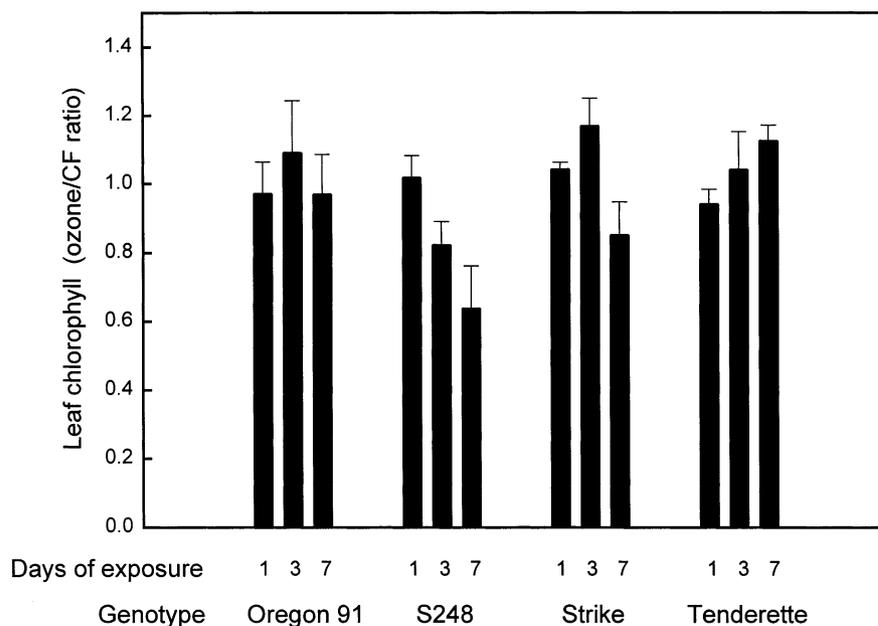
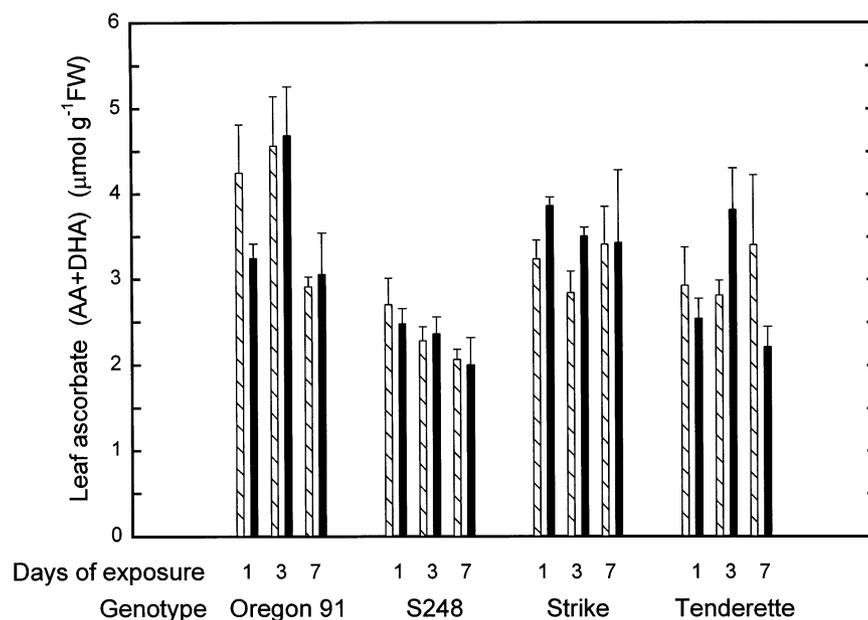


Fig. 1. Ozone effect on leaf chlorophyll content. Leaf chlorophyll was measured from one plant per chamber and ozone/CF ratios were calculated for each experimental block. Values represent the mean ± SE for three replicates on each harvest date.

Fig. 2. Total leaf ascorbate (AA + DHA) in control (hatch bars) and ozone-treated (solid bars) plants. Values represent the average  $\pm$  SE for three replicates on each harvest date.



bate increased to 2–3% of total leaf ascorbate in CF controls of Oregon 91 and S248 by the end of the experiment. Apoplast ascorbate levels were higher in Strike on day 1 (approximately 70 nmol g<sup>-1</sup> fresh weight) and remained constant during the experimental period (Fig. 3). Strike partitioned approximately 2% of total leaf ascorbate into the apoplast under CF conditions (Fig. 4). Tenderette contained the highest apoplast ascorbate levels (70–150 nmol g<sup>-1</sup> fresh weight) observed under CF conditions (Fig. 3).

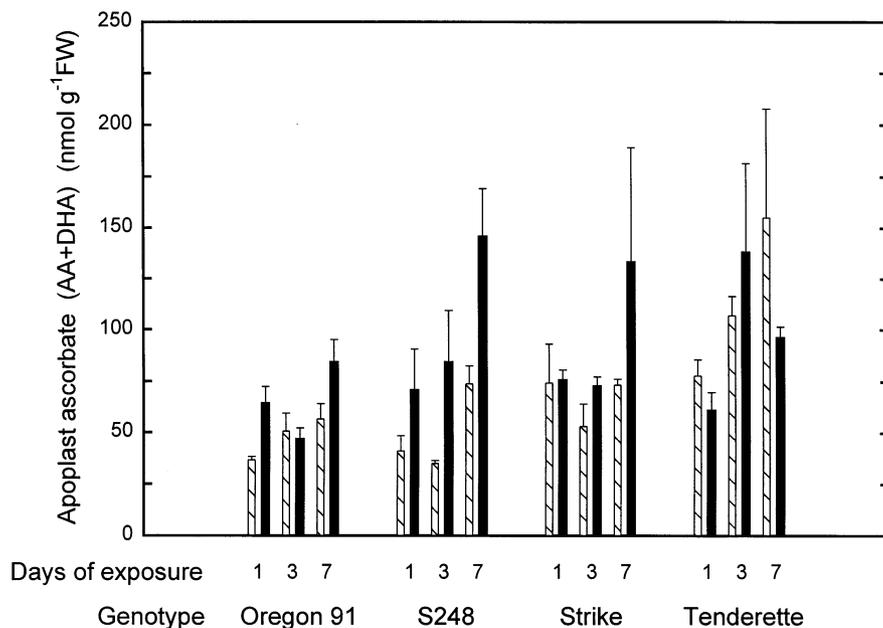
Ozone treatment caused apoplast ascorbate levels to increase in three of the genotypes (Fig. 3). The largest change occurred in S248, where apoplast ascorbate increased 2-fold in ozone-treated plants relative to CF controls, representing the partitioning of approximately 7% of total leaf ascorbate

into the apoplast by the end of the 7-day exposure (Fig. 4). A similar pattern was observed in Oregon 91 and Strike, but the magnitude of the response was smaller. A different pattern was observed in Tenderette, where similar or slightly lower levels of apoplast ascorbate were found in ozone-treated plants compared to CF controls (Fig. 3). Tenderette consistently partitioned 3–4% of total leaf ascorbate into the apoplast compartment in both ozone and CF treatments (Fig. 4).

#### Ascorbate redox status

AA extracted from leaf tissue was primarily in the reduced form. All genotypes exhibited leaf AA/(AA + DHA) ratios

Fig. 3. Total apoplast ascorbate (AA + DHA) in control (hatch bars) and ozone-treated (solid bars) plants. Measurements of leaf weight before and after infiltration with 100 mM KCl and following the IWF centrifugation step were used to calculate the recovery of the infiltrated solution (typically 70–80%). The recovery percentage for each sample was used in the calculation of apoplast ascorbate content so that the reported values are normalized to reflect 100% recovery. Values represent the average  $\pm$  SE for three replicates on each harvest date.



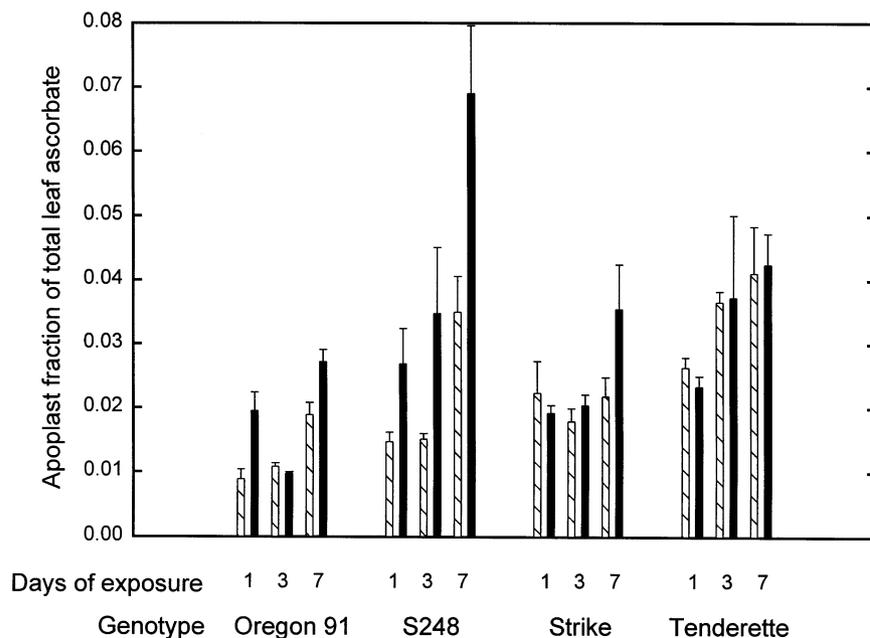


Fig. 4. Partitioning of ascorbate (AA + DHA) between the apoplast and cytoplasm in control (hatch bars) and ozone-treated (solid bars) plants. The apoplast fraction of total leaf ascorbate was calculated by dividing apoplast ascorbate content (Fig. 3) by leaf ascorbate content (Fig. 2). Values represent the average  $\pm$  SE for three replicates on each harvest date.

of 0.9 or greater in both CF and ozone-treated plants. No significant differences were observed when genotype  $\times$  ozone treatment interactions were analyzed.

AA/(AA + DHA) ratios were lower in the apoplast than for the corresponding leaf tissue. The apoplast AA/(AA + DHA) ratio was approximately 0.6 for Strike and Tenderette, with no significantly different ozone response observed for the two genotypes. The apoplast AA/(AA + DHA) ratio for Oregon 91 and S248 was 0.3–0.5, which was significantly lower than Strike and Tenderette. Only S248 exhibited a significant ozone response in apoplast AA/(AA + DHA) ratio. The apoplast AA/(AA + DHA) ratio increased in ozone-treated S248 plants to approximately 0.8 by the end of the 7-day ozone exposure.

## Discussion

AA (vitamin C) has the potential to react directly with ozone or serve as a substrate for enzymes (e.g., ascorbate peroxidase) that remove ozone breakdown products, such as hydrogen peroxide (Runeckles and Chevone 1992). This important antioxidant metabolite is frequently measured in studies of plant responses to ozone (Nouchi 1993, Bender et al. 1994) and in comparisons of plants with different ozone sensitivities (Lee et al. 1984, Guzy and Heath 1993). Conclusions are somewhat mixed regarding the response of total leaf AA to ozone stress, possibly because of differences in ozone exposure regimes (acute versus chronic). However, a study with an *Arabidopsis* AA-deficient mutant clearly showed that a minimum level of ascorbate is critical for ozone tolerance (Conklin et al. 1996). The mutant study suggested that a threshold ascorbate level in the range of 1–2  $\mu\text{mol g}^{-1}$  fresh weight is required to provide at least partial tolerance. In the present study, total leaf AA content

was in the range of 2–4  $\mu\text{mol g}^{-1}$  fresh weight, but no relationship was observed between leaf ascorbate content and ozone tolerance. Although the most ozone-sensitive genotype S248 had the lowest leaf ascorbate content, the highest leaf ascorbate level was observed in the other sensitive genotype Oregon 91 (Fig. 2). By comparison, the more tolerant genotypes Strike and Tenderette had intermediate leaf ascorbate levels. Additionally, total leaf ascorbate content was not affected by ozone treatment (Fig. 2). Perhaps total leaf ascorbate becomes a factor in ozone tolerance only when levels fall below a threshold that is not clearly defined at present.

Localization of ascorbate in the cell wall is considered to be an important factor in ozone detoxification (Moldau 1998). AA has been identified in the apoplast of several plant species (Castillo and Greppin 1988, Takahama and Oniki 1992, Luwe et al. 1993, Luwe and Heber 1995) and there is evidence that apoplast ascorbate increases when plants are exposed to ozone (Castillo and Greppin 1988, Luwe and Heber 1995). In this study, apoplast AA was measured in sensitive and tolerant snap beans exposed to moderate ozone levels under field conditions. A range of apoplast AA content was observed and genetic variation was found in the ozone response (Figs. 3 and 4). The most ozone-sensitive genotype S248 initially contained relatively small amounts of AA in the apoplast under CF conditions, but accumulated relatively high levels of apoplast ascorbate in response to ozone treatment. Oregon 91 and Strike also exhibited an ozone-induced increase in apoplast AA, but the effect was smaller than in S248. In contrast, no ozone-induced increase in apoplast AA was observed in Tenderette, an ozone-tolerant genotype. In general, apoplast AA increased following ozone treatment, but the magnitude of the response was proportional to the ozone sensitivity of the

plant. For the genotypes/ozone combinations chosen for this study, the ozone response ranged from a large increase in apoplast AA (e.g., sensitive S248) to no effect (e.g., tolerant Tenderette). Based on these observations, tolerant plants would require higher levels of ozone to affect the steady-state level of apoplast AA.

Ascorbate partitioning in ozone-tolerant Tenderette was distinct from the other genotypes. Tenderette partitioned higher levels of AA (3–4% of total leaf ascorbate) into the apoplast under CF conditions (Fig. 4). The additional apoplast AA was presumably available to react with the ozone entering the leaf. Although very speculative, the Tenderette results suggest that elevated apoplast AA is one factor in ozone tolerance. High constitutive levels of apoplast AA may be more effective for minimizing ozone injury than transporting AA into the apoplast after damage to the plasma membrane has already begun. Future studies are planned to compare Tenderette to other tolerant genotypes to test this hypothesis.

Whether elevated apoplast AA was the result of a constitutive (e.g., Tenderette) or an induced (e.g., S248) effect, this study showed that plants regulate the apoplast/cytoplasm partitioning of AA. The mechanism does not appear to be passive diffusion, because apoplast AA levels ranging from 1 to 7% of total leaf ascorbate (Fig. 4) were observed in leaves that contained similar amounts of total AA (Fig. 2). The biochemical basis for this mechanism may involve specific carriers in the plasma membrane. There is evidence for a carrier system that transports AA and DHA between the cytoplasm and apoplast (Rautenkranz et al. 1994, Horemans et al. 1996). The regulation of such a system by environmental signals (e.g., ozone stress) or genetic potential could explain the range in AA partitioning observed in this study. If partitioning of AA into the apoplast is a factor in ozone tolerance, then the ability to manipulate this carrier system may be important for the production of stress-tolerant plants.

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