RESEARCH PAPER

Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (Glycine max)

Kelly M. Gillespie¹, Alistair Rogers²,³ and Elizabeth A. Ainsworth¹,⁴,*

¹ Physiological and Molecular Plant Biology Program, University of Illinois, Urbana-Champaign, 1201 W. Gregory Drive, Urbana, IL 61801, USA
² Department of Environmental Sciences, Brookhaven National Laboratory, Upton, NY 11973-5000, USA
³ Department of Crop Sciences, University of Illinois, Urbana-Champaign, 1201 W. Gregory Drive, Urbana, IL 61801, USA
⁴ Global Change and Photosynthesis Research Unit, USDA/ARS, Urbana, IL 61801, USA

* To whom correspondence should be addressed. E-mail: lisa.ainsworth@ars.usda.gov

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Abstract

Soybeans (Glycine max Merr.) were grown at elevated carbon dioxide concentration ([CO₂]) or chronic elevated ozone concentration ([O₃]; 90 ppb), and then exposed to an acute O₃ stress (200 ppb for 4 h) in order to test the hypothesis that the atmospheric environment alters the total antioxidant capacity of plants, and their capacity to respond to an acute oxidative stress. Total antioxidant metabolism, antioxidant enzyme activity, and antioxidant transcript abundance were characterized before, immediately after, and during recovery from the acute O₃ treatment. Growth at chronic elevated [O₃] increased the total antioxidant capacity of plants, while growth at elevated [CO₂] decreased the total antioxidant capacity. Changes in total antioxidant capacity were matched by changes in ascorbate content, but not phenolic content. The growth environment significantly altered the pattern of antioxidant transcript and enzyme response to the acute O₃ stress. Following the acute oxidative stress, there was an immediate transcriptional reprogramming that allowed for maintained or increased antioxidant enzyme activities in plants grown at elevated [O₃]. Growth at elevated [CO₂] appeared to increase the response of antioxidant enzymes to acute oxidative stress, but dampened and delayed the transcriptional response. These results provide evidence that the growth environment alters the antioxidant system, the immediate response to an acute oxidative stress, and the timing over which plants return to initial antioxidant levels. The results also indicate that future elevated [CO₂] and [O₃] will differentially affect the antioxidant system.

Key words: Antioxidant metabolism, ascorbate, dehydroascorbate reductase, glutathione reductase, oxidative stress, ozone pollution.

Introduction

Two aspects of global climate change that directly impact plant productivity are a rising atmospheric carbon dioxide concentration ([CO₂]) and a rising tropospheric ozone concentration ([O₃]) (Ainsworth et al., 2008). Atmospheric [CO₂] is projected to continue rising to at least 550 ppb by 2050 (Solomon et al., 2007). The current annual average [O₃] ranges from 20 ppb to 45 ppb across the globe, which is roughly double the concentration that preceded the Industrial Revolution (Vingarzan, 2004). Background [O₃] is predicted to continue increasing by 0.5–2% per year over the next century, mainly due to increases in precursor emissions from anthropogenic sources (Solomon et al., 2007). While CO₂ is well mixed in the atmosphere, O₃ is a spatially and temporally heterogeneous pollutant and local concentrations depend heavily on upwind precursor emissions and local O₃-generating environmental conditions. Short periods of very high [O₃] can occur in rural areas and have the potential to cause marked damage to foliage (The Royal Society, 2008).
Ozone diffuses into the leaf apoplast via the stomata where it is rapidly converted into other reactive oxygen species (ROS) that signal a diverse metabolic response (Long and Naidu, 2002; Kangasjarvi et al., 2005). Ozone stress has been characterized as either acute or chronic, depending on the [O₃] and the exposure duration (Sandermann, 1996; Fiscus et al., 2005). While the actual concentration and duration threshold for O₃ damage varies from species to species and even among genotypes of the same species (Burkey et al., 2000), it is commonly accepted that acute damage results from a very high concentration of O₃ (>150 ppb) over a short period of time, and chronic O₃ damage results from a lower concentration of exposure over a longer period of time. In general, acute O₃ damage has been well characterized and mimics the biochemical defence response of plants to pathogen attack (Overmyer et al., 2003; Kangasjarvi et al., 2005). In contrast, the mechanisms leading to chronic O₃ damage are less well characterized, but physiological symptoms include: decreased photosynthetic productivity, decreased Rubisco activity and chlorophyll content, lower stomatal conductance, leaf chlorosis, accelerated senescence, and a general decrease in green leaf area and plant productivity (Morgan et al., 2003; Ashmore, 2005; Fuhrer, 2009). Although the molecular and biochemical basis for tolerance to chronic O₃ is unknown, it is thought that the endogenous antioxidative metabolism plays a key role in quenching ROS generated from chronic O₃ exposure (Burkey et al., 2003). In addition to abiotic sources, plants produce ROS as a by-product of primary metabolic processes such as chloroplastic and mitochondrial electron transport (Foyer and Noctor, 2003). In parallel, plants utilize an array of ROS detoxification processes, collectively called antioxidant metabolism, to maintain cellular redox balance (Foyer and Noctor, 2005). Changes in environmental factors can easily disturb the steady-state redox balance by causing a rapid increase in ROS generation. The cellular redox state has been proposed to be an environmental sensor and signal among various aspects of plant metabolism (Fedoroff, 2006; Noctor, 2006). When a plant senses small changes in redox balance, an acclimation response is induced, and irreversible damage is avoided. Recently, there has been evidence suggesting that plants have a physiological ‘memory’ of a stress event by existing in a primed metabolic state to activate cellular defences more efficiently during a future stress (Conrath et al., 2006). Most of this evidence comes from plant–pathogen interaction research, but plants also responded with a faster and increased calcium signal to osmotic stress when pre-treated with H₂O₂ (Knight et al., 1998). Furthermore, plantlets generated from O₃-treated calli displayed increased oxidative stress tolerance (Nagendra-Prasad et al., 2008), and H₂O₂ seed treatment led to increased salt tolerance in wheat seedlings (Wahid et al., 2007). Therefore, there is evidence that exposure to an abiotic stress can predispose plants to improved tolerance to a subsequent abiotic stress event.

The prevailing view is that elevated [O₃] causes an up-regulation of antioxidant metabolism in plants (Ranieri et al., 1996, 2000; Scebba et al., 2003; Puckette et al., 2007; Xu et al., 2008; Olbrich et al., 2009). However, the direct evidence for this up-regulation is variable, dependent on the duration and method of O₃ fumigation, and the species and components of antioxidant metabolism investigated (Burkey et al., 2000; Robinson and Britz, 2000; Iglesias et al., 2006). Likewise, reports investigating the effects of growth at elevated [CO₂] show contrasting responses of individual components of the antioxidant system (Rao et al., 1995; Polle et al., 1997; Pritchard et al., 2000; Di Toppi et al., 2002) and there is recent evidence of increased oxidative stress in plants grown at elevated [CO₂] (Qiu et al., 2008).

This study investigated how the growth environment alters antioxidant metabolism and response to an acute oxidative stress. Soybeans were grown at chronic elevated [CO₂], chronic elevated [O₃], or control [CO₂] and [O₃] to test the hypothesis that growth at elevated [CO₂] or elevated [O₃] alters the total antioxidant capacity of plants, and therefore alters the capacity to respond to an acute oxidative stress. It is also hypothesized that the growth environment will change the timing over which plants will return to steady-state antioxidant levels. With the aim of developing a more holistic understanding of the effects of growth environment on the antioxidant system, this study investigated antioxidant metabolism at the metabolite, enzyme, and transcript levels.

### Materials and methods

#### Leaf material and growth chamber conditions

Soybean (*Glycine max*, cv. Pioneer 93B15) seeds were planted, four to a pot, in soil-less planting mix (Sunshine Professional Peat-Lite Mix LC1, SunGro Horticulture, Canada). Plants were maintained at a photosynthetic photon flux density (PPFD) of 300 μmol m⁻² s⁻¹ in a 10 h light/14 h dark cycle, at 25 °C and 22 °C, respectively. Six growth chambers were used and one of three atmospheric treatments, control, elevated [CO₂], or elevated [O₃], was randomly assigned to two chambers. The control treatment was 400 ppm [CO₂] and 0 ppb [O₃], the elevated CO₂ treatment was 650 ppm [CO₂] and 0 ppb [O₃], and the chronic elevated O₃ treatment was 90 ppb [O₃] for 6 h daily and 400 ppm [CO₂]. Ozone was produced by a variable output UV-C light bulb ballast (HVC 560 ozone generator, Crystal Air, Langley, Canada), and a custom multiport sampling system was used to measure and control chamber CO₂ and O₃ concentrations. CO₂ was continuously monitored with a CO₂ gas analyser (SBA4, PP Systems, Amesbury, MA, USA) and O₃ was monitored with an O₃ analyser (Thermo Electron 49i, Thermo Scientific, Waltham, MA, USA). The CO₂ fumigation system averaged 409 ppm in the control and elevated [CO₂] treatments, control, elevated [CO₂], or elevated [O₃], was randomly assigned to two chambers. The control treatment was 400 ppm [CO₂] and 0 ppb [O₃], the elevated CO₂ treatment was 650 ppm [CO₂] and 0 ppb [O₃], and the chronic elevated O₃ treatment was 90 ppb [O₃] for 6 h daily and 400 ppm [CO₂]. Ozone was produced by a variable output UV-C light bulb ballast (HVC 560 ozone generator, Crystal Air, Langley, Canada), and a custom multiport sampling system was used to measure and control chamber CO₂ and O₃ concentrations. CO₂ was continuously monitored with a CO₂ gas analyser (SBA4, PP Systems, Amesbury, MA, USA) and O₃ was monitored with an O₃ analyser (Thermo Electron 49i, Thermo Scientific, Waltham, MA, USA). The CO₂ fumigation system averaged 409 ppm in the control and elevated [CO₂] treatment and 653 ppm in the elevated [O₃] treatment. The O₃ fumigation system averaged 93 ppm in the elevated [O₃] treatment and 3 ppm in the control and elevated [CO₂] treatment.

Fourteen days after planting (DAP), soybeans were thinned to two uniform plants per pot. Pots were well watered, with weekly additions of 5 mM potassium nitrate. At midday, 32 DAP, the second trifoliate leaf of three plants per chamber was sampled for initial measurements of antioxidant parameters. The measurements made in the subsequent days were also performed on the second trifoliate at midday to avoid any potential diurnal variation in measured parameters. At 33 DAP, all plants received an acute 200 ppb O₃ treatment for 4 h, ending at midday. Leaf tissue was sampled from three plants per chamber, in two duplicate chambers.
immediately following the acute O₃ treatment and again 24 h and 48 h post-treatment. At each time point and from each treatment, leaf tissue from three plants was excised for metabolite and enzyme analysis, and three whole leaflets, from different plants, were sampled and pooled for gene expression analysis. The leaf tissue was immediately plunged into liquid nitrogen and maintained at −80°C until analysis. After a plant was sampled, it was removed from the chamber.

**Antioxidant metabolite and enzyme assays**

Total antioxidant capacity expressed as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents was measured with an oxygen radical absorption capacity (ORAC) assay, which measures inhibition of peroxyl radical-induced oxidations and provides a general measure of antioxidant capacity (Gillespie et al., 2007). Levels of reduced (ASA) and oxidized ascorbic acid (DHA) were measured using a ω-ω-bipyridyl-based colorimetric assay (Gillespie and Ainsworth, 2007). Total phenolic content was measured by a Folin–Ciocalteu assay (Ainsworth and Gillespie, 2007).

The activities of six antioxidant enzymes, ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.8.1.7), dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and superoxide dismutase (SOD; EC 1.15.1.1), were measured in a common extract. Leaf discs (381 mm², ~20 mg fresh weight (FW)) were macerated in microcentrifuge tubes at −80°C until individual assays were initiated. All samples and controls were prepared using the boiled enzyme handling robot (Janus, Perkin Elmer, Waltham, MA, USA) to allow rapid, accurate pipetting and the ability to initiate and stop reactions simultaneously in all 96 wells of the assay plate. In order to express enzyme activity on a protein basis, the protein content of the extracts was determined using the Pierce Protein Determination Kit (Pierce, Rockford, IL, USA).

**Catalase**

An established assay that measures CAT activity by determining H₂O₂ consumption (Summermatter et al., 1995) was adapted for automation in 96-well format. In quadruplicate, 5–10 µl of enzyme extract was diluted with assay buffer (50 mM Na₃PO₄, pH 6.5) to a total volume of 50 µl and loaded on the 96-well assay plate with 50 µl of standards (0, 3.5, 7, and 35 mM H₂O₂). A 50 µl aliquot of assay buffer was added to standard wells and the reaction in the sample wells initiated by addition of 50 µl of 35 mM H₂O₂. At the moment of reaction initiation the concentration of the reagents in the wells was 16.67 mM Na₃PO₄, 11.67 mM H₂O₂. After a 1 min incubation at 25°C, reactions were stopped simultaneously in one half of the assay plate by addition of 50 µl of 15% (w/v) trichloroacetic acid (TCA). After an additional 2 min, TCA was added to the other half of the assay plate, stopping all remaining reactions. The amount of H₂O₂ remaining after incubation with CAT was determined by transferring the sample to a new 96-well determination plate and mixing it with 100 µl of determination mix (1 g l⁻¹ ABTS, 0.8 µM l⁻¹ peroxidase). The determination plate was incubated for 10 min at room temperature and absorbance at 410 nm measured using a 96-well plate spectrophotometer (SynergyHT, Biotek, Winooski, VT, USA). Comparison of the amount of H₂O₂ consumed between the two TCA additions allowed the calculation of CAT activity. The use of stopped assays, rather than direct monitoring of the decrease in H₂O₂ (Aebi, 1984), allows accurate definition of the incubation period. This approach has been described previously (Summermatter et al., 1995) and was used for previous soybean experiments (Pritchard et al., 2000).

**Glutathione reductase**

An existing assay for GR activity based on measuring the rate of reduction of oxidized glutathione by NADPH oxidation was adapted for high throughput and automation in a 96-well format (Pohle et al., 1990; Pritchard et al., 2000). The assay plate was prepared by loading 15–30 µl of enzyme extract, and blanks (extract buffer) in quadruplicate. Half of the samples received 98 µl of control buffer (50 mM tricine, pH 7.8, 0.5 mM EDTA) and the other half received 98 µl of assay buffer (50 mM tricine, pH 7.8, 0.5 mM EDTA, 0.25 mM oxidized glutathione, prepared immediately before use). At the moment of reaction initiation, the concentrations of the reagents in the wells were as follows: 49 mM tricine, 0.49 mM EDTA, 0.245 mM oxidized glutathione, and 0.15 mM NADPH. The assay plate was equilibrated to 25°C and the reaction started with the addition of 2 µl of 7.5 mM NADPH. The oxidation of NADPH was followed at 340 nm for 10 min. The rate of NADPH oxidation in the absence of oxidized glutathione was subtracted to account for non-enzymatic oxidation of NADPH by the extract. Both enzymatic and non-enzymatic rates were corrected by the rate of NADPH oxidation in the absence of leaf extract.

**Dehydroascorbate reductase**

An existing assay for DHAR activity based on determining the rate of reduction of DHA to ASA was adapted for high throughput and automation in a 96-well format (Asada, 1984; Pritchard et al., 2000). An 88 µl aliquot of ASA standards (0, 0.1, 0.5, and 1.0 mM in 50 mM KH₂PO₄, 50 mM K₂HPO₄, 0.5 mM EDTA, pH 7.8), 15 µl of blanks (extraction buffer), and 15 µl of enzyme extracts were loaded onto a 96-well assay plate capable of absorbance measurements at UV wavelengths. An 83 µl aliquot of assay buffer (50 mM KH₂PO₄, 50 mM K₂HPO₄, 0.5 mM EDTA, pH 7.8, 2 mM reduced glutathione) was added to the wells containing extracts and blanks. An aliquot of each extract was boiled at 95°C for 10 min to denature all enzymes and a second plate was prepared as described above using the boiled enzyme extracts. Assays were started by adding 2 µl of 7.5 mM DHA to sample and blank wells. At the moment of reaction initiation, the concentrations of the reagents in the wells were as follows: 49 mM KH₂PO₄, 49 mM K₂HPO₄, 0.44 mM EDTA, 1.74 mM oxidized glutathione, and 0.15 mM NADPH. The reduction of DHA to ASA was followed at 265 nm for 10 min. The rate and final absorbance were recorded. DHAR activity was determined by subtracting the rate of non-enzymatic reduction of DHA (boiled extract) from the rate of enzymatic reduction (unboiled extracts).

**Monodehydroascorbate reductase**

Existing methods for measuring MDHAR activity (Dalton et al., 1986) were adapted for high-throughput and automation in a 96-well format. MDHAR was determined by following the rate of reduction of monodehydroascorbate (MDHA) to ASA by NADH oxidation. MDHA was produced in vitro by the action of ascorbate oxidase on ASA in the presence of O₂. Previously MDHA reduction in the absence of extract, ASA, and ascorbate oxidase was found to be negligible (Pohle et al., 1990). This was confirmed, and a common blank (extract buffer) was used to increase throughput. Aliquots (10 µl) of the enzyme extracts were added to a 96-well plate that included two 10 µl blanks. An 88 µl aliquot of assay buffer (50 mM KH₂PO₄, 50 mM K₂HPO₄, pH 7.8, 0.5 mM EDTA, 1.74 mM oxidized glutathione, and 0.15 mM NADPH) was added to the wells containing enzyme extracts. An additional 2 min, TCA was added to the other half of the assay plate, stopping all remaining reactions. The amount of MDHA remaining after incubation with CAT was determined by transferring 3 µl from the assay plate to a new 96-well determination plate and mixing it with 100 µl of determination mix (1 g l⁻¹ ABTS, 0.8 µM l⁻¹ peroxidase). The determination plate was incubated for 10 min at room temperature and absorbance at 410 nm measured using a 96-well plate spectrophotometer (SynergyHT, Biotek, Winooski, VT, USA). Comparison of the amount of MDHA consumed between the two TCA additions allowed the calculation of MDHAR activity. The use of stopped assays, rather than direct monitoring of the decrease in MDHA (Aebi, 1984), allows accurate definition of the incubation period. This approach has been described previously (Summermatter et al., 1995) and was used for previous soybean experiments (Pritchard et al., 2000).
7.8, 0.25 mM NADH, 1.5 mM sodium ascorbate) was added to all wells except those containing the NADH standards (98 µl of 0.1, 0.25, and 0.5 mM NADH in 50 mM KH2PO4, 50 mM K2HPO4, pH 7.8, 1.5 mM sodium ascorbate). The assay plate was equilibrated to 25 °C and the reaction initiated by adding 2 µl of ascorbate oxidase (0.01 U µl⁻¹ in 50 mM KH2PO4, 50 mM K2HPO4) to each well. At the moment of reaction initiation, the concentrations of the reagents in the wells were as follows: 49 mM KH2PO4, 49 mM K2HPO4, 0.245 mM NADH, 1.47 mM sodium ascorbate, and 0.002 U µl⁻¹ of APX. Standards were 0, 0.098, 0.245, and 0.49 mM NADH. MDHAR activity was determined from the rate of NADH oxidation measured at 340 nm over 10 min.

**Superoxide dismutase**

SOD activity was measured using a previously optimized 96-well assay (Ewing and Janero, 1995). Briefly, SOD activity was measured as the percentage inhibition of total nitroblue tetrazolium (NBT) oxidation and compared with a standard curve of SOD purified from horseradish. A 25 µl aliquot of extract or SOD standard was loaded on a 96-well microplate followed by 173 µl of assay buffer 50 mM KH2PO4, 50 mM K2HPO4, pH 7.8, 0.4 mM EDTA, 0.2 mM NBT, 0.3 mM NADH). Immediately before beginning the kinetic assay, 2 µl of 0.33 mM phenazine methosulphate (PMS) was added to every well. At the moment of reaction initiation, the concentrations of the reagents in the wells were as follows: 43.25 mM KH2PO4, 43.25 mM K2HPO4, 0.35 mM EDTA, 0.17 mM NBT, 0.26 mM NADH, and 0.003 mM PMS. The oxidation of NBT was followed at 560 nm and the SOD activity was determined by comparing the inhibition of NBT oxidation in the plant extracts with that of the purified SOD.

A unit of SOD activity was defined as the amount of SOD required to inhibit cytochrome c reduction by 50% in a coupled system with xanthine oxidase at pH 7.8 at 25 °C.

**Ascorbate peroxidase**

Existing methods for measuring APX activity by determining the rate of ASA oxidation by H2O2 were adapted for 96-well plates (Asada, 1984; Jahnke et al., 1991). Duplicate 10 µl aliquots of extract and two blanks were loaded on a 96-well assay plate capable of absorbance measurements at UV wavelengths. This was followed by the addition of 80 µl of assay buffer [50 mM KH2PO4, 50 mM K2HPO4, pH 7.8, 0.5 mM ascorbate, 0.2 mM diethylene-triaminepentaacetic acid (DTPA)]. Duplicate 100 µl SOD standards were added to each plate (0, 0.1, 0.5, and 1.0 mM ascorbate in 50 mM KH2PO4, 50 mM K2HPO4, pH 7.8). The assay was initiated by adding 10 µl of 20 mM H2O2 to all wells except those containing standards. At the moment of reaction initiation, the concentration of the reagents in the wells was as follows: 45 mM KH2PO4, 45 mM K2HPO4, 0.45 mM ascorbate, 0.18 mM DTPA, and 2 mM H2O2. The rate of ASA oxidation was determined by subtracting oxidation rates in the absence of extract, and quantified with a standard curve. APX is typically assayed in extracts containing ascorbate (Jahnke et al., 1991). However, including ASA in the extraction buffer would interfere with the other enzyme assays. Therefore, the effect of ASA in the extraction buffer on recoverable APX activity was tested. Values for APX determined with and without ASA in the extract buffer were indistinguishable from each other (data not shown).

**Gene expression analysis**

Total RNA was extracted following the methods of Bilgin et al. (2009). The quantity and quality of RNA samples was determined with a spectrophotometer (Nanodrop 1000, Thermo Fischer Scientific) and a microfluidic visualization tool (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). Contaminating DNA was removed with a DNA-free DNase treatment (Applied Biosystems/Ambion, Austin, TX, USA) following the manufacturer’s instructions. cDNA was prepared from 3 µg of RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT) primers according to the manufacturer’s instructions. qRT-PCR was performed using a quantitative real-time PCR system (7900 HT, Applied Biosystems) using SYBR Green JumpStart Taq ReadyMix (Sigma) and 100 nM of each gene-specific primer in a 384-well plate. An initial denaturing step at 95 °C for 10 min was followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers for genes annotated with antioxidant function and subcellular localization are listed in Table 1. Soybean is a paleopolyploid that has experienced two genome duplication events in its evolutionary history resulting in nearly 75% of the genes present in multiple highly homologous copies (Schmutz et al., 2010). As such, single gene primer specificity was of high importance and any homologous genes that could not be resolved were left out of this analysis. F-box protein, NCBI CD397253, was used as the control gene in these experiments (Libault et al., 2008). After completion of PCR amplification, a dissociation curve was run to check for DNA contamination and to ensure that a single gene product was amplified. The baseline-corrected delta-Rn values were used to calculate the PCR efficiency (E) and the cut-off value (Ct) in the log-linear phase of the PCR using LinReg PCR software (Ruijter et al., 2009). The normalized expression level for each gene (G) was determined using the following equation: G = (Econtrol - Ci-control)/(Egene - Ci-gene). Two replicate reactions were run for each sample, and their E values were averaged. Data are expressed relative to control grown plants at time 0 h.

**Statistical analysis**

For all parameters, a completely randomized, repeated measures, mixed model analysis of variance (PROC MIXED, SAS v9.2, SAS Institutes) with the Satterthwaite option was used with atmosphere mixed model analysis of variance (PROC MIXED, SAS v9.2, SAS Institutes) with the Satterthwaite option was used with atmosphere.
Increase over pre-spike levels by 72 h (P < 0.0001; Fig. 1A) 4 h after the O3 spike, although this change was not reflected in ASA pool size or phenolic content (Fig. 1B, C). After 48 h, ambient-grown plants increased total antioxidant capacity by 88% compared with pre-spikes levels (P=0.0003), but returned to pre-spikes levels by 72 h (Fig. 1A). The increase in total antioxidant capacity at 48 h was mirrored by a 78% increase in ASA at 48 h over the pre-spikes level (P < 0.0001), which lessened to a 27% increase over pre-spikes levels by 72 h (P=0.0298; Fig. 1B). Plants grown at elevated [O3] sustained high total antioxidant capacity for 48 h following the acute O3 spike, but levels dropped by 46% by 72 h (Fig. 1A). The increase in total antioxidant capacity immediately following the acute O3 spike that was measured in the elevated [CO2]-grown plants subsided so that by 72 h it was not different from pre-spike levels (Fig. 1A). The stimulation of total antioxidant capacity in the elevated [CO2]-grown plants was not reflected in changes in ASA pool size or phenolic content throughout the entire experimental time frame (Fig. 1B, C).

### Antioxidant recycling enzymes

Consistent with the observation of a greater ASA pool before the acute stress in elevated [O3]-grown plants,
DHAR, an enzyme responsible for recycling DHA to ASA, showed 132% higher activity in the elevated [O$_3$] plants ($P = 0.0090$; Table 2). Conversely, GR activity in elevated [O$_3$]-grown plants was 43% lower than GR activity in ambient-grown plants ($P = 0.0089$; Table 2). GR was the only enzyme to exhibit a response to growth at elevated [CO$_2$], with a 29% decrease in rate compared with ambient-grown plants ($P = 0.0454$; Table 2).

Following the acute O$_3$ spike, the activity of enzymes that recycle ASA was increased in plants grown under ambient conditions. DHAR activity increased by 250% ($P = 0.0003$; Fig. 2A, Time 24 h) and MDHAR activity increased by 162% ($P < 0.0001$; Fig. 2B, Time 24 h). Direct ROS-scavenging enzymes, APX, CAT, and SOD, responded differently. APX activity decreased by 20% ($P = 0.0086$; Fig. 2D), CAT activity increased by 90% ($P = 0.0280$; Fig. 2E), and SOD activity increased by 40% ($P = 0.0003$; Fig. 2F).

The stimulation in CAT activity dropped back to pre-spike levels by 72 h (Fig. 2E). DHAR and MDHAR activity dropped after 48 h, but a secondary stimulation was recorded at 72 h (Fig. 2A, B). While GR, SOD, and APX activity all decreased immediately following the O$_3$ spike, the post-spike recovery of these activity levels varied. GR activity increased at 24 h back to pre-spike levels, but a second drop in activity was recorded at 72 h (Fig. 2C). Decreased SOD activity was sustained throughout the remainder of the experiment and APX activity decreased even further at 48 h and remained low up to 72 h (Fig. 2F).

Plants grown under chronic elevated [O$_3$] displayed an immediate response to the acute O$_3$ spike similar to the control plants in all enzymes except GR. A 200% increase in MDHAR activity ($P > 0.0001$; Fig. 2B) along with a 30% decrease in APX activity ($P = 0.0002$; Fig. 2D) and a 38% decrease in SOD activity ($P = 0.0008$; Fig. 2F) mirrored the response of the control-grown plants. However, DHAR...
activity only increased by 60% \( (P=0.0454; \text{ Fig. 2A}) \) compared with the 250% increase in control plants, and GR activity, which did not change in the control plants, increased by 84% \( (P=0.0061; \text{ Fig. 2C}) \). Also, the significant increase in CAT activity measured in the control plants did not occur in the O₃-grown plants. In fact, CAT activity was significantly lower 48 h following the acute spike and remained low up to 72 h (Fig. 2E). Elevated [O₃] plants were the only group to display an increase in GR activity immediately following the acute O₃ spike, and that activity slowly returned to pre-spike levels by 72 h (Fig. 2C).

Plants grown with elevated [CO₂] responded with the largest changes in antioxidant enzyme activity immediately following the acute O₃ spike. DHAR activity increased by 700% \( (P>0.0001; \text{Fig. 2A}) \), MDHAR activity increased by 200% \( (P>0.0001; \text{Fig. 2B}) \) and CAT activity increased by 450% \( (P>0.0001; \text{Fig. 2E}) \), compared with pre-spike activity levels. Conversely, SOD activity decreased by 41% \( (P=0.0141; \text{Fig. 2F}) \). In the recovery period following the acute O₃ spike, CAT activity returned to pre-spike levels by 48 h and remained low up to 72 h (Fig. 2E). DHAR activity began to decrease at 48 h, but remained higher than pre-spike levels up to 72 h (Fig. 2A). Plants grown at elevated [CO₂] did not display the same immediate drop and sustained low APX activity observed in the control and [O₃]-grown plants. Instead, APX activity in elevated [CO₂]-grown plants decreased at 48 h but returned to pre-spike activity levels by 72 h (Fig. 2D).

### Antioxidant transcript response

This experiment measured total enzyme activity, so the contributions of different isoforms to the total activity level were not distinguishable. In order to distinguish the contribution of various isoforms localized in different cellular components, transcript levels for 24 genes annotated with antioxidant function and cellular localization were quantified (Table 1). Sixteen genes showed a significant growth environment (atmosphere), time, or interaction effect (Table 3), and are shown in Fig. 3. Before the acute O₃ treatment (Time 0 in Fig. 3), elevated [CO₂] decreased the abundance of transcripts encoding chloroplastic Cu-Zn SOD (Fig. 3D), SOD copper chaperone (Fig. 3E), GPX7 (Fig. 3F), and cytosolic GR1 (Fig. 3J). Elevated [O₃] decreased the transcript abundance of two chloroplastic Cu-Zn SODs (Fig. 3B, D) and GPX7 (Fig. 3F). However, the transcript abundance for ascorbate oxidase (AOX; Fig. 3L) and MDHAR (Fig. 3P) was higher in plants grown at elevated [O₃].

Immediately following the acute O₃ spike, the transcript abundance of three chloroplastic Cu-Zn SOD enzymes decreased by 73, 69, and 57% (Fig. 3B–D) in plants grown under control conditions. Transcript abundance for two of these genes remained low up to 72 h, but the other chloroplast Cu-Zn SOD increased 360% at 72 h (Fig. 3B). APX4 (Fig. 3H) increased by 77% over the pre-spike level by 72 h. Conversely, transcript abundance for two antioxidant enzymes targeted to the cytosol increased immediately following the acute O₃ spike: GR1 (Fig. 3J) and MDHAR2 (Fig. 3N). Transcript abundance for these genes returned to pre-spike levels by 48 h (Fig. 3I, N).

The immediate decrease in transcript abundance for chloroplastic antioxidant enzymes following the acute O₃ spike was not present in the elevated [O₃]-grown plants; however, there was an increase in transcripts coding for cytosolic antioxidant enzymes (Fig. 3A, J, N). Similarly, there was an increase in MDHAR1 (Fig. 3M), which is putatively targeted to the apoplast, as well as the peroxisome and chloroplast. Transcript abundance for these genes returned to pre-spike levels by 48 h. In addition, the transcript level of cell wall-located AOX increased by 50% immediately after the acute O₃ spike in elevated [O₃]-grown plants; the increase in CAT activity measured in the control plants did not occur in the O₃-grown plants. Instead, APX activity in elevated [O₃]-grown plants decreased at 48 h but returned to pre-spike levels by 72 h (Fig. 2C). GHG (AJ272035) was higher in plants grown under control conditions. Transcript abundance for two of these genes returned to pre-spike levels by 48 h. In addition, the transcript level of cell wall-located AOX increased by 50% immediately after the acute O₃ spike in elevated [O₃]-grown plants; the increase in CAT activity measured in the control plants did not occur in the O₃-grown plants. Instead, APX activity in elevated [O₃]-grown plants decreased at 48 h but returned to pre-spike levels by 72 h (Fig. 2C).

### Table 3. Analysis of variance of antioxidant pools, enzymes, and GenBank IDs of transcripts measured for soybeans grown under control conditions (400 ppm [CO₂], 0 ppb [O₃]), elevated [CO₂] (650 ppm [CO₂], 0 ppb [O₃]), and elevated [O₃] (400 ppm [CO₂], 90 ppb [O₃]), before and after an acute (200 ppb) O₃ spike

<table>
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\( P \)-values in italics are marginally significant. See Table 1 for descriptions of GenBank IDs.
A large increase in the transcript abundance for the Cu-Zn SOD copper chaperone was also observed in elevated \([\text{O}_3]\)-grown plants immediately following the acute \(\text{O}_3\) spike (Fig. 3E). AOX and Cu-Zn SOD copper chaperone transcript levels dropped by 48 h, so that, by 72 h, levels were no longer different among \([\text{CO}_2]\), \([\text{O}_3]\), and control-grown plants (Fig. 3E, L).

Plants grown at elevated \([\text{CO}_2]\) did not display many of the changes in transcript abundance for cytosolic and chloroplastic antioxidant enzymes following the acute \(\text{O}_3\) spike that were observed in the elevated \([\text{O}_3]\)- and control-grown plants. Levels of abundance of transcripts encoding cytosolic enzymes did not increase as much or at the same time. For example, transcript abundance of GR1 increased slightly, immediately following the acute \(\text{O}_3\) spike, and continued to increase for 48 h following the spike (Fig. 3J). Furthermore, transcript abundance of cytosolic MDHAR did not increase significantly following the \(\text{O}_3\) spike in soybean grown at elevated \([\text{CO}_2]\) (Fig. 3N). Other differences in transcript abundance in plants grown at elevated \([\text{CO}_2]\) include increased peroxisomal MDHAR4 (Fig. 3O) and MDHAR (Fig. 3P) 48–72 h following the \(\text{O}_3\) spike.

**Discussion**

**Chronic growth environment alters antioxidant metabolism**

Growth at elevated \([\text{CO}_2]\) or elevated \([\text{O}_3]\) significantly altered the total antioxidant capacity, measured as the inhibition of peroxyl radical-induced oxidations, of soybean leaves. Growth at elevated \([\text{CO}_2]\) decreased total antioxidant capacity, while elevated \([\text{O}_3]\) increased total antioxidant capacity. These changes are consistent with the expectations from previous studies, which have suggested increased oxidative stress in elevated \([\text{O}_3]\) (Kangasjarvi et al., 2005), and decreased perceived oxidative stress at elevated \([\text{CO}_2]\), perhaps due to lower rates of ROS production from increased carboxylation rates and decreased oxygenation and subsequent photorespiration (Bowes, 1991;
Pritchard et al., 2000; Long et al., 2004). The changes in total antioxidant capacity were consistent with changes in ASA content, but phenolic content was unaffected by elevated [CO\textsubscript{2}] or elevated [O\textsubscript{3}]. ASA is one of the most abundant antioxidants in plant tissue (Dalton et al., 1986; Pritchard et al., 2000), and high levels of ASA are essential to oxidative stress tolerance (Robinson and Britz, 2000; Smirnoff, 2000; Conklin and Barth, 2004; Chen and Gallie, 2005). Therefore, it is not surprising that ASA levels were significantly higher in plants grown in elevated [O\textsubscript{3}] On the other hand, there was no significant change in total phenolic content of soybean exposed to elevated [CO\textsubscript{2}] or elevated [O\textsubscript{2}]. Previous studies have found increased phenolic content in plants exposed to [O\textsubscript{3}] (Kangasjarvi et al., 1994; Saviranta et al., 2010); however, there is also evidence that individual phenolic compounds can respond differently to [O\textsubscript{3}] (Saviranta et al., 2010), which the total phenolic content assay used in this study would not detect.

The activities of most of the antioxidant enzymes investigated in this study were not affected by growth at elevated [CO\textsubscript{2}] or elevated [O\textsubscript{3}] (Table 2). However, DHAR activity was >2-fold greater in plants grown at elevated [O\textsubscript{2}] compared with controls (Table 2). DHAR recycles DHA to ASA and regulates redox balance in cells (Chen et al., 2003), and the increased activity is consistent with the observation of a greater ASA pool in elevated [O\textsubscript{3}]-grown plants. On the other hand, GR activity was significantly lower in plants grown at elevated [O\textsubscript{3}] compared with controls (Table 2). GR is responsible for reducing glutathione (GSH), which provides reducing power to DHAR. In pea, GR is localized in the cytosol, chloroplast, and mitochondria (Edwards et al., 1990), with the chloroplastic GR contributing ~80% to the total cellular GR activity. Therefore, a reduction in total GR activity does not directly indicate a reduction in activity of all isoforms or the availability of sufficient GSH to power DHAR activity. Additionally, increased DHAR activity may not require a matching increase in GR activity since GR activity is greater than DHAR activity (Table 2). The \( k_m \) of GR for GSSG is approximately two orders of magnitude smaller than the \( k_m \) of DHAR for GSH (Chang et al., 2009), and the \( k_m \) of GR was significantly lower in peas grown at elevated [O\textsubscript{3}] (Edwards et al., 1994). Therefore, ASA formation by DHAR is unlikely to be limited by the supply of reduced glutathione.

Decreased total antioxidant capacity and ASA pool size in plants grown at elevated [CO\textsubscript{2}] was accompanied by decreased GR activity (Fig. 2C). Consistent with lower total antioxidant capacity, transcript abundance for GR genes (L11632, AW459932, and CF807683) was lower in elevated [CO\textsubscript{2}]-grown plants (Time 0, Fig. 3I, J, K). Other antioxidant enzymes and transcripts coding for various isoforms of each enzyme were not significantly changed by growth at either elevated [CO\textsubscript{2}] or elevated [O\textsubscript{3}] compared with control conditions. For CAT and SOD, this may reflect the very high capacities of these enzymes, which catalyse initial steps in ROS scavenging. Having an excess capacity for O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} scavenging would be highly desirable since the consequences of a slow response to superoxides are severe (Foyer et al., 1994).

Response to and recovery from an acute oxidative stress

Immediately following a 200 ppb O\textsubscript{3} spike for 4 h, plants grown under all environmental conditions shifted their antioxidant systems to accommodate the increase in ROS. The activity of DHAR and the transcript level and activity of MDHAR, two ASA recycling enzymes, was increased immediately following the O\textsubscript{3} spike in all environments (Fig. 2). Relative increases in DHAR and MDHAR enzyme activity were greatest in plants exposed to elevated [CO\textsubscript{2}], which also showed increased total antioxidant capacity, measured by the ORAC assay (Fig. 1). Plants grown at elevated [O\textsubscript{3}] increased GR activity immediately following the O\textsubscript{3} spike, with transcript levels of both chloroplastic/mitochondrial and cytosolic GR up-regulated. Although protein content was not investigated in this study, these data support the viewpoint that the increase in GR activity in plants grown at elevated [O\textsubscript{3}] and exposed to the O\textsubscript{3} spike was due to the synthesis of new isoforms, as previously suggested (Edwards et al., 1994; Rao et al., 1995). Direct ROS-scavenging enzymes responded to the O\textsubscript{3} spike with shifts in activity toward the recycling and preservation of the ASA pool with increased scavenging by CAT, especially in elevated [CO\textsubscript{2}], and decreased production of H\textsubscript{2}O\textsubscript{2} by SOD (Fig. 2). APX, which is regulated by its substrate, H\textsubscript{2}O\textsubscript{2} (Lu et al., 2005), also showed lower activity following the O\textsubscript{3} spike.

The apoplast is often considered to be the first line of defence against O\textsubscript{3} (Ranieri et al., 1996; Burkey et al., 2003) and, in this study, the transcript abundance of the cell wall-localized AOX was significantly higher in plants grown at elevated [O\textsubscript{3}] and increased in all plants following the acute [O\textsubscript{3}] spike (Fig. 3L). However, reducing equivalents are absent from the apoplast (Pignocchi and Foyer, 2003), suggesting that any reduced ASA required in the apoplast must be generated and reduced in the cytosol and then transported across the cell membrane. Thus, the cytosolic antioxidant system is integral to the ability of the apoplast to quench ROS (Pignocchi and Foyer, 2003; Chen and Gallie, 2005, 2006, 2008; Eltayeb et al., 2006). The transcript data from this experiment support this general finding, in that the abundance of cytosol-targeted enzymes was up-regulated following the spike (Fig. 3A, J, N). On the other hand, transcript abundance for three chloroplastic Cu-Zn SOD enzymes decreased in plants grown at control and elevated [O\textsubscript{3}] following an acute O\textsubscript{3} spike, which is consistent with previous observations (Mahalingam et al., 2005). The dampened transcriptional response of plants grown at elevated [CO\textsubscript{2}] (Fig. 3) suggests that either less oxidative stress was perceived or plants grown under elevated [O\textsubscript{3}] or control conditions were primed to respond more quickly to the oxidative stress.

It was anticipated that the acute O\textsubscript{3} stress would cause an abrupt change in the antioxidant system, which would then
return to its original state by the end of the experiment (48 h after the acute stress). This general pattern was apparent in some of the transcript data (Fig. 3A, E, G, I, J, K, L, M, N) and for some of the enzymes (CAT, APX, and SOD), but it was not seen in other antioxidant enzymes (DHAR, MDHAR, and GR), total antioxidant capacity, or ASA or phenolic content. Previous research has shown that members of antioxidant gene families are differentially regulated in response to stress (Milla et al., 2003; Secenji et al., 2010), and the present results support these findings. However, agreement between changes in antioxidant enzyme activity and transcript abundance was not always apparent, and more research with specific knock-out mutants would be needed to determine where changes in enzyme activity were directly related to transcript abundance. It is worth noting that for many enzymes, changes in transcript abundance and enzyme activity are not synchronized. Observing such correlations is largely dependent upon the pool size and turnover time of both the enzyme and the transcript (Gibon et al., 2004, 2006). Phenolics play a key role in antioxidant defence, but are costly to manufacture and may not be synthesized as quickly as small antioxidant molecules (Grace, 2006), which could explain the relatively stable content measured in this experiment. Transcript levels of GR and MDHAR localized to the cytosol did not increase as much or as rapidly in plants grown at elevated [CO2] and exposed to acute O3 stress, which different components of the plant antioxidant system recover from an acute O3 spike also differed considerably, suggesting distinct mechanisms of control.

Concluding remarks

Previous research demonstrated that growth at elevated [O3] caused a general up-regulation of antioxidant metabolism in plants (Ranieri et al., 1996, 2000; Scebbia et al., 2003; Puckette et al., 2007; Xu et al., 2008; Olbrich et al., 2009). However, direct evidence for this up-regulation has been variable and dependent on the duration and method of O3 fumigation, and the components of antioxidant metabolism investigated (Burkey et al., 2000; Robinson and Britz, 2000; Iglesias et al., 2006). Likewise, reports investigating the effects of growth at elevated [CO2] showed contrasting responses of individual components of the antioxidant system (Rao et al., 1995; Polle et al., 1997; Pritchard et al., 2000; Di Toppi et al., 2002). In this study, it was demonstrated that growth in a chronic elevated [O3] environment increased total antioxidant capacity, while chronic elevated [CO2] decreased total antioxidant capacity. The growth environment also significantly altered the pattern of antioxidant transcript and enzyme response to an acute oxidative stress. Growth at chronic elevated [O3] increased the basal levels of components of the antioxidant system, which were then unchanged or only slightly increased following an acute oxidative stress, suggesting that growth at chronic elevated [O3] allowed for a primed antioxidant system. Growth at elevated [CO2] decreased the basal level of antioxidant capacity, increased the response of the existing antioxidant enzymes, but dampened and delayed the transcriptional response, suggesting a different regulation of the antioxidant system. The time frame across which different components of the plant antioxidant system recover from an acute O3 spike also differed considerably, suggesting distinct mechanisms of control.

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