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RESEARCH PAPER

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

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Abstract

Soybeans (*Glycine max* Merr.) were grown at elevated carbon dioxide concentration ([CO_2]) or chronic elevated ozone concentration ([O_3]; 90 ppb), and then exposed to an acute O_3 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 O_3 treatment. Growth at chronic elevated [O_3] increased the total antioxidant capacity of plants, while growth at elevated [CO_2] 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 O_3 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 [O_3]. Growth at elevated [CO_2] 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 [CO_2] and [O_3] 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 ([CO_2]) and a rising tropospheric ozone concentration ([O_3]) (Ainsworth *et al.*, 2008). Atmospheric [CO_2] is projected to continue rising to at least 550 ppb by 2050 (Solomon *et al.*, 2007). The current annual average [O_3] ranges from 20 ppb to 45 ppb across the globe, which is roughly double the concentration that preceded the Industrial Revolution (Vingarzan, 2004). Background [O_3] 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 CO_2 is well mixed in the atmosphere, O_3 is a spatially and temporally heterogeneous pollutant and local concentrations depend heavily on upwind precursor emissions and local O_3-generating environmental conditions. Short periods of very high [O_3] 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 [O_3] and the exposure duration (Sandermann, 1996; Fiscus *et al.*, 2005). While the actual concentration and duration threshold for O_3 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 O_3 (>150 ppb) over a short period of time, and chronic O_3 damage results from a lower concentration of exposure over a longer period of time. In general, acute O_3 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 O_3 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 O_3 is unknown, it is thought that the endogenous antioxidative metabolism plays a key role in quenching ROS generated from chronic O_3 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 H_2O_2 (Knight *et al.*, 1998). Furthermore, plantlets generated from O_3-treated calli displayed increased oxidative stress tolerance (Nagendra-Prasad *et al.*, 2008), and H_2O_2 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 [O_3] 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 O_3 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 [CO_2] 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 [CO_2] (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 [CO_2], chronic elevated [O_3], or control [CO_2] and [O_3] to test the hypothesis that growth at elevated [CO_2] or elevated [O_3] 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 [CO_2], or elevated [O_3], was randomly assigned to two chambers. The control treatment was 400 ppm [CO_2] and 0 ppb [O_3], the elevated CO_2 treatment was 650 ppm [CO_2] and 0 ppb [O_3], and the chronic elevated O_3 treatment was 90 ppb [O_3] for 6 h daily and 400 ppm [CO_2]. Ozone was produced by a variable output UV-C light bulb ballast (HVAC 560 ozone generator, Crystal Air, Langley, Canada), and a custom multiport sampling system was used to measure and control chamber CO_2 and O_3 concentrations. CO_2 was continuously monitored with a CO_2 gas analyser (SBA4, PP Systems, Amesbury, MA, USA) and O_3 was monitored with an O_3 analyser (Thermo Electron 49i, Thermo Scientific, Waltham, MA, USA). The CO_2 fumigation system averaged 409 ppm in the control and elevated [O_3] treatment and 653 ppm in the elevated [CO_2] treatment. The O_3 fumigation system averaged 93 ppb in the elevated [O_3] treatment and 3 ppb in the control and elevated [CO_2] 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 O_3 treatment for 4 h, ending at midday. Leaf tissue was sampled from three plants per chamber, in two duplicate chambers

immediately following the acute O_3 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-carboxcylic 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 an α-α'-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 mm^2, ~20 mg fresh weight (FW)] were macerated in microcentrifuge tubes using tungsten carbide beads in 50 mM KH_2PO_4, 50 mM K_2HPO_4, pH 7.8. Samples were centrifuged at 14 000 *g* for 5 min at 4 °C. An aliquot of the supernatant was removed for the assessment of SOD, APX, and MDHAR. Approximately 5 mg of poly(vinylpolypyrrolidone) (PVPP) was added to the remaining supernatant and pellet; the mixture was shaken and re-centrifuged. An aliquot of this second supernatant was used for the determination of CAT, DHAR, and GR activity. The omission of PVPP from the aliquot used to assay SOD, APX, and MDHAR was based on the original protocols that were adapted for these assays (Ewing and Janero, 1995; Pritchard *et al.*, 2000). The extracts and temperature-sensitive reagents were maintained at 4 °C until individual assays were initiated. All assays were completed within 2 h of extraction using a liquid 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 plates. 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 H_2O_2 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 Na_3PO_4, 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 H_2O_2). 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 H_2O_2. At the moment of reaction initiation the concentration of the reagents in the wells was 16.67 mM Na_3PO_4, 11.67 mM H_2O_2. 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) tricholoroacetic 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 H_2O_2 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 U ml^{-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 H_2O_2 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 H_2O_2 (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 (Polle *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 receive 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). A 100 µl aliquot of ASA standards (0, 0.1, 0.5, and 1.0 mM in 50 mM KH_2PO_4, 50 mM K_2HPO4, 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 KH_2PO_4, 50 mM K_2HPO4, 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 KH_2PO_4, 49 mM K_2HPO_4, 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 extracts) 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 O_2. Previously MDHA reduction in the absence of extract, ASA, and ascorbate oxidase was found to be negligible (Polle *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 KH_2PO_4, 50 mM K_2HPO4, pH

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, 0.1, 0.25, and 0.5 mM NADH in 50 mM KH_2PO_4, 50 mM K_2HPO4, 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 KH_2PO_4, 50 mM K_2HPO4) to each well. At the moment of reaction initiation, the concentrations of the reagents in the wells were as follows: 49 mM KH_2PO_4, 49 mM K_2HPO_4, 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 KH_2PO_4, 50 mM K_2HPO_4, 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 KH_2PO_4, 43.25 mM K_2HPO_4, 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 H_2O_2 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 KH_2PO_4, 50 mM K_2HPO_4, pH 7.8, 0.5 mM ascorbate, 0.2 mM diethylenetriaminepentaacetic acid(DTPA)]. Duplicate 100 µl ASA standards were added to each plate (0, 0.1, 0.5, and 1.0 mM ascorbate in 50 mM KH_2PO_4, 50 mM K_2HPO_4, pH 7.8). The assay was initiated by adding 10 µl of 20 mM H_2O_2 to all wells except those containing standards. At the moment of reaction initiation, the concentration of the reagents in the wells were as follows: 45 mM KH_2PO_4, 45 mM K_2HPO_4, 0.45 mM ascorbate, 0.18 mM DTPA, and 2 mM H_2O_2. 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 extract 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 quantitiative 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 insure 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 (C_t) 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: $G=(E_{control}{}^{Ct-control})/(E_{gene}{}^{Ct-gene})$. Two replicate reactions were run for each sample, and their E^C 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 as a fixed effect. To assess differences among the parameters before the acute spike, an analysis of variance was run on the data at 0 h. For the time series data, time was treated as a repeated measure with an auto-regressive co-variance matrix structure. Chamber was not a significant source of variation and therefore statistical analyses for antioxidant metabolite and enzyme activity were performed on data from individual plants (n=6) and lsmeans were reported ± 1 SE. For gene expression data, only one pooled sample was analysed per chamber (n=2). All statistics were performed on the control gene-normalized expression data. Effects were considered significant at P <0.05.

Results

Antioxidant metabolites

Growth at elevated [O_3] significantly increased the total quenching capacity of available antioxidant metabolites (ORAC) by 24% in comparison with ambient conditions (P=0.0214; Table 2). This was reflected in a 42% larger ASA pool (P=0.0028; Table 2), but not in any significant change in phenolic content (Table 2). In contrast, growth at elevated [CO_2] significantly decreased the total antioxidant capacity by 20% compared with that of ambient-grown soybean (P=0.0392; Table 2). The ASA and phenolics pools were 15% smaller at elevated [CO_2], although those changes were not statistically significant (Table 2).

Changes in total antioxidant capacity, ASA pool size, and phenolic content were examined following an acute O_3 spike (Fig. 1). Immediately following the acute O_3 spike, total antioxidant capacity, ASA pool size, and phenolic content remained unchanged from pre-spike levels in the plants grown in ambient conditions and elevated [O_3].

Table 1. Real-time PCR targets, GenBank identifier, and primers for antioxidant transcript analysis

Target descriptions for soybean genes are based on peptide homologues in *Arabidopsis thaliana*.

Target description	GenBank ID	Forward primer	Reverse primer
AOX, cell wall	AB083032	TGGGTATGGGAAGGGAAAGT	ATGAAAAGCCCACACTCCAG
APX4	CD404038	CCAATATGGGAGAAGGCAAG	AGCAACATCAGGATCAGTGG
DHAR3, chloroplast	BE823473	CAATGTCCGCTGTGAGAGTT	AGGAGGAACGGAAGACATTG
GPX, mitochondria	AI939064	AAGAATGCAGCGCCACTTTA	TCGATTTTCAGAGGTGAGGTG
GPX6, chloroplast, mitochondria, cytosol	CF809067	AATCTCGCCGATTACAAAGG	CATGGGAATGCCAGAATTT
GPX7, chloroplast	CD414500	TGGACCATTTACAACTCCAGTG	GGAGACGTTGTTGGTGGGTA
GR, chloroplast, mitochondria	L11632	AGGCTGACTTTGATGCCACT	CTGCAGCTTTTGCTTGAGAG
GR1, cytosol	AW459932	AAGGTTCTCGGAGCCTCAAT	CACTGACCGCATGGTTACAA
GR	CF807683	GCTGTTGGTGTTGAGCTTGA	AAGGCCACCGGAGTAAGATT
Homoglutathione synthetase, chloroplast	AJ272035	GCCTGGTTTTGGAGTGGTAG	AGAAACAGCAATGCCTCCAT
MDHAR, cytosol	BU761097	GTTGTTGGGGTCTTTCTGGA	GCAAACCACCAAGCATTTTT
MDHAR	BG237135	GTAAGCTGAAAGGAGTTCTTCTTGA	AGTTTGGCTTTATCAATCAGAGG
MDHAR1, peroxisome, chloroplast, apoplast	BU084390	CTGGAAGGTGGAACTCCTGA	GCAAACCACCAAGCATCTTT
MDHAR4, peroxisome	BM525801	ACAATGGTCTTCCCTGAGGA	CCTTCCCATTGGAGTCAAAA
MDHAR4, peroxisome	BG362963	CCTGCATTGAGCAAAGGATT	TGGATTTAACTCCGGTTCCA
SOD [Cu-Zn], copper chaperone, chloroplast	AF329816	GCTGAGGGTGGCTGATCTTA	GGTGCCATCACACGTACAGA
SOD [Fe], chloroplast, mitochondria, plasma membrane	AF108084	CCTGGGATGCAGTGAGTTCT	TATTCCTCATGACGCCATCC
SOD [Cu-Zn], cytosol	AW101841	GAGAATCGTCATGCTGGTGA	CAGGATCAGCATGGACAACA
SOD [Cu-Zn], chloroplast	BE331642	AATGCCGATGGTAATGCTTC	AGGAGCACCTGATTGTCCAC
SOD2 [Cu-Zn], chloroplast	BI968043	CCACTCTCTGGCCCTAATTC	ATGCTGGAGTCAAACCAACC
SOD2 [Cu-Zn], chloroplast	BM188117	TTTGAGCACTGGAAATGCTG	GAAGGATCGTGCTTCAGAGC
SOD2 [Fe], chloroplast	CD409698	TGTAAACCCCCTTGTTTGGA	AGCTGACTGCATCCCAAGAC
SOD1 [Fe], chloroplast, mitochondria, plasma membrane	M64267	TTACCAGTGCATGATGCTGA	CAAAAGATGTGCCTGCTTACA
SOD3 [Cu-Zn], cytosol	CD412753	AGTGACAGGGTTGTCCCAAG	CATGGCGCTTATCATCTGAA

Table 2. The effect of growth at elevated [CO_2] or elevated [O_3] on soybean antioxidant capacity, metabolite content, and enzyme activity

Plants were grown under control conditions (400 ppm [CO_2], 0 ppb [O_3]), elevated [CO_2] (650 ppm [CO_2], 0 ppb [O_3]), or chronic elevated [O_3] (400 ppm [CO_2], 90 ppb [O_3]), and sampled before the acute O_3 spike. Statistically significant differences are indicated by different letters (P <0.05).

	Control	Elevated [CO_2]	Elevated [O_3]
ORAC (mmol TE g DW^{-1})	0.33±0.021 a	0.26±0.021 b	0.40±0.021 c
ASA (mmol g DW^{-1})	0.016±1.1e^{-3} a	0.013±1.2e^{-3} a	0.022±1.2e^{-3} b
Phenolics (mg GA g DW^{-1})	21.8±1.45 a	18.5±1.32 a	19.0±1.32 a
DHAR (nmol DHA min^{-1} mg $protein^{-1}$)	7.9±2.1 a	7.3±2.4 a	18.3±2.4 b
MDHAR (nmol MDA min^{-1} mg $protein^{-1}$)	28.7±1.9 a	25.2±2.3 a	29.7±2.3 a
GR (nmol GSSG min^{-1} mg $protein^{-1}$)	206.3±18.8 a	146.2±18.8 b	116.7±21.1 b
APX (nmol ASA min^{-1} mg $protein^{-1}$)	306.7±16.1 a	299.5±14.4 a	333.0±14.4 a
CAT (μmol H_2O_2 min^{-1} mg $protein^{-1}$)	9.8±3.8 a	6.3±3.9 a	14.8±3.9 a
SOD (U mg $protein^{-1}$)	180.6±6.9 a	161.7±8.0 a	180.7±8.0 a

However, the elevated [CO_2]-grown plants showed a 60% increase in total antioxidant capacity (P <0.0001; Fig. 1A) 4 h after the O_3 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-spike levels (P=0.0003), but returned to pre-spike 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-spike level (P <0.0001), which lessened to a 27% increase over pre-spike levels by 72 h (P=0.0298; Fig. 1B). Plants grown at elevated [O_3] sustained high total antioxidant capacity for 48 h following the acute O_3 spike, but levels dropped by 46% by 72 h (Fig. 1A). The increase in total antioxidant capacity immediately following the acute O_3 spike that was measured in the elevated [CO_2]-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 [CO_2]-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 [O_3]-grown plants,

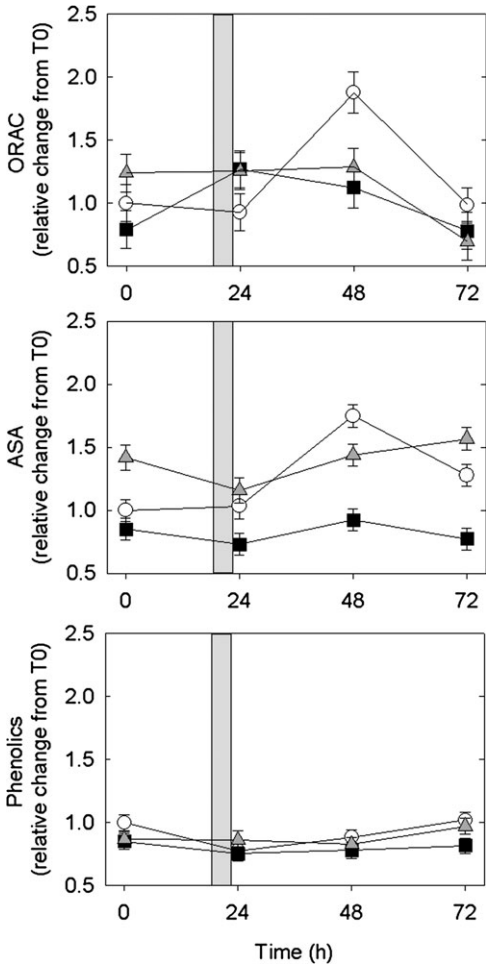

Fig. 1. The effects of atmospheric growth condition on the response of soybean total antioxidant capacity (A), reduced ascorbic acid (B), and phenolic content (C) to an acute O_3 spike (200 ppb [O_3] for 4 h). Total antioxidant capapcity was measured by the ORAC assay in Trolox equivalents (TEs). Phenolic content was measured as gallic acid (GA) equivalents. Growth conditions were control (open circles; 400 ppm [CO_2] and 0 ppb [O_3]), elevated CO_2 (filled squares; 650 ppm [CO_2] and 0 ppb [O_3]), and elevated O_3 (shaded triangles; 400 ppm [CO_2] and 90 ppb [O_3]). Graphs show relative change of each parameter compared with the control treatment at time 0 h (see Table 2) ± 1 SE; the shaded bar represents the timing of the 4 h acute O_3 spike.

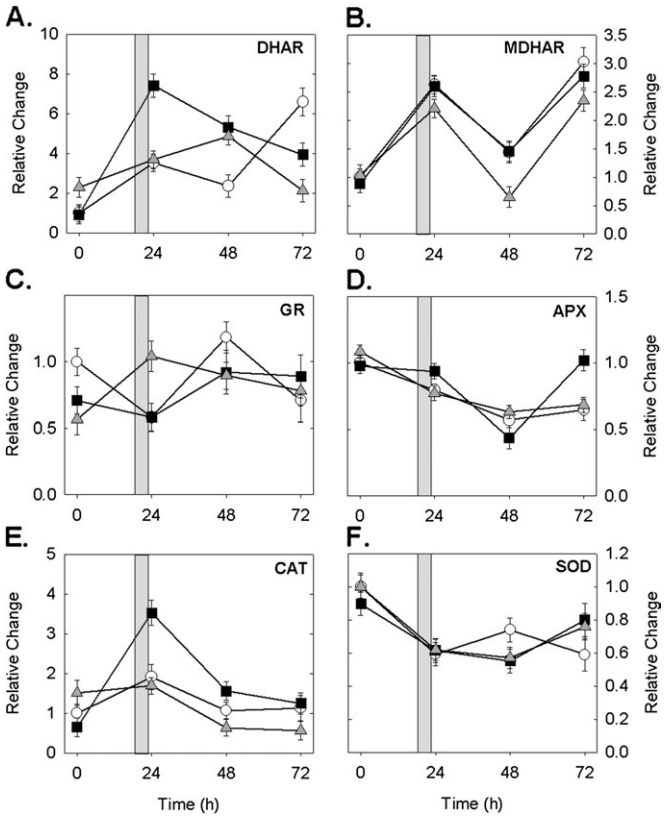

Fig. 2. The effects of atmospheric growth condition on the activity levels of six different antioxidant enzymes: dehydroascorbate reductase (DHAR; A), monodehydroascorbate reductase (MDHAR; B), glutathione reductase (GR; C), ascorbate peroxidase (APX; D), catalase (CAT; E), and superoxide dismutase (SOD; F) before and after an acute O_3 spike (200 ppb for 4 h). Symbols are as described for Fig. 1. All panels report the relative change of each parameter compared with the control treatment at time 0 h, ± 1 SE; the shaded bar represents the timing of the 4 h acute O_3 spike.

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; 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; Fig. 2C). Also, the significant increase in CAT activity measured in the control plants did not occur in the O_3-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 [O_3]-grown plants were the only group to display an increase in GR activity immediately following the acute O_3 spike, and that activity slowly returned to pre-spike levels by 72 h (Fig. 2C).

Plants grown with elevated [CO_2] responded with the largest changes in antioxidant enzyme activity immediately following the acute O_3 spike. DHAR activity increased by 700% (P >0.0001; Fig. 2A), MDHAR activity increased by 200% (P >0.0001; Fig. 2B), and CAT activity increased by 450% (P >0.0001; Fig. 2E), compared with pre-spike activity levels. Conversely, SOD activity decreased by 41% (P=0.0141; Fig. 2F). In the recovery period following the acute O_3 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 [CO_2] did not display the same immediate drop and sustained low APX activity observed in the control and [O_3]-grown plants. Instead, APX activity in elevated [CO_2]-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 O_3 treatment (Time 0 in Fig. 3), elevated [CO_2] 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 [O_3] 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 [O_3].

Immediately following the acute O_3 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 O_3 spike: GR1 (Fig. 3J) and MDHAR2 (Fig. 3N). Transcript abundance for these genes returned to pre-spike levels by 48 h (Fig. 3J, N).

The immediate decrease in transcript abundance for chloroplastic antioxidant enzymes following the acute O_3 spike was not present in the elevated [O_3]-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 O_3 spike in elevated [O_3]-grown

Table 3. Analysis of variance of antioxidant pools, enzymes, and GenBank IDs of transcripts measured for soybeans grown under control conditions (400 ppm [CO_2], 0 ppb [O_3]), elevated [CO_2] (650 ppm [CO_2], 0 ppb [O_3]), and elevated [O_3] (400 ppm [CO_2], 90 ppb [O_3]), before and after an acute (200 ppb) O_3 spike

P-values in italics are marginally significant. See Table 1 for descriptions of GenBank IDs.

	Atmosphere	Time	Atmosphere×time
ORAC	NS	0.0001	0.0109
Phenolic content	0.0023	0.0292	NS
ASA	<0.0001	<0.0001	0.0012
DHA	NS	<0.0001	NS
CAT	0.0065	<0.0001	0.0065
SOD	NS	<0.0001	NS
APX	NS	<0.0001	0.0043
GR	NS	0.0496	0.0068
DHAR	0.0085	<0.0001	<0.0001
MDHAR	0.0023	<0.0001	NS
Transcripts			
AOX (AB083032)	<0.0001	0.0157	0.0364
APX4 (CD404038)	*0.0537*	0.0114	NS
DHAR3 (BE823473)	NS	NS	NS
GPX (AI939064)	NS	0.0153	NS
GPX6 (CF809067)	NS	NS	NS
GPX7 (CD414500)	NS	0.0005	0.0462
GR (L11632)	NS	*0.0723*	NS
GR1 (AW459932)	*0.0687*	<0.0001	0.0077
GR (CF807683)	0.0188	0.0021	0.0210
HGS (AJ272035)	NS	NS	NS
MDHAR (BU761097)	*0.0748*	0.0015	NS
MDHAR (BG237135)	NS	NS	0.0268
MDHAR1 (BU084390)	NS	0.0101	NS
MDHAR4 (BM525801)	NS	NS	NS
MDHAR4 (BG362963)	0.0312	0.0018	0.0165
SOD chaperone (AF329816)	0.0088	0.0160	0.0158
SOD-Fe (AF108084)	NS	NS	NS
SOD (AW101841)	NS	0.0166	NS
SOD (BE331642)	0.0013	0.0002	0.0021
SOD2 (BI968043)	*0.0751*	NS	NS
SOD2 (BM188117)	0.0143	*0.0505*	NS
SOD2 (CD409698)	NS	NS	NS
SOD1 (M64267)	NS	NS	NS
SOD3 (CD412753)	NS	NS	NS

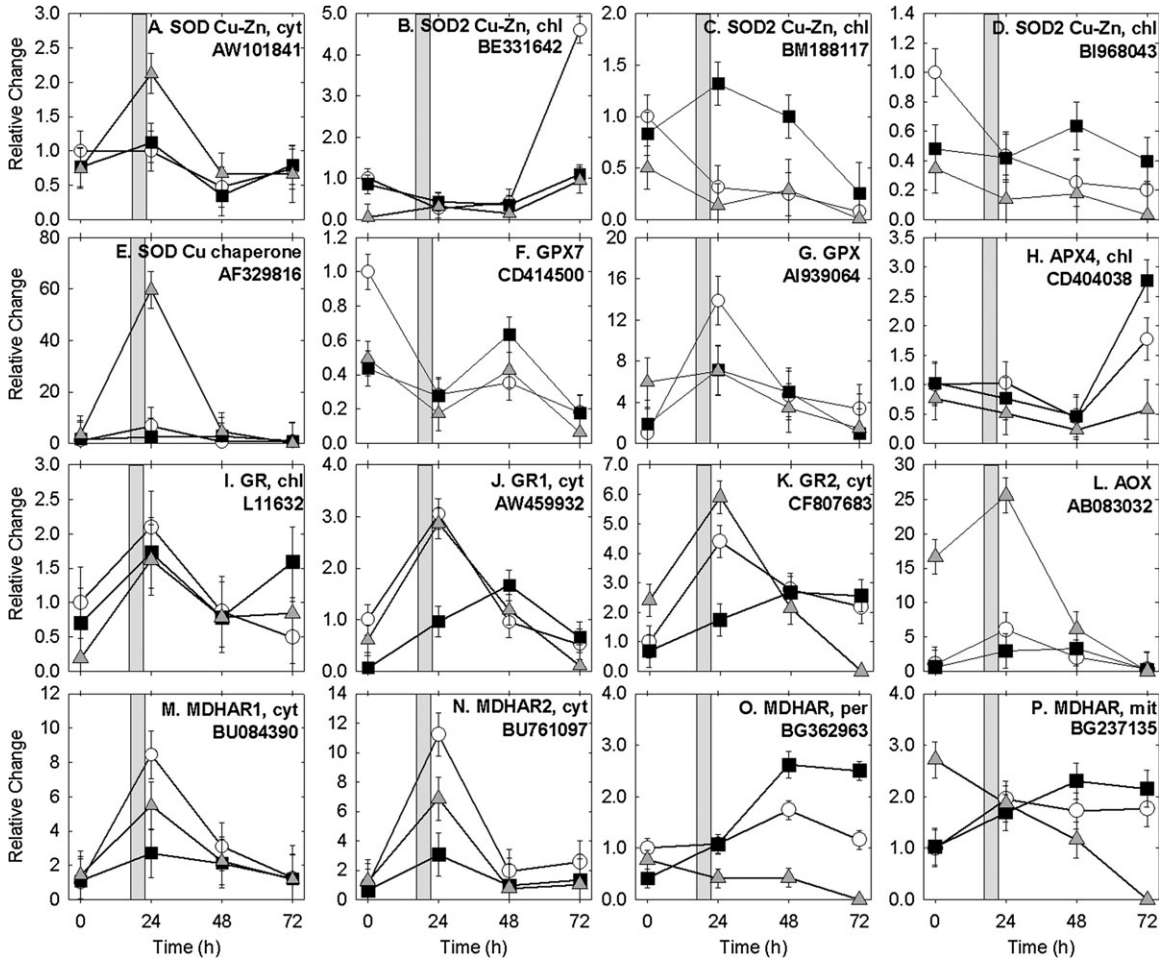

Fig. 3. The effects of atmospheric growth condition on the response of soybean transcripts involved in antioxidant metabolism before and after an acute O_3 spike (200 ppb for 4 h). Each transcript is labelled with its annotated function and subcellular localization, if known, as well as the GenBank identification number (cyt, cytosol; chl, chloroplast; per, peroxisome; mit, mitochondria). Further information about the transcripts can be found in Table 1. Symbols are as described for Fig. 1. All panels report the relative change of each transcript compared with the control treatment at time 0 h, ± 1 SE; the shaded bar represents the timing of the 4 h acute O_3 spike.

plants (Fig. 3L). A large increase in the transcript abundance for the Cu-Zn SOD copper chaperone was also observed in elevated [O_3]-grown plants immediately following the acute 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 [CO_2]-, [O_3]-, and control-grown plants (Fig. 3E, L).

Plants grown at elevated [CO_2] did not display many of the changes in transcript abundance for cytosolic and chloroplastic antioxidant enzymes following the acute O_3 spike that were observed in the elevated [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 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 O_3 spike in soybean grown at elevated [CO_2] (Fig. 3N). Other differences in transcript abundance in plants grown at elevated [CO_2] include increased peroxisomal MDHAR4 (Fig. 3O) and MDHAR (Fig. 3P) 48–72 h following the O_3 spike.

Discussion

Chronic growth environment alters antioxidant metabolism

Growth at elevated [CO_2] or elevated [O_3] significantly altered the total antioxidant capacity, measured as the inhibition of peroxyl radical-induced oxidations, of soybean leaves. Growth at elevated [CO_2] decreased total antioxidant capacity, while elevated [O_3] increased total antioxidant capacity. These changes are consistent with the expectations from previous studies, which have suggested increased oxidative stress in elevated [O_3] (Kangasjarvi *et al.*, 2005), and decreased perceived oxidative stress at elevated [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_2] or elevated [O_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_3]. On the other hand, there was no significant change in total phenolic content of soybean exposed to elevated [CO_2] or elevated [O_3]. Previous studies have found increased phenolic content in plants exposed to [O_3] (Kangasjarvi *et al.*, 1994; Saviranta *et al.*, 2010); however, there is also evidence that individual phenolic compounds can respond differently to [O_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_2] or elevated [O_3] (Table 2). However, DHAR activity was >2-fold greater in plants grown at elevated [O_3] 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_3]-grown plants. On the other hand, GR activity was significantly lower in plants grown at elevated [O_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_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_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_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_2] or elevated [O_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_2 and H_2O_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_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_3 spike in all environments (Fig. 2). Relative increases in DHAR and MDHAR enzyme activity were greatest in plants exposed to elevated [CO_2], which also showed increased total antioxidant capacity, measured by the ORAC assay (Fig. 1). Plants grown at elevated [O_3] increased GR activity immediately following the O_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_3] and exposed to the O_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_3 spike with shifts in activity toward the recycling and preservation of the ASA pool with increased scavenging by CAT, especially in elevated [CO_2], and decreased production of H_2O_2 by SOD (Fig. 2). APX, which is regulated by its substrate, H_2O_2 (Lu *et al.*, 2005), also showed lower activity following the O_3 spike.

The apoplast is often considered to be the first line of defence against O_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_3] and increased in all plants following the acute [O_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_3] following an acute O_3 spike, which is consistent with previous observations (Mahalingam *et al.*, 2005). The dampened transcriptional response of plants grown at elevated [CO_2] (Fig. 3) suggests that either less oxidative stress was perceived or plants grown under elevated [O_3] or control conditions were primed to respond more quickly to the oxidative stress.

It was anticipated that the acute O_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 [CO_2] and exposed to acute O_3 stress (Fig. 3J, N). In fact, peak transcript abundance was measured 24–48 h following the spike, rather than immediately (4 h) after. Such a sluggish transcriptional response has been suggested as a measure of ozone sensitivity in cultivars of *Arabidopsis* and *Medicago* (Mahalingam *et al.*, 2005; Puckette *et al.*, 2008). If that is accurate, it suggests that plants grown at elevated [CO_2] were more sensitive to the acute O_3 spike compared with plants grown in ambient or chronic elevated [O_3].

Concluding remarks

Previous research demonstrated that growth at elevated [O_3] caused a general 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, direct evidence for this up-regulation has been variable and dependent on the duration and method of O_3 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 [CO_2] 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 [O_3] environment increased total antioxidant capacity, while chronic elevated [CO_2] 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 [O_3] 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 [O_3] allowed for a primed antioxidant system. Growth at elevated [CO_2] 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 O_3 spike also differed considerably, suggesting distinct mechanisms of control.

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