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Greater antioxidant and respiratory metabolism in field-grown soybean exposed to elevated O_3 under both ambient and elevated CO_2

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ABSTRACT

Antioxidant metabolism is responsive to environmental conditions, and is proposed to be a key component of ozone (O_3) tolerance in plants. Tropospheric O_3 concentration ($[O_3]$) has doubled since the Industrial Revolution and will increase further if precursor emissions rise as expected over this century. Additionally, atmospheric CO_2 concentration ($[CO_2]$) is increasing at an unprecedented rate and will surpass 550 ppm by 2050. This study investigated the molecular, biochemical and physiological changes in soybean exposed to elevated $[O_3]$ in a background of ambient $[CO_2]$ and elevated $[CO_2]$ in the field. Previously, it has been difficult to demonstrate any link between antioxidant defences and O_3 stress under field conditions. However, this study used principle components analysis to separate variability in $[O_3]$ from variability in other environmental conditions (temperature, light and relative humidity). Subsequent analysis of covariance determined that soybean antioxidant metabolism increased with increasing $[O_3]$, in both ambient and elevated $[CO_2]$. The transcriptional response was dampened at elevated $[CO_2]$, consistent with lower stomatal conductance and lower O_3 flux into leaves. Energetically expensive increases in antioxidant metabolism and tetrapyrrole synthesis at elevated $[O_3]$ were associated with greater transcript levels of enzymes involved in respiratory metabolism.

Key-words: *Glycine max*; climate change; free air concentration enrichment (FACE); principal components analysis.

INTRODUCTION

Emissions from fossil fuel burning have dramatically increased since the Industrial Revolution, resulting in elevated concentrations of carbon dioxide ($[CO_2]$) and ozone ($[O_3]$) in the troposphere (Prentice *et al*. 2001; Royal Society 2008). These two atmospheric constituents have opposing effects on C_3 plants; elevated $[O_3]$ typically decreases photosynthesis, growth and crop yield (Ashmore 2005; Fiscus, Booker & Burkey 2005), whereas elevated $[CO_2]$ typically stimulates productivity of C_3 plants (Ainsworth & Long 2005). Even if emission rates are stabilized or reduced, a continued increase in atmospheric $[CO_2]$ is committed (Meehl *et al*. 2007), and under current emission scenarios, background tropospheric $[O_3]$ is also predicted to increase in the coming decades (Royal Society 2008). Therefore, identifying mechanisms that improve stress tolerance traits while enabling crops to exploit rising $[CO_2]$ for yield enhancement is a critical step towards adapting crops to future growing environments (Ainsworth, Rogers & Leakey 2008a; Mittler & Blumwald 2010).

Significant progress has been made towards understanding plant biochemical and molecular mechanisms regulating protective and repair processes in response to oxidative stress induced by elevated $[O_3]$ (Sharma *et al*. 1996; Pell, Schlaghaufer & Arteca 1997; Sandermann *et al*. 1998; Overmyer *et al*. 2000, 2008; Rao *et al*. 2000; Foyer & Noctor 2005; Brosché *et al*. 2010; Street *et al*. 2011). Briefly, O_3 reacts rapidly with components of the cell and breaks down to form other reactive oxygen species (ROS; Kangasjärvi *et al*. 1994). These ROS trigger downstream responses including hormone cascades (Overmyer, Brosché & Kangasjärvi 2003; Kangasjärvi, Jaspers & Kollist 2005) and increased antioxidant metabolism (Kangasjärvi *et al*. 1994; Conklin & Barth 2004). Much of this understanding comes from plants exposed to acute O_3 stress, which contrasts with the stress experienced by field-grown crops in two important ways. First, in many regions, today and in the future, crops will experience mean seasonal $[O_3]$ (40–80 ppb) that are associated with chronic symptoms of accelerated leaf senescence and reduced carbon balance, productivity and yield (Morgan, Ainsworth & Long 2003; Ashmore 2005; Betzelberger *et al*. 2010), but not the programmed cell death, tissue damage and severe growth impairment associated with acute O_3 exposures often used in experimentation (>200 ppb; Kangasjärvi *et al*. 2005). Second, the dynamic environmental conditions plants experience in the field, along with their typically greater metabolic rates, mean they routinely deal with greater oxidative stress than plants grown under relatively low light in controlled environment

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conditions. Given that antioxidant metabolism is known to be primed by previous oxidative stress events (Knight, Brandt & Knight 1998; Gillespie, Rogers & Ainsworth 2011), this may partly explain why the transcriptional response of field-grown plants to stress is not easily duplicated in controlled environments (Miyazaki *et al*. 2004; Pasquer *et al*. 2005). Consequently, this study primarily aims to identify the key transcriptional and biochemical components of soybean response to elevated [O_3] under field conditions in one of the primary regions of production, the Midwest United States.

The nature of plant responses to future, elevated [O_3] will be significantly modified by the co-occurrence of elevated [CO_2]. Growth at elevated [CO_2] decreases many of the negative effects of elevated [O_3] on plant physiology; however, the molecular mechanisms for amelioration of O_3 damage at elevated [CO_2] are not well understood. One hypothesis suggests that decreases in stomatal conductance (g_s) as a result of growth at elevated [CO_2] lead to smaller fluxes of O_3 into the leaf and less subsequent damage, without a need for large changes in antioxidant metabolism (McKee, Eiblmeier & Polle 1997; McKee *et al*. 2000; Wustman *et al*. 2001). A second hypothesis suggests that an increase in photoassimilate availability at elevated [CO_2] supports increased investment in ROS detoxification and repair of damaged components in order to reduce the negative effects of elevated [O_3] (Rao, Hale & Ormrod 1995). This second hypothesis implies that antioxidant metabolism is limited by C availability at ambient [CO_2], and that this limitation is at least partially relieved at elevated [CO_2]. The negative correlation between the capacity of antioxidant metabolism and photosynthesis in 8 soybean cultivars grown at elevated [O_3] suggests a trade-off between C gain and defence at ambient [CO_2] (Betzelberger *et al*. 2010). However, the two hypotheses are not mutually exclusive, and the 'protective' effect of elevated [CO_2] likely depends upon the concentrations of both O_3 and CO_2.

Although it is more relevant to crop production and improvement, investigating the mechanisms of interaction between elevated [CO_2] and [O_3] in the field is complicated by three major factors. First, there is variability in other environmental parameters, which also influence C metabolism, ROS production and detoxification. Second, O_3 is highly reactive and its concentration in the atmosphere varies over time and space (Royal Society 2008). Third, the magnitude of the effects of elevated [CO_2] and elevated [O_3] on plant physiology has been shown to be influenced by the developmental stage of the plant (Morgan *et al*. 2004), as well as environmental conditions such as temperature and water deficit (Bernacchi *et al*. 2006; Leakey *et al*. 2006). Nonetheless, genomic technologies are increasingly applied to plants grown under ecologically relevant treatments (Taylor *et al*. 2005; Ainsworth *et al*. 2006; Druart *et al*. 2006; Leakey *et al*. 2009a,b; Tallis *et al*. 2010; Travers *et al*. 2010). The ability to collect large data sets across biological scales from a field system is a powerful approach, and when combined with reduction modelling techniques that simplify the environmental inputs (Janes & Yaffe 2006), allows mechanisms of response to be determined in ecologically and agriculturally relevant contexts. Here we demonstrate the power of such an approach to address important uncertainties about the response of antioxidant metabolism to the combination of elevated [CO_2] and elevated [O_3].

In this study, the response of soybean to elevated [O_3] was assessed in the field throughout the 2007 growing season, under both ambient and elevated [CO_2] to address uncertainty in the response of antioxidant metabolism to chronic elevated [O_3] and the associated metabolic adjustments necessary to support changes in antioxidant metabolism. Combining transcript profiling with metabolomics and enzyme activity profiling allowed us to test for transcriptional reprogramming of antioxidant capacity and also identify regulatory genes putatively coordinating changes in antioxidant pathways as well as any associated changes in primary and secondary metabolism. Because environmental variability of field systems is known to influence the transcriptional, biochemical and physiological plant response, principle components analysis (PCA) was used to collapse 10 co-varying environmental measures, including [O_3], into two orthogonal principle components (PC) that explained 76% of the environmental variation (Table 1). Analysis of co-variance was subsequently used to model the effects of the two PCs (Littel *et al*. 2006), and to determine how [CO_2] influenced those effects. This approach has provided a definitive test of the effects of [O_3] within two atmospheric [CO_2], while simultaneously accounting for the variability in other environmental factors to demonstrate the impact of elevated [CO_2] and elevated [O_3] on the plant antioxidant system under open-field conditions. Transcript profiling revealed a significant positive correlation between [O_3] and the abundance of transcripts encoding components of the TCA cycle and mitochondrial electron transport pathways. Night-time respiratory CO_2 efflux was discovered to increase with growth [O_3] on both an area and mass basis in subsequent experiments on soybean grown at a range of [O_3].

Table 1. The eigenvectors and eigenvalues of the correlation matrix for the first two principle components

Environmental variable	PC 1	PC2
T_{max}	–0.19	0.51
T_{min}	–0.16	0.03
RH_{max}	–0.11	0.54
PCMI	–0.03	0.25
PAR_{max}	0.18	–0.54
1 h max [O_3] 14 d	0.41	0.20
8 h ave [O_3] 14 d	0.42	0.15
14 d AOT40	0.42	0.14
1 h max [O_3]	0.43	0.10
8 h ave [O_3]	0.44	0.05
Eigenvalue	5.0	2.6
Percent variability	50.4	26.2
Cumulative percent variability	50.4	76.6

Each principle component is an orthogonal, linear combination of the 10 original environmental variables. See text for abbreviations.

MATERIALS AND METHODS

Field site description

Soybean (*Glycine max*, cv. Pioneer 93B15) was grown at the Soybean Free Air Concentration Enrichment (SoyFACE) experimental facility (http://www.soyface.illinois.edu) during 2007. The field site, agronomic practices, CO_2 and O_3 fumigation methods have been described previously (Morgan *et al*. 2004; Rogers *et al*. 2004). The experiment was designed as a randomized complete-block design ($n = 4$). Within each block, one ring was maintained at ambient $[CO_2]$ and ambient $[O_3]$, elevated $[CO_2]$ (550 ppm) and ambient $[O_3]$, ambient $[CO_2]$ and elevated $[O_3]$ (2× ambient), or both elevated $[CO_2]$ (550 ppm) and elevated $[O_3]$. Treatments were chosen to simulate atmospheric conditions predicted for 2050 (Prentice *et al*. 2001).

Fumigation started on 4 June 2007, shortly after crop emergence, and ended on 21 September 2007, after the soybeans were mature. Across the 2007 growing season, daytime average $[CO_2]$ was 386 ppm in the ambient $[CO_2]$ treatment and 552 ppm in the elevated $[CO_2]$ treatment. Based on 1 min averages, the $[CO_2]$ in the elevated $[CO_2]$ plots was within 10% of the target concentration for 80% of the time. Average 8 h (1000–1800 h) $[O_3]$ was 46.3 ppb and 82.5 ppb in the ambient and elevated $[O_3]$ plots, respectively (Betzelberger *et al*. 2010). Elevated $[O_3]$ was within 10% of the target concentration for 70% of the time.

Sampling for photosynthetic, biochemical and transcriptional analyses

Mature soybean leaves in sun were assessed on five dates in 2007 throughout the growing season (Fig. 1). At midday, photosynthetic gas exchange under growth conditions was measured on three plants per plot using open gas-exchange systems (LI-6400; Li-Cor Inc., Lincoln, NE, USA) as described in (Leakey *et al*. 2009b). Measurements on all individuals were made at growth $[CO_2]$. Photosynthetic photon flux density (PPFD) and T_{air} were recorded at the top of the canopy prior to starting the sampling procedure at each time point. Then throughout that time point the

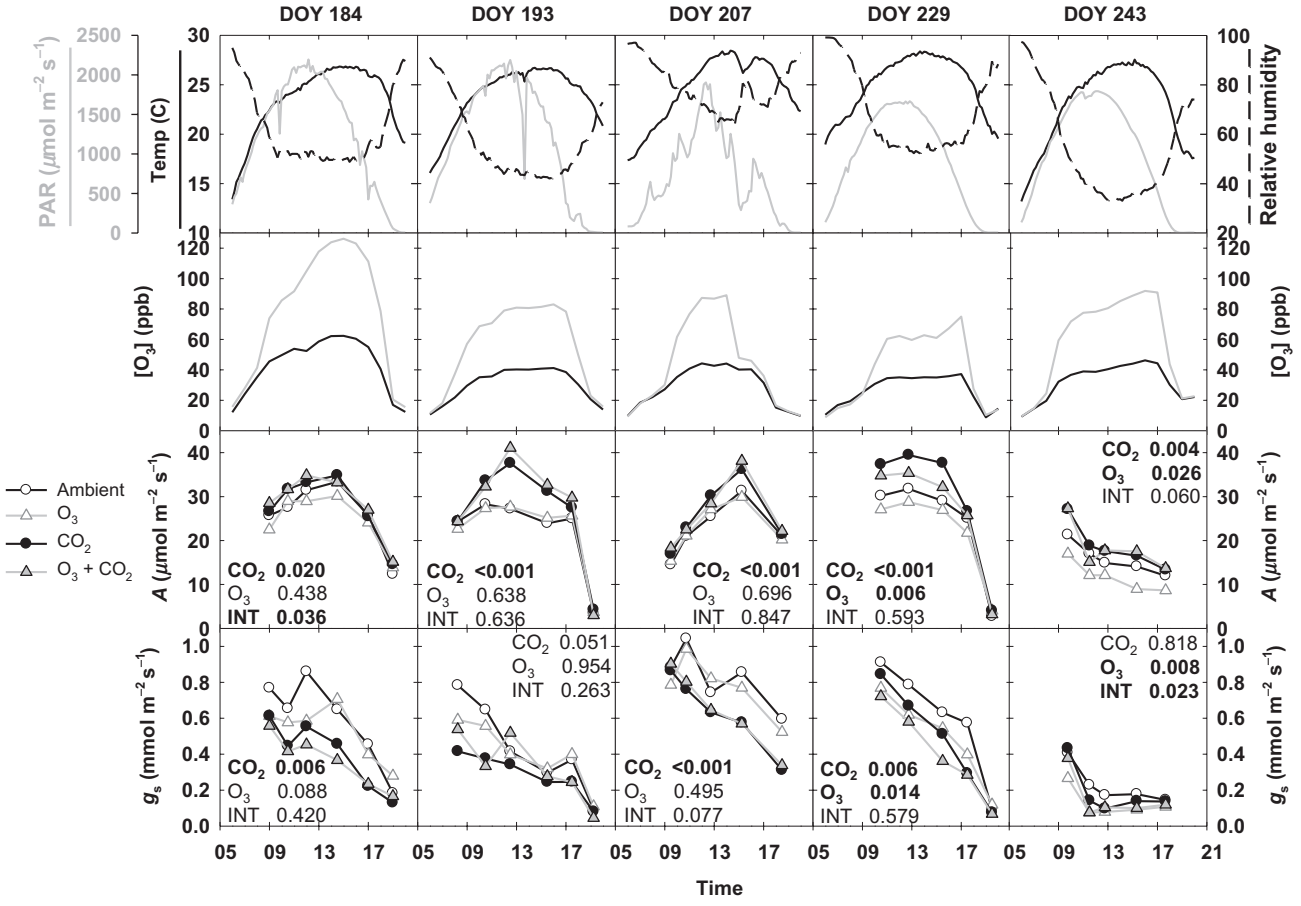

Figure 1. Diurnal course of photosynthetically active radiation (PAR), air temperature (Temp), relative humidity, hourly $[O_3]$ in ambient air (black line) and elevated (grey line) $[O_3]$ treatment, photosynthesis (A) and stomatal conductance (g_s) measured on 5 d in 2007 at the SoyFACE experiment. A and g_s were collected on the most recently fully expanded soybean leaf at the top of the canopy. Growth conditions were ambient $[CO_2]$ and $[O_3]$ (open circle, black line), elevated $[O_3]$ (open triangle, grey line), elevated $[CO_2]$ (closed circle, black line) and elevated $[CO_2]$ and elevated $[O_3]$ (closed triangle, black line). Each point is the mean of the replicate plots ($n = 4$) at that time. P-values indicate statistical significance of CO_2, O_3, and $CO_2 \times O_3$ interaction (INT) effects. DOY, day of year.

conditions were replicated in each leaf chamber by the red-blue LED light source and Peltier heat sinks. The water vapour concentration of air entering the chamber was not controlled and therefore tracked ambient conditions. Directly after the photosynthetic measurements, tissue was excised from three to five plants per plot for determination of chlorophyll, soluble carbohydrates, starch, protein and total amino acid content (Ainsworth *et al*. 2007), total antioxidant capacity (Gillespie, Chae & Ainsworth 2007) reduced and oxidized ascorbic acid content (Gillespie & Ainsworth 2007), total phenolic content (Ainsworth & Gillespie 2007), and activity of six enzymes involved in antioxidant recycling (Gillespie *et al*. 2011). Simultaneously, six leaflets from separate plants were excised, immediately plunged into liquid nitrogen, and stored at –80 °C until RNA was extracted for global transcript analysis using soybean genechips (Affymetrix, Santa Clara, CA, USA). RNA extraction, hybridization and data processing were performed as described in Leakey *et al*. (2009b).

Statistical analysis of photosynthetic, biochemical and transcriptional analyses

Photosynthesis and stomatal conductance were tested with a randomized complete block mixed model analysis of variance (ANOVA), using the Kenward–Rogers option (PROC MIXED, SAS 9.2; SAS Institute, Cary, NC, USA; Leakey *et al*. 2009b). In the model, CO_2, O_3 and time were fixed effects and block was a random effect. A P-value < 0.05 was the threshold for significance and each day was analysed independently (Fig. 1).

The ANOVA approach is limited because it considers $[O_3]$ to be a fixed variable, when in fact, $[O_3]$ is dynamic and varies in both ambient and elevated plots in each day of sampling (Fig. 1). Moreover, other environmental variables (including light, temperature and relative humidity; Fig. 1) have strong effects on plant metabolism, vary over the growing season and are highly correlated (Janes & Yaffe 2006; Travers *et al*. 2010; Supporting Information Fig. S1). Therefore, PCA was used to collapse 10 environmental variables into two PCs that accounted for 76% of the variability in the environment data (Table 1). The 10 environmental variables included: maximum daytime temperature (T_{max}), minimum nighttime temperature (T_{min}), maximum relative humidity (RH_{max}), Palmer Crop Moisture Index (PCMI), maximum photosynthetically daily active radiation (PAR_{max}), maximum 1 h $[O_3]$ averaged over 14 d prior to sampling (14 d 1 h Max $[O_3]$), 8 h (1000–1800 h) average $[O_3]$ for the 14 d prior to sampling (14d 8 h Ave $[O_3]$), 14 d accumulated ozone over a threshold concentration of 40 ppb (14 d AOT40), 1 h maximum $[O_3]$ on the day of sampling (1 h Max $[O_3]$) and 8 h average $[O_3]$ on the day of sampling (8 h Ave $[O_3]$). Ozone metrics were chosen because leaf development data collected at SoyFACE suggested that the measured leaves unfolded ~14 d prior to sampling. AOT40 was calculated according to Mauzerall & Wang (2001).

The five different metrics of $[O_3]$ loaded heavily onto PC 1 ($PC1_{O3}$), whereas T_{max}, RH_{max} and PAR_{max} loaded heavily onto $PC2_{ENV}$ (Table 1). Negative values of $PC1_{O3}$ correspond to low $[O_3]$ whereas positive and high values correspond to high $[O_3]$. The five sampling dates displayed a negative correlation between RH_{max} and PAR_{max} (Supporting Information Fig. S1), such that the least humid days were the brightest. Therefore, $PC2_{ENV}$ accounted for this relationship with greater PAR_{max} loading heavily on negative values and greater RH_{max} and temperature loading heavily on the positive values (Table 1).

The PCA multivariate technique effectively explains and parses the day-to-day variability in field environmental conditions from the day-to-day variability of $[O_3]$ into orthogonal PCs. These PC values were used in all subsequent analyses of biochemical parameters and transcript changes. Biochemical parameters and transcript levels were tested with a mixed model analysis of covariance (SAS 9.2). In all tests, CO_2 was treated as a fixed effect whereas $PC1_{O3}$ and $PC2_{ENV}$ were treated as covariates. Because a single test does not have enough degrees of freedom to determine whether the slopes of the covariate models were different from zero and different between $[CO_2]$ treatments, an equal slopes model was used to test for differences among the slopes between CO_2 treatments and an unequal slopes model was used to determine whether the individual slopes at both CO_2 concentrations were different from zero (Littel *et al*. 2006). The slope of the regression line between the dependant variable and $PC1_{O3}$ or $PC2_{ENV}$ at both levels of CO_2 was obtained from the solution for the unequal model outputs. A P-value < 0.05 was used to reject the hypothesis that the slopes at either ambient or elevated $[CO_2]$ equalled zero. Using the equal slopes model, a P-value < 0.1 was used to reject the hypothesis that the slope at ambient $[CO_2]$ equalled the slope at elevated $[CO_2]$.

Nighttime rates of leaf respiratory CO_2 efflux

In 2009 and 2010, soybean were grown in eight FACE plots, each at a different daytime target $[O_3]$ (40, 55, 70, 85, 110, 130, 160, 200 ppb). Dark respiration of mature leaves at the top of the canopy was measured during the first 4 h of the night on two dates in the 2009 (27 July and 10 August) and 2010 field seasons (14 July and 26 August). In 2009, rates of respiratory CO_2 efflux of detached leaves were measured at 25 °C using a laboratory-based gas exchange system, as described by Leakey *et al*. (2009b). In 2010, rates of respiratory CO_2 efflux from attached leaves were measured in the field at ambient temperature using a custom built leaf chamber designed for use with the LI-6400 gas exchange system (Li-Cor Biosciences) using standard mounting hardware as well as a standard leaf thermocouple and extended chamber exhaust tube. The upper and lower chamber sections were machined from aluminium to internal dimensions of $12 \times 8.5 \times 1$ cm (W × L × D). Internal corners were rounded to maximize chamber mixing and were coated with nickel polytetrafluoroethylene (PTFE) (Teflon™) plating to minimize adsorption and absorption of CO_2 and water.

The chamber was tightly sealed by applying pressure with three spring clamps to pinch the upper and lower chamber sections together and compress a rubber o-ring sandwiched between the outer walls. A shallow channel in the leading edge of the chamber held the leaf petiole and was sealed with non-stick putty (Qubitac sealant; Qubit Systems, Kingston, Canada). Leaves were sealed in the chamber and allowed to equilibrate for 1–3 min before measurements were logged. The large leaf area measured using these custom chambers (78 cm^2 on average) resulted in large [CO_2] differentials (21 ppm on average), which greatly improved the signal : noise ratio of the instrument relative to when smaller leaf areas are enclosed in the standard chambers for this gas exchange analyser (6 cm^2). Images were collected for determination of leaf area before leaves were oven dried and weighed. The relationship between rates of respiratory CO_2 efflux (mass and area-basis) and growth [O_3] were tested using non-linear regression (Sigmaplot, Systat Software Inc. Chicago, IL, USA).

RESULTS

Leaf physiological responses to elevated [CO_2] and [O_3], and PCA of environmental variables

Elevated [O_3] decreased A by 9% on average across the growing season, although this decrease was largely driven by the 26% decrease in A on the last sampling date, 31 August (Fig. 1). Elevated [CO_2] increased A by 15% compared with ambient [CO_2], and the magnitude of the response varied from 5% on 3 July to 23% on 17 August (Fig. 1). On most dates, the photosynthetic response to the combination treatment was similar to the elevated [CO_2] treatment alone (Fig. 1). Growth at elevated [O_3] decreased g_s by 14% on average throughout the growing season. Again, this was caused by a large decrease (46%) in g_s towards the end of the growing season. Growth at elevated [CO_2] decreased g_s by 26% consistently throughout the growing season, and the response in the combination treatment (elevated [CO_2] + elevated [O_3]) was similar to that in elevated [CO_2] alone (Fig. 1). Elevated [CO_2] significantly increased specific leaf weight (SLW) on a consistent basis over the growing season under both ambient and elevated [O_3] (Fig. 2). However, there was no effect of elevated [O_3] on SLW either as a main effect ($P = 0.73$) or in interaction with CO_2 ($P = 0.47$) or day of year ($P = 0.32$).

These physiological data show that the effects of [O_3] accumulated over the growing season (Fig. 1), and the impacts of [O_3] on leaf-level processes are caused by a combination of the current [O_3] and the cumulative O_3 dose. Additionally, the magnitude of the effect of elevated [CO_2] on soybean photosynthesis varied with environmental conditions (Fig. 1; Bernacchi *et al.* 2006). Therefore, to more appropriately characterize the O_3 treatment and the environmental variation, PCA was used to condense correlated O_3 metrics and environmental variables (Supporting Information Fig. S1) into two dimensions (Table 1). The O_3 metrics loaded heavily onto the first PC ($PC1_{O3}$), whereas other environmental variables including temperature, relative humidity and light loaded heavily onto the second PC ($PC2_{ENV}$; Table 1). $PC1_{O3}$ accounted for 50.4% and and $PC2_{ENV}$ for 26.2% of the variability in 10 environmental variables (Table 1).

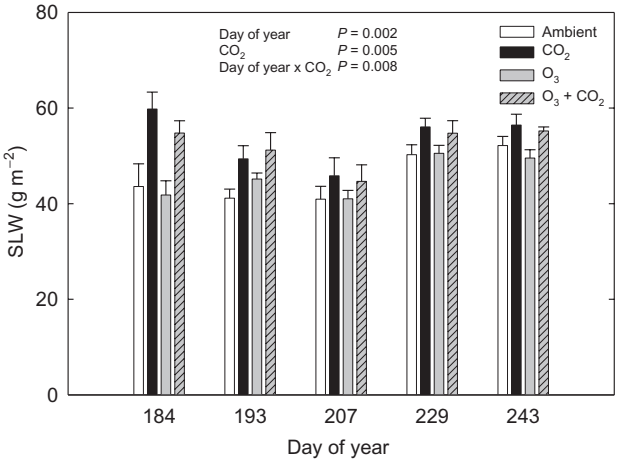

Figure 2. Specific leaf weight (SLW) of mature sun leaves of soybean grown at ambient [CO_2] and ambient [O_3] (white bars), elevated [CO_2] and ambient [O_3] (black bars), ambient [CO_2] and elevated [O_3] (plain grey bars), and elevated [CO_2] and elevated [O_3] (grey hatched bars) on five dates during the 2007 growing season. Each point is the mean (±SE) of the replicate plots measured at that time ($n = 4$). P-values for treatments with statistically significant effects are shown.

Transcriptional and biochemical responses of antioxidant metabolism

Total antioxidant capacity was significantly lower in plants grown at elevated [CO_2] compared with ambient [CO_2] (Table 2), and was positively correlated with $PC1_{O3}$ at ambient [CO_2], indicating that total antioxidant capacity increased with increasing [O_3] (Supporting Information Fig. S2; Table 3). The correlation between total antioxidant capacity and $PC1_{O3}$ at elevated [CO_2] was weaker and only marginally significant (Supporting Information Fig. S2; Table 3). Likewise, ascorbate (ASA) content was positively correlated with $PC1_{O3}$ at ambient [CO_2], but not at elevated [CO_2] (Table 3). Consistent with antioxidant capacity, monodehydroascorbate reductase (MDHAR) and superoxide dismutase (SOD) activity were lower in plants grown at elevated [CO_2] (Table 2). The only antioxidant enzyme activity that was significantly correlated with $PC1_{O3}$ was catalase (CAT), which showed a negative correlation with $PC1_{O3}$ at both ambient and elevated [CO_2] (Table 3).

Genes associated with antioxidant metabolism were a major feature of the transcriptional response to elevated [O_3] (Supporting Information Table S1). A significantly larger fraction of genes involved in antioxidant metabolism were correlated with $PC1_{O3}$ at ambient [CO_2] (19%) compared with transcripts associated with other functions (14%; $P = 0.038$, one-tailed Fisher's exact test; Fig. 3).

Table 2. Least-squared means (lsmeans) ± 1 standard error reported for each variable at ambient [CO_2] and elevated [CO_2] after controlling for the co-variation in $PC1_{O3}$ and $PC2_{ENV}$

	Ambient [CO_2]	Elevated [CO_2]	*P* value
APX (nmol ASA min^{-1} mg $protein^{-1}$)	93.1 ± 7.4	100 ± 7.3	0.510
SOD (Units mg $protein^{-1}$)	**144 ± 5.2**	**125 ± 5.2**	**0.0131**
CAT (μmol H_2O_2 min^{-1} mg $protein^{-1}$)	18.3 ± 1.7	20.9 ± 1.7	0.292
DHAR (nmol DHA min^{-1} mg $protein^{-1}$)	15.4 ± 1.1	14.8 ± 1.1	0.703
MDHAR (nmol MDA min^{-1} mg $protein^{-1}$)	**37.9 ± 3.4**	**27.5 ± 3.3**	**0.0308**
GR (nmol GSSG min^{-1} mg $protein^{-1}$)	75.7 ± 8.7	79.4 ± 8.8	0.764
ORAC (nmol TE g DW^{-1})	**0.77 ± 0.04**	**0.66 ± 0.04**	**0.0482**
Phenolics (mg GA g DW^{-1})	17.0 ± 1.0	16.4 ± 1.0	0.679
ASA (mmol g DW^{-1})	0.041 ± 0.001	0.039 ± 0.001	0.141
DHASA (mmol g DW^{-1})	**0.007 ± 0.004**	**0.006 ± 0.004**	**0.081**
Chlorophyll *a* (μmol m^{-2})	523 ± 15.1	532 ± 15.1	0.640
Chlorophyll *b* (μmol m^{-2})	103 ± 3.0	105 ± 3.0	0.592
Total chlorophyll (μmol m^{-2})	626 ± 17.9	638 ± 17.9	0.630
Glucose (mmol m^{-2})	**1.02 ± 0.1**	**2.04 ± 0.1**	**<0.0001**
Sucrose (mmol m^{-2})	**2.02 ± 0.1**	**2.84 ± 0.1**	**<0.0001**
Fructose (mmol m^{-2})	**0.85 ± 0.07**	**1.25 ± 0.07**	**0.0002**
Starch (mmol m^{-2})	**28.3 ± 2.8**	**54.1 ± 2.8**	**<0.0001**
Protein (g m^{-2})	**9.83 ± 0.2**	**9.41 ± 0.2**	**0.0787**
Amino acids (mmol m^{-2})	**3.09 ± 0.09**	**3.53 ± 0.09**	**0.00121**

Bold pairs indicate significant differences between ambient and elevated [CO_2] at $P < 0.1$. See text for abbreviations.

Similarly, at elevated [CO_2], a larger fraction of antioxidant metabolism genes were correlated with $PC1_{O3}$ (18%) compared with transcripts associated with other functions (11%; $P = 0.0012$, one-tailed fisher's exact test; Fig. 3). Moreover, for 46 of the 56 genes that were significantly correlated with $PC1_{O3}$ at ambient [CO_2] and for 44 of the 52 genes that were significantly correlated with $PC1_{O3}$ at elevated [CO_2], the correlation was positive, indicating greater transcript abundance at elevated [O_3]. The transcript abundance for the majority of the genes coding for various isoforms of the antioxidant recycling enzymes were positively correlated with $PC1_{O3}$ in plants grown at ambient and elevated [CO_2] (Fig. 4; Supporting Information Fig. S3 and Table S1), despite the lack of corresponding changes in

Table 3. Results of the analysis of co-variance (ANCOVA) for biochemical parameters

	$PC1_{O3}$		$PC2_{ENV}$	
	Slope at amb [CO_2], *P*	Slope at ele [CO_2], *P*	Slope at amb [CO_2], *P*	Slope at ele [CO_2], *P*
APX	4.02, 0.222	3.87, 0.246	–3.17, 0.484	4.08, 0.367
SOD	–3.36, 0.154	0.99, 0.676	**–16.5, <0.001**	**–6.7, 0.044**
CAT	–1.34, 0.0819	–1.69, 0.029	4.28, <0.001	4.74, <0.001
DHAR	–0.07, 0.886	0.29, 0.587	1.54, 0.028	1.60, 0.025
MDHAR	–0.17, 0.911	–0.34, 0.820	–1.66, 0.422	–3.08, 0.135
GR	6.39, 0.109	–0.70, 0.863	–7.30, 0.177	–13.9, 0.013
ORAC	0.06, 0.003	0.032, 0.087	–0.014, 0.575	0.015, 0.550
Phenolics	–0.09, 0.843	–0.46, 0.319	0.52, 0.408	1.18, 0.064
ASA	**0.0009, 0.043**	**–0.0003, 0.493**	–0.00009, 0.877	0.00003, 0.968
DHASA	0.00002, 0.930	–0.0001, 0.469	0.0007, 0.006	0.0009, <0.001
Chl *a*	–7.20, 0.287	–1.88, 0.783	–5.49, 0.559	–9.02, 0.341
Chl *b*	–1.78, 0.179	–0.76, 0.571	0.47, 0.797	0.34, 0.853
Total Chl	–9.00, 0.263	–2.64, 0.745	–5.01, 0.653	–8.68, 0.440
Glucose	0.02, 0.726	–0.08, 0.206	–0.034, 0.695	0.06, 0.492
Sucrose	0.12, 0.064	0.23, <0.001	**–0.38, <0.001**	**–0.67, <0.001**
Fructose	**0.04, 0.172**	**–0.042, 0.204**	–0.06, 0.190	0.004, 0.923
Starch	0.71, 0.564	1.35, 0.280	**–3.35, 0.053**	**–9.42, <0.001**
Protein	–0.14, 0.064	–0.18, 0.016	0.38, <0.001	0.19, 0.065
Amino acids	–0.003, 0.933	0.017, 0.693	–0.07, 0.241	–0.14, 0.021

The slope of the regression between $PC1_{O3}$ or $PC2_{ENV}$ and the measured variable at either ambient (amb [CO_2]) or elevated [CO_2] (ele [CO_2]) is displayed along with the *P*-value, which denotes whether the slope is significantly different from zero. Grey boxes highlight significant slopes at $P < 0.1$. Pairs highlighted in bold denote slopes that are significantly different between ambient and elevated [CO_2]. See text for abbreviations.

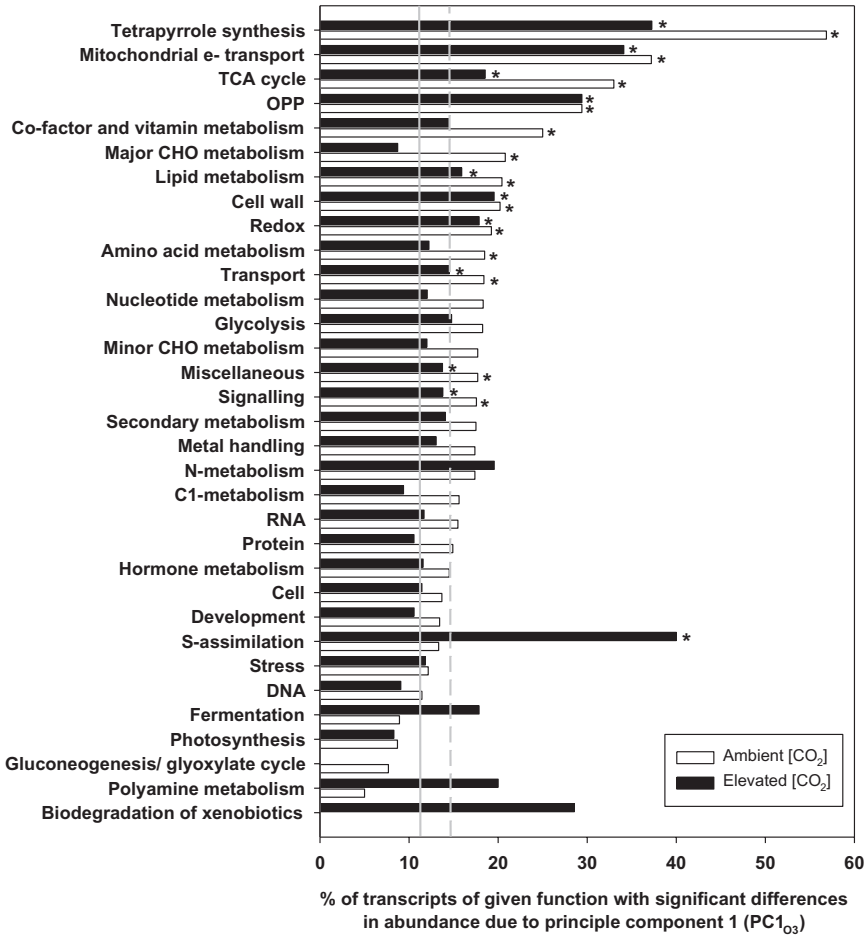

Figure 3. Percentage of transcripts with different cellular and metabolic functions that displayed significant differences in abundance because of principle component 1 ($PC1_{O3}$) in ambient [CO_2] (open bars) and elevated [CO_2] (closed bars). The solid grey line is the average number of all transcripts tested that responded significantly to $PC1_{O3}$ in the elevated [CO_2] treatment (11%) and the dashed grey line is the average number that responded significantly in ambient [CO_2] (15%). Functional categories with significantly more genes responding than average were tested with the Fisher's exact test and are indicated with an asterisk. OPP, oxidative pentose phosphate pathway.

enzyme activity. In contrast, there were no significant correlations between $PC1_{O3}$ and transcripts coding for CAT, which was the only enzyme whose activity displayed a significant negative correlation with $PC1_{O3}$ at both ambient and elevated [CO_2] (Fig. 4; Supporting Information Fig. S3; Table 3). Two genes coding for chloroplast localized Fe-SOD were negatively correlated with $PC1_{O3}$ at ambient [CO_2] (Fig. 4). However, genes coding for chloroplastic and cytosolic Cu-Zn SOD were positively correlated with $PC1_{O3}$ as was a SOD copper chaperone (Fig. 4). These responses were generally reproduced at elevated [CO_2] with three transcripts coding for Fe-SOD negatively correlated with $PC1_{O3}$, and three transcripts for Cu-Zn SOD positively correlated with $PC1_{O3}$ (Supporting Information Fig. S3).

This analysis also effectively parsed the effects of [O_3] from the effects of the other environmental factors that alter antioxidant metabolism. The activity levels of four antioxidant enzymes were more responsive to $PC2_{ENV}$ than $PC1_{O3}$ (Table 3). SOD and GR activity were negatively correlated with $PC2_{ENV}$, whereas CAT and DHAR activity were positively correlated with $PC2_{ENV}$ (Table 3). In addition, a significantly larger portion of genes associated with redox metabolism were affected by $PC2_{ENV}$ (55% at ambient [CO_2] and 48% at elevated [CO_2]) compared with the average response of transcripts with other functions (38% at ambient [CO_2] and 35% at elevated [CO_2]; $P < 0.0001$, one-tailed Fisher's exact test; Supporting Information Fig. S4).

Tetrapyrrole synthesis gene expression and chlorophyll content

Transcripts associated with tetrapyrolle synthesis were positively correlated with $PC1_{O3}$ at both ambient [CO_2] and elevated [CO_2] (Fig. 3). In ambient [CO_2], a significantly larger portion of tetrapyrrole synthesis genes were correlated with $PC1_{O3}$ (57%) compared with the portion of genes responding that correspond to other functional categories (14%; $P < 0.0001$, one-tailed Fisher's exact test; Fig. 3). The same was true at elevated [CO_2], where 37% of tetrapyrrole synthesis genes were correlated with $PC1_{O3}$ compared with 11% of genes with other functions ($P < 0.0001$, one-tailed Fisher's exact test; Fig. 3). Specifically, transcript abundance for genes encoding the three key regulatory enzymes in chlorophyll biosynthesis was positively correlated with $PC1_{O3}$ at both ambient and elevated [CO_2]. These three steps were: (1) the activation of glutamate by glutamyl tRNA synthetase; (2) the conversion of glutamate-1-semialdehyde (GSA) to 5-aminolevulinic acid (ALA) by GSA aminotransferase; and (3) the first step in the chlorophyll branch,

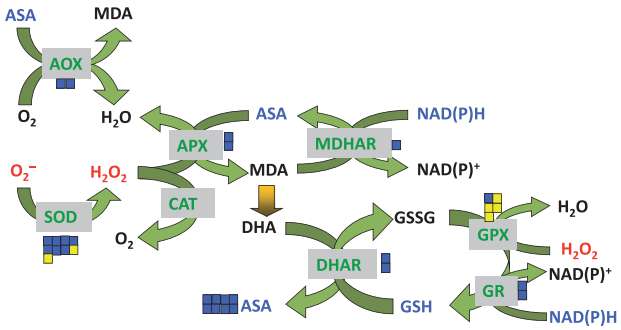

Figure 4. Transcripts coding for antioxidant recycling components that were significantly affected by ozone ($PC1_{O3}$) at ambient [CO_2]. Each box represents a unique transcript encoding an enzyme or protein structure. Blue boxes denote a significant positive correlation between transcript abundance and $PC1_{O3}$; yellow boxes denote a significant negative correlation between transcript abundance and $PC1_{O3}$. Reactive oxygen species are shown in red text, reduced metabolites in blue text and oxidized metabolites in black text. Reduced ascorbic acid (ASA), monodehydroascorbate (MDA), dehydroascorbate (DHA), reduced glutathione (GSH), oxidized glutathione (GSSG), ascorbate oxidase (AOX), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX). Details of the transcriptional response and gene annotations are in Supporting Information Table S1.

which consists of an ATP-dependant insertion of Mg^{2+} into protoporphyrin IX by magnesium chelatase (Fig. 5 and Supporting Information Fig. S5; Tanaka & Tanaka 2007). Chlorophyll content showed no relationship with $PC1_{O3}$ at ambient or elevated [CO_2] (Table 3); however, the coordinated positive correlation of the genes involved in chlorophyll synthesis suggests that chlorophyll turnover may have been accelerated by elevated [O_3].

Respiration gene expression and carbohydrate content

Transcript categories related to respiration had consistently larger percentages of genes correlated with $PC1_{O3}$, including TCA metabolism (33% compared with 14% at ambient CO_2, $P < 0.0001$ and 19% compared with 11% at elevated CO_2, $P = 0.0356$, one-tailed Fisher's exact test; Fig. 3) and mitochondrial electron transport (38% compared with 14% at ambient CO_2, $P < 0.0001$ and 34% compared with 11% at elevated CO_2, $P < 0.0001$, one-tailed Fisher's exact test; Fig. 3). The vast majority of transcripts associated with TCA metabolism and mitochondrial electron transport displayed a positive correlation with $PC1_{O3}$ (Supporting Information Table S1), indicating up regulation at greater [O_3]. However, important exceptions to this general trend were the abundance of transcripts encoding ATP-citrate lyase and the soybean alternative oxidase 2b (AOX2b), which showed negative correlations with $PC1_{O3}$ at ambient [CO_2] (Fig. 6; Supporting Information Table S1). The gene coding for PEPCase, which carboxylates PEP to pyruvate, was positively correlated with $PC1_{O3}$ at both ambient and elevated [CO_2] (Fig. 6; Supporting Information Fig. S6). Glucose, fructose, sucrose, starch, and amino acid content were greater at elevated [CO_2] (Table 2), but only sucrose content was positively correlated with $PC1_{O3}$. Protein content was negatively correlated with $PC1_{O3}$ at both ambient and elevated [CO_2] (Table 3).

Rates of respiratory CO_2 efflux

To assess whether greater expression of respiratory genes was associated with greater respiratory flux, nighttime respiratory CO_2 efflux from mature, sun leaves was assessed on two dates in 2009 and again 2010, when soybean was grown at a range of [O_3] (40–200 ppb) using FACE technology. There was a statistically significant relationship between rate of respiration on an area basis and growth [O_3] on three out of four sampling dates (Fig. 7). In mid-season 2009, this relationship was a positive, linear correlation. Near the end of the growing season in 2009 and 2010 when leaves in the highest [O_3] treatments showed early senescence, the relationship was parabolic with rates of respiration initially rising with greater [O_3], peaking at ~150 ppb and then decreasing. Rates of respiration expressed on a mass basis were also consistently positively related to growth [O_3], with the exception of the 200 ppb treatment at the end of the season in 2010 (Supporting Information Fig. S7).

DISCUSSION

The response of field grown soybean to elevated [O_3] at ambient and elevated [CO_2] was investigated in this study by combining genomic, biochemical and physiological analyses across multiple growing seasons. Intensive sampling and integrating datasets across biological levels allows for the discovery of key mechanisms that drive plant physiological responses to climate change (Leakey *et al.* 2009b; Stitt, Sulpice & Keurentjes 2010). However, each sampling date had a unique set of environmental conditions that influenced the parameters being measured (Fig. 1). This variability in the environment has been shown to alter the genomic (Miyazaki *et al.* 2004) and physiological (Bernacchi *et al.* 2006) responses of plants to climate change treatments. This study parsed the effects of environmental fluctuations from climate change treatments in order to identify the mechanism with which plants respond to elevated [CO_2] and [O_3] under field conditions. Two statistical techniques were used to partition the environmental variation and isolate changes that were dependent on the climate change treatments. PCA collapsed 10 co-varying environmental conditions into two orthogonal PCs that separated the variability in [O_3] from the variability in light, temperature and relative humidity (Table 1). Analysis of co-variance was subsequently used to model the effects of the continuous variables, $PC1_{O3}$ and $PC2_{ENV}$, and determine how those modelled effects changed between ambient and elevated [CO_2] (Littel *et al.* 2006). Using this approach,

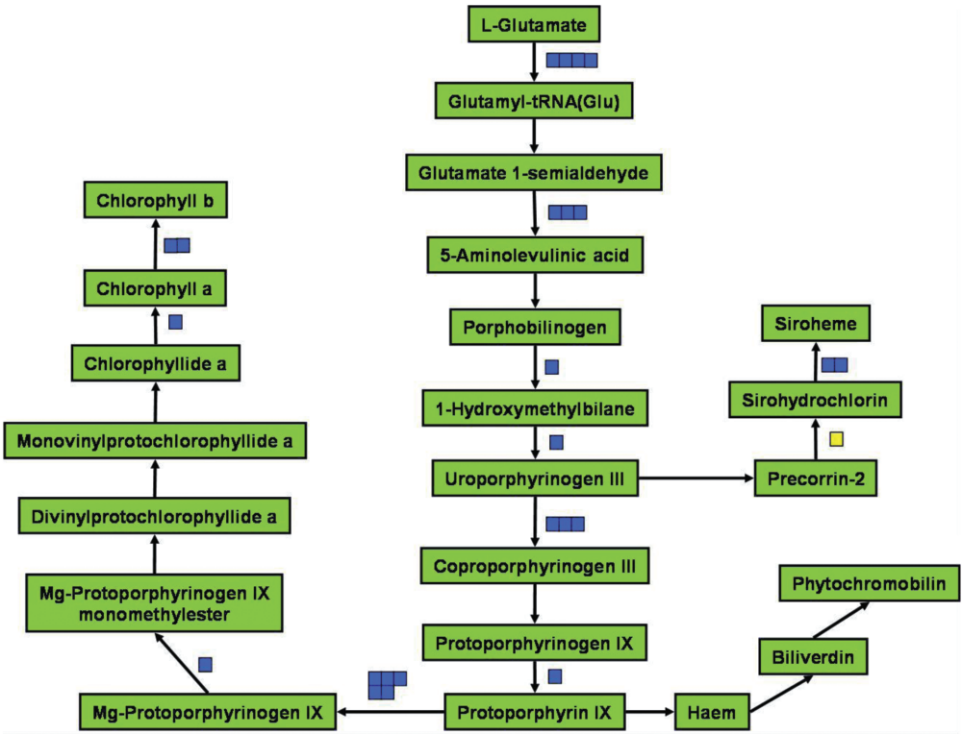

Figure 5. Transcripts coding for components of tetrapyrrole synthesis significantly affected by ozone ($PC1_{O3}$) at ambient [CO_2]. Each box represents a unique transcript encoding an enzyme or protein structure. Blue boxes denote a significant positive correlation between transcript abundance and $PC1_{O3}$. Yellow boxes denote a significant negative correlation between transcript abundance and $PC1_{O3}$. Three key regulatory steps in this pathway are: (1) the activation of glutamate by glutamyl tRNA synthetase; (2) the conversion of glutamate-1-smialdehyde to 5-aminolevulinic acid (ALA) by GSA aminotransferase; and (3) an ATP dependant insertion of Mg^{2+} into protoporphyrin IX by magnesium chelatase (Tanaka & Tanaka 2007). Details of the transcriptional response and gene annotations are in Supporting Information Table S1.

transcriptional reprogramming to increase the capacity of antioxidant metabolism and tetrapyrolle synthesis was demonstrated in soybean grown at elevated [O_3] at both ambient [CO_2] or elevated [CO_2]. The increased demand for energy and carbon skeletons from these protective and repair pathways appears to have been met by transcriptional reprogramming to increase respiratory capacity.

Growth at increasing levels of [O_3], as captured by $PC1_{O3}$, was positively correlated with total antioxidant capacity, ASA content and transcript abundance for a number of genes encoding enzymes in the antioxidant recycling system (Fig. 4; Table 3; Supporting Information Fig. S3 and Table S1). This result was consistent regardless of CO_2 environment and supports previous reports that elevated [O_3] increases antioxidant metabolism (Fiscus *et al.* 2005; Heath 2008). There were no effects of [O_3] on SLW, indicating that treatment effects were not an indirect consequence of changes in leaf structure. However, there were no correlations between $PC1_{O3}$ and the maximum activity levels of any antioxidant enzyme (Table 3), suggesting that enzyme turnover may have been faster in elevated [O_3], and maintenance of activity was supported by greater transcript abundance, which promoted more rapid translation. Greater levels of protein misfolding and fragmentation have been reported previously for plants challenged with elevated [O_3] (Cho *et al.* 2008), which would subsequently lead to increased rates of protein turnover. In this study, 87 transcripts involved in protein degradation were significantly correlated with $PC1_{O3}$ in plants grown in both ambient and elevated [CO_2] (Supporting Information Table S2). Over 75% of those transcripts showed a positive correlation with $PC1_{O3}$, providing further evidence of greater protein turnover at elevated [O_3].

The ability to distinguish the response of each component of the antioxidant system to [O_3] ($PC1_{O3}$) from the response to other environmental signals ($PC2_{ENV}$) identified the components that are largely influenced by [O_3] in a field system. Total antioxidant capacity and ASA correlated with $PC1_{O3}$, whereas the total activities of many antioxidant enzymes correlated with $PC2_{ENV}$ (Table 3). Previous research on a tobacco mutant with impaired respiratory metabolism showed that maintenance of antioxidant metabolites was uncoupled from changes in enzyme activity (Dutilleul *et al.* 2003). Thus, both environmental stress (elevated [O_3]) and genetic mutations (Dutilleul *et al.* 2003) can induce re-orchestration of the cellular antioxidant system. The negative correlation between SOD and GR, and $PC2_{ENV}$ indicated that activity of these enzymes increased with greater maximum photosynthetically active radiation. This is consistent with the strong correlation between light

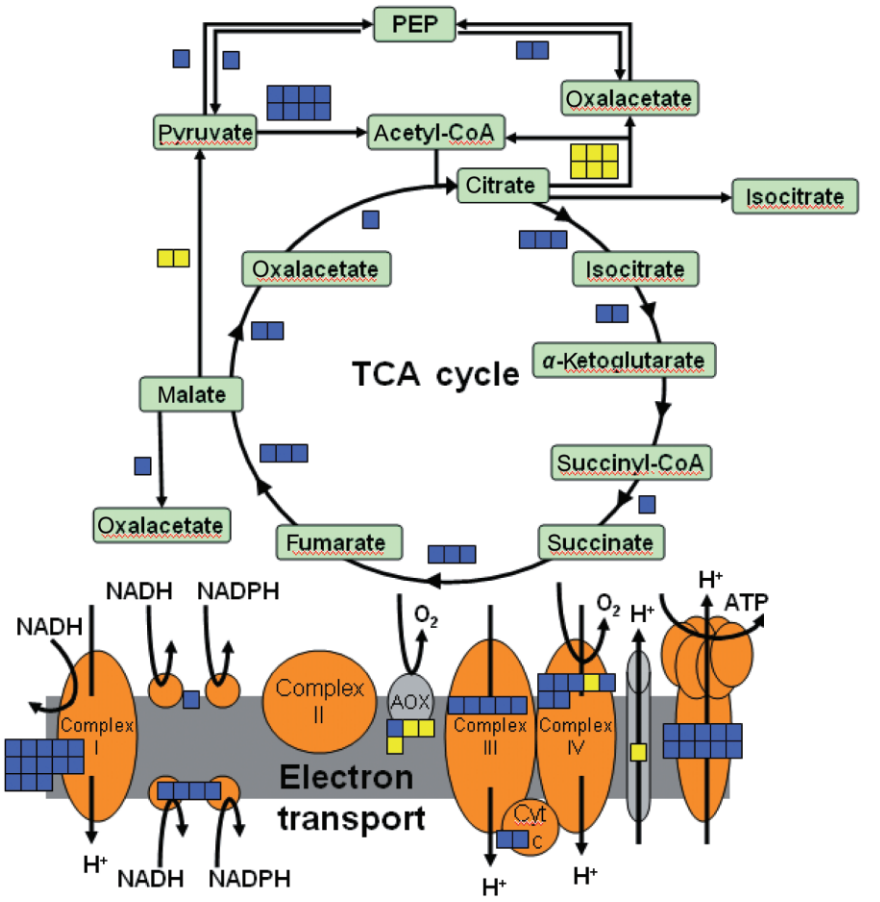

Figure 6. Transcripts coding for components of the TCA cycle and mitochondrial electron transport significantly affected by ozone ($PC1_{O3}$) at ambient [CO_2]. Each box represents a unique transcript encoding an enzyme or protein structure. Blue boxes denote a significant positive correlation between transcript abundance and $PC1_{O3}$. Yellow boxes denote a significant negative correlation between transcript abundance and $PC1_{O3}$. Details of the transcriptional response and gene annotations are in Supporting Information Table S1.

intensity and activity of antioxidant enzymes, namely SOD, which has been reported for a number of plant species (Logan *et al*. 2006). The positive correlation of CAT with $PC2_{ENV}$ suggested that CAT activity was lower in higher light, which is consistent with understanding that CAT is light sensitive (Schmidt, Dehne & Feierabend 2002) and CAT turnover rates increase at higher photon flux densities (Hertwig, Streb & Feierabend 1992). Therefore, breeding efforts aimed at improving soybean O_3 tolerance might focus on cultivars with the ability to maintain stable antioxidant enzyme activity levels while increasing metabolite antioxidant capacity.

In addition to increases in antioxidant metabolism with elevated [O_3], the gene expression results imply a coordinated up-regulation of tetrapyrrole synthesis in both ambient and elevated [CO_2] (Fig. 5; Supporting Information Fig. S5). Although up-regulation of senescence-associated genes and accelerated leaf senescence at elevated [O_3] has been widely reported in the literature (Gupta *et al*. 2005; Heath 2008; Kontunen-Soppela *et al*. 2010), coordinated changes in genes involved in chlorophyll biosynthesis have not been widely reported. However, despite the changes in transcript abundance for chlorophyll biosynthesis genes, there was no increase in chlorophyll content at elevated [O_3]. Therefore, the results suggest a possible increase in chlorophyll turnover in relatively young leaves exposed to elevated [O_3]. Leaf senescence is commonly characterized by chlorophyll degradation (Woo *et al*. 2004; Zimmermann & Zentgraf 2005), and this study did not investigate any products of chlorophyll degradation, nor was there a correlation between [O_3] and the genes coding for enzymes in the chlorophyll degradation pathway. Still, the coordinated up-regulation of transcripts encoding the three major control points of chlorophyll biosynthesis, glutamyl tRNA synthetase, GSA aminotransferase and magnesium chelatase (Tanaka & Tanaka 2007), is compelling and warrants further investigation.

The increase in antioxidant metabolism and chlorophyll synthesis with increasing [O_3] would be energetically expensive and a corresponding increase in rates of dark respiration as well as a coordinated up-regulation of genes involved in mitochondrial respiration was observed (Fig. 6; Supporting Information Fig. S6 and Table S1). This physiological and transcriptional evidence is consistent with previous research measuring greater respiration rates in plants exposed to elevated [O_3] (Skarby, Troeng & Bostrom 1987; Amthor 1988; Volin & Reich 1996; Kellomaki & Wang 1998; Biswas *et al*. 2008). In addition, we measured greater transcript levels for PEPcase at increasing [O_3] in both ambient and elevated [CO_2] (Fig. 6; Supporting Information Fig. S6). Up-regulation of PEPcase activity and content at elevated [O_3] has been suggested as a key metabolic shift for increasing reducing power for antioxidant metabolism (Dizengremel *et al*. 2008). Previous FACE research has demonstrated

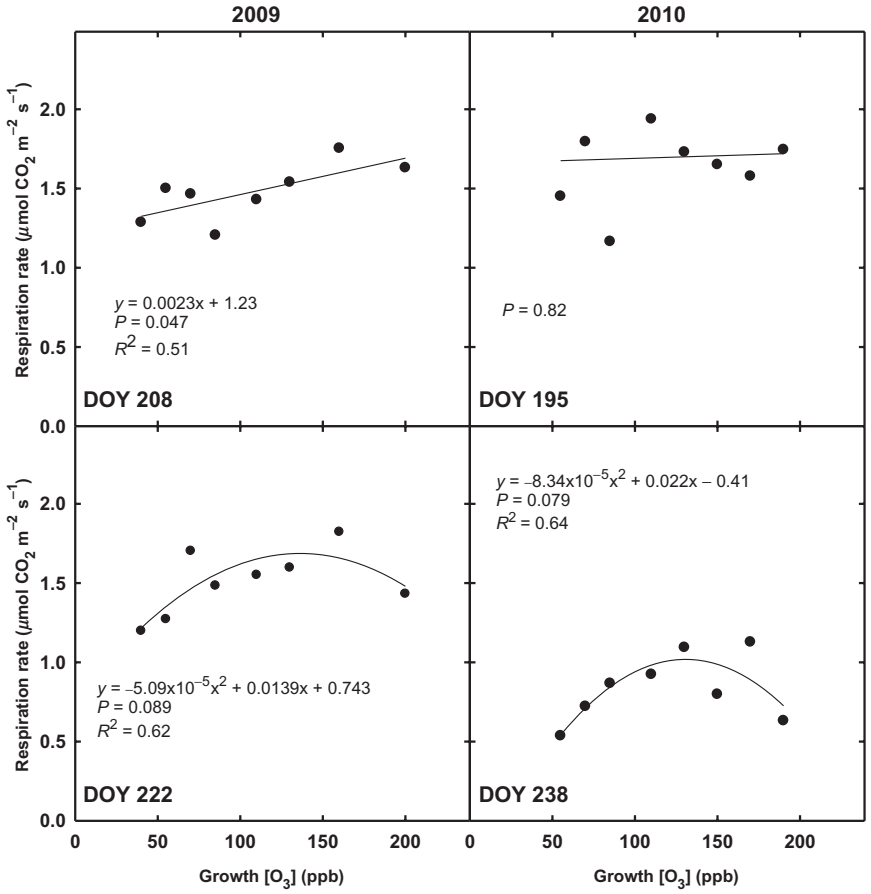

Figure 7. Scatterplot of nighttime rates of respiratory CO_2 efflux per unit leaf area from mature leaves grown at $[O_3]$ ranging from 40 to 200 ppb. Data were collected during the first 4 h of after sunset on day of year (DOY) 208 and 222 in 2009 and DOY 195 and 238 in 2010. The line of best fit and statistical results from regression analysis are shown on each panel.

that growth at elevated $[CO_2]$ increased respiration rates and altered the abundance of transcripts encoding enzymes throughout the respiratory pathway (Leakey *et al*. 2009b). As in Leakey *et al*. (2009b), our results showed that growth at elevated $[CO_2]$ increased soluble carbohydrate and starch pools, which provide substrate for increased respiration. Yet even at elevated $[CO_2]$, respiratory transcripts were further increased with higher $[O_3]$ suggesting that both the greater C availability and the increased demand for antioxidant metabolism were driving greater rates of respiration in the combination of climate change factors. Interestingly, transcripts encoding ATP-citrate lyase and the mitochondrial AOX2b were negatively correlated with elevated $[O_3]$ (PC1; Fig. 6; Supporting Information Fig. S6 and Table S1). The cytosolic ATP-citrate lyase catalyses the formation of acetyl-CoA, which is an intermediate precursor for a number of secondary compounds and phytochemicals (Fatland *et al*. 2002). Down-regulating ATP-citrate lyase content or activity at elevated $[O_3]$ may preferentially shunt carbon skeletons into the TCA cycle and mitochondria for energy production. Within the mitochondria, AOX reduces the ATP-yield of respiration by circumventing the proton-pumping sites of complexes III and IV of the mitochondrial electron transport chain (Plaxton & Podesta 2006). Therefore, down-regulating AOX2b at elevated $[O_3]$ may favor ATP formation needed for antioxidant metabolism and defence. AOX was also one of the most down-regulated transcripts in both *Populus deltoides* and *P. trichocarpa* in response to elevated $[O_3]$ (Street *et al*. 2011). Taken together, elevated $[O_3]$ appears to increase the need for energy for metabolically costly antioxidant and chlorophyll metabolism.

The transcriptional response of antioxidant metabolism to increasing $[O_3]$ was similar in ambient and elevated $[CO_2]$ environments (Fig. 4; Supporting Information Fig. S3). If photoassimilate availability were limiting increases in antioxidant capacity at ambient $[CO_2]$, the increase in abundance of transcripts encoding genes involved in antioxidant metabolism and the resultant changes in antioxidant capacity would be expected to be equal or greater in elevated $[CO_2]$ at a consistent O_3 dose. The overall transcriptional response to elevated $[O_3]$ was dampened by 19% when soybean was grown at elevated $[CO_2]$ compared with ambient $[CO_2]$ (Fig. 8). This is similar in magnitude to the reduced O_3 flux into the leaf expected from the 26% lower g_s observed at elevated $[CO_2]$. If the small discrepancy in the magnitude of these two responses were caused by greater C availability at elevated $[CO_2]$ allowing greater transcriptional reprogramming, it would likely be expressed as greater increases in transcript abundance encoding components of the antioxidant and respiratory pathways and smaller changes in transcript

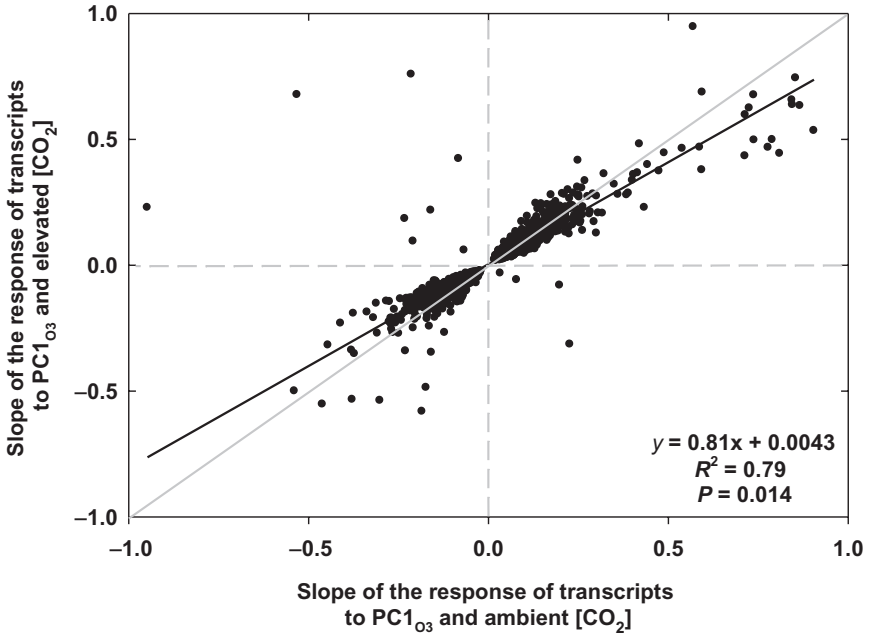

Figure 8. The magnitude of the significant changes in transcripts to $PC1_{O3}$ (slope) between ambient and elevated $[CO_2]$. The solid grey line indicates the 1:1 line and the black line indicates the best fit from linear regression. The slope of the regression line is significantly different from the 1:1 line, tested by a *t*-test ($P = 0.014$).

abundance associated with cellular stress and damage. However, there was no evidence for such pathway-specific responses. Therefore, the dampened response of antioxidant metabolism at elevated $[CO_2]$ may be best explained by lower flux of $[O_3]$ into the leaf because of stomatal closure (Fig. 1; McKee, Farage & Long 1995; Fiscus *et al*. 1997; Karnosky *et al*. 2003; Paoletti & Grulke 2005; Vapaavuori *et al*. 2009)

In addition to characterizing coordinated transcriptional reprogramming of antioxidant, chlorophyll biosynthesis and respiratory pathways at elevated $[O_3]$, this study identified many putative regulators of the metabolic re-orchestration. Over 2400 transcripts were significantly correlated with $PC1_{O3}$ in soybeans grown at both ambient and elevated $[CO_2]$ (Supporting Information Table S2). These include transcripts known to be related to O_3 signalling (Ludwikow & Sadowski 2008), transcriptional regulation and hormone metabolism (Supporting Information Table S2). Specifically, OPR1, which encodes a 12-oxophytodienoic acid reductase, was positively correlated with $PC1_{O3}$. OPR1 is also induced by senenscence, wounding, UV-C, jasmonic acid and salicylic acid (He & Gan 2001; He *et al*. 2002; Blanco *et al*. 2005). NPC4, a non-specific phospholipase involved in lipid remodelling under stress (Nakamura *et al*. 2009; Peters *et al*. 2010), was also positively correlated with $[O_3]$. Geminivirus rep interacting kinase 2 (GRIK2; Shen, Reyes & Hanley-Bowdoin 2009) and calcineurin B-like-interacting protein kinase 15 (CIPK15; Weinl & Kudla 2009) both activate sucrose non-fermenting-1-related kinases (SnRK1), a central regulator of carbon metabolism (Halford & Hey 2009) and were negatively correlated with $PC1_{O3}$. Activation of SnRK1 under conditions of C starvation has been shown to drive transcriptional down-regulation of biosynthetic pathways (Baena-Gonzalez & Sheen 2008). Lower expression of GRIK2 and CIPK15 is therefore consistent with deactivation of SnRK1 causing transcriptional up-regulation of chlorophyll biosynthesis and antioxidant metabolism. Further analysis is required to test if these and the other regulatory genes identified in this study are required to drive the metabolic changes observed in soybean exposed to elevated $[O_3]$. Reverse genetic screens for altered phenotypic response to $[O_3]$ of the putative regulatory elements identified in this study have the potential to advance mechanistic understanding of response. This is currently most feasible in controlled environment experiments on *Arabidopsis* (O'Malley & Ecker 2010). However, investment in larger, gridded FACE experimental facilities would make such experiments possible on major food crops under field conditions (Ainsworth *et al*. 2008b). Such facilities would also expand current capabilities to perform quantitative genetics studies as a complimentary approach to discovery of key genes controlling plant response to elevated $[O_3]$ (Ainsworth *et al*. 2008b; Street *et al*. 2011).

This study used a novel statistical approach to analyse data on field-grown soybean exposed to elevated $[O_3]$, elevated $[CO_2]$, and the combination of both elevated $[CO_2]$ and elevated $[O_3]$. Soybean antioxidant metabolism was up-regulated at high $[O_3]$ and the response was dampened by growth at elevated $[CO_2]$. This research also established that some components of antioxidant metabolism were more sensitive to changes in the light, temperature and relative humidity than to $[O_3]$. Genes involved in tetrapyrrole synthesis increased with higher $[O_3]$, which provided evidence for accelerated chlorophyll turnover in elevated $[O_3]$. Finally, transcriptional profiling and measurements of respiratory CO_2 efflux suggested that greater antioxidant metabolism and chlorophyll metabolism at elevated $[O_3]$ were supported by increased respiratory metabolism.

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REFERENCES

Ainsworth E.A. & Gillespie K.M. (2007) Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nature Protocols* **2,** 875–877.

Ainsworth E.A. & Long S.P. (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *The New Phytologist* **165,** 351–372.

Ainsworth E.A., Rogers A., Vodkin L.O., Walter A. & Schurr U. (2006) The effects of elevated CO_2 concentration on soybean gene expression. An analysis of growing and mature leaves. *Plant Physiology* **142,** 135–147.

Ainsworth E.A., Rogers A., Leakey A.D.B., Heady L.E., Gibon Y., Stitt M. & Schurr U. (2007) Does elevated atmospheric CO_2 alter diurnal C uptake and the balance of C and N metabolites in growing and fully expanded soybean leaves? *Journal of Experimental Botany* **58,** 579–591.

Ainsworth E.A., Rogers A. & Leakey A.D.B. (2008a) Targets for crop biotechnology in a future high-CO_2 and high-O_3 world. *Plant Physiology* **147,** 13–19.

Ainsworth E.A., Beier C., Calfapietra C., *et al*. (2008b) Next generation of elevated [CO_2] experiments with crops: a critical investment for feeding the future world. *Plant, Cell & Environment* **31,** 1317–1324.

Amthor J.S. (1988) Growth and maintenance respiration in leaves of bean (*Phaseolus vulgaris* l.) exposed to ozone in open-top chambers in the field. *The New Phytologist* **110,** 319–325.

Ashmore M.R. (2005) Assessing the future global impacts of ozone on vegetation. *Plant, Cell & Environment* **28,** 949–964.

Baena-Gonzalez E. & Sheen J. (2008) Convergent energy and stress signaling. *Trends in Plant Science* **13,** 474–482.

Bernacchi C.J., Leakey A.D.B., Heady L.E., *et al*. (2006) Hourly and seasonal variation in photosynthesis and stomatal conductance of soybean grown at future CO_2 and ozone concentrations for 3 years under fully open-air field conditions. *Plant, Cell & Environment* **29,** 2077–2090.

Betzelberger A.M., Gillespie K.M., McGrath J.M., Koester R.P., Nelson R.L. & Ainsworth E.A. (2010) Effects of chronic elevated ozone concentration on antioxidant capacity, photosynthesis and seed yield of 10 soybean cultivars. *Plant, Cell & Environment* **33,** 1569–1581.

Biswas D.K., Xu H., Li Y.G., Sun J.Z., Wang X.Z., Han X.G. & Jiang G.M. (2008) Genotypic differences in leaf biochemical, physiological and growth responses to ozone in 20 winter wheat cultivars released over the past 60 years. *Global Change Biology* **14,** 46–59.

Blanco F., Garreton V., Frey N., Dominguez C., Perez-Acle T., Van der Straeten D., Jordana X. & Holuigue L. (2005) Identification of NPR-1 dependent and independent genes early induced by salicylic acid treatment in *Arabidopsis*. *Plant Molecular Biology* **59,** 927–944.

Brosché M., Merilo E., Mayer F., Pechter P., Puzorjova I., Brader G., Kangasjärvi J. & Kollist H. (2010) Natural variation in ozone sensitivity among *Arabidopsis thaliana* accessions and its relation to stomatal conductance. *Plant, Cell & Environment* **33,** 914–925.

Cho K., Shibato J., Agarwal G.K., *et al*. (2008) Integrated transcriptomics, proteomics and metabolomics analyses to survey ozone responses in the leaves of rice seedling. *Journal of Proteome Research* **7,** 2980–2998.

Conklin P.L. & Barth C. (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant, Cell & Environment* **27,** 959–970.

Dizengremel P., Le Thiec D., Bagard M. & Jolivet Y. (2008) Ozone risk assessment for plants: central role of metabolism-dependent changes in reducing power. *Environmental Pollution* **156,** 11–15.

Druart N., Rodriguez-Buey M., Barron-Gafford G., Sjodin A., Bhalerao R. & Hurry V. (2006) Molecular targets of elevated CO_2 in leaves and stems of *Populus deltoides*: implications for future tree growth and carbon sequestration. *Functional Plant Biology* **33,** 121–131.

Dutilleul C., Garmier M., Noctor G., Mathieu C., Chetrit P., Foyer C.H. & de Paepe R. (2003) Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. *The Plant Cell* **15,** 1212–1226.

Fatland B.L., Ke J.S., Anderson M.D., Mentzen W.I., Cui L.W., Allred C.C., Johnston J.L., Nikolau B.J. & Wurtele E.S. (2002) Molecular characterization of a heteromeric ATP-citrate lyase that generates cytosolic acetyl-coenzyme a in *Arabidopsis*. *Plant Physiology* **130,** 740–756.

Fiscus E.L., Reid C.D., Miller J.E. & Heagle A.S. (1997) Elevated CO_2 reduces O_3 flux and O_3-induced yield losses in soybeans: possible implications for elevated CO_2 studies. *Journal of Experimental Botany* **48,** 307–313.

Fiscus E.L., Booker F.L. & Burkey K.O. (2005) Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. *Plant, Cell & Environment* **28,** 997–1011.

Foyer C.H. & Noctor G. (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell* **17,** 1866–1875.

Gillespie K.M. & Ainsworth E.A. (2007) Measurement of reduced, oxidized and total ascorbate content in plants. *Nature Protocols* **2,** 871–874.

Gillespie K.M., Chae J.M. & Ainsworth E.A. (2007) Rapid measurement of total antioxidant capacity in plants. *Nature Protocols* **2,** 867–870.

Gillespie K.M., Rogers A. & Ainsworth E.A. (2011) Long-term exposure to elevated ozone or elevated carbon dioxide alters antioxidant capacity and response to acute oxidative stress. *Journal of Experimental Botany* **62,** 2667–2678. doi:10.1093/jxb/erq435.

Gupta P., Duplessis S., White H., Karnosky D.F., Martin F. & Podila G.K. (2005) Gene expression patterns of trembling aspen trees following long-term exposure to interacting elevated CO_2 and tropospheric O_3. *The New Phytologist* **167,** 129–142.

Halford N.G. & Hey S.J. (2009) SNF1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. *Biochemical Journal* **419,** 247–259.

He Y. & Gan S. (2001) Identical promoter elements are involved in regulation of the OPR1 gene by senescence and jasmonic acid in *Arabidopsis*. *Plant Molecular Biology* **47,** 595–605.

He Y., Fukushige H., Hildebrand D.F. & Gan S. (2002) Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. *Plant Physiology* **128,** 876–884.

Heath R.L. (2008) Modification of the biochemical pathways of plants induced by ozone: what are the varied routes to change? *Environmental Pollution* **155,** 453–463.

Hertwig B., Streb P. & Feierabend J. (1992) Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. *Plant Physiology* **100,** 1547–1553.

Janes K.A. & Yaffe M.B. (2006) Data-driven modelling of signal-transduction networks. *Nature Reviews Molecular Cell Biology* **7,** 820–828.

Kangasjärvi J., Talvinen J., Utriainen M. & Karjalainen R. (1994) Plant defense systems induced by ozone. *Plant, Cell & Environment* **17,** 783–794.

Kangasjärvi J., Jaspers P. & Kollist H. (2005) Signaling and cell death in ozone-exposed plants. *Plant, Cell & Environment* **28,** 1021–1036.

Karnosky D.F., Zak D.R., Pregitzer K.S., *et al*. (2003) Tropospheric O_3 moderates responses of temperate hardwood forests to elevated CO_2: a synthesis of molecular to ecosystem results from the aspen face project. *Functional Ecology* **17,** 289–304.

Kellomaki S. & Wang K.Y. (1998) Growth, respiration and nitrogen content in needles of Scots pine exposed to elevated ozone and carbon dioxide in the field. *Environmental Pollution* **101,** 263–274.

Knight H., Brandt S. & Knight M.R. (1998) A history of stress alters drought calcium signalling pathways in *Arabidopsis*. *The Plant Journal* **16,** 681–687.

Kontunen-Soppela S., Riikonen J., Ruhanen H., *et al.* (2010) Differential gene expression in senescing leaves of two silver birch genotypes in response to elevated CO_2 and tropospheric ozone. *Plant, Cell & Environment* **33,** 1016–1028.

Leakey A.D.B., Uribelarrea M., Ainsworth E.A., Naidu S.L., Rogers A., Ort D.R. & Long S.P. (2006) Photosynthesis, productivity, and yield of maize are not affected by open-air elevation of CO_2 concentration in the absence of drought. *Plant Physiology* **140,** 779–790.

Leakey A.D.B., Ainsworth E.A., Bernard S.M., *et al*. (2009a) Gene expression profiling: opening the black box of plant ecosystem responses to global change. *Global Change Biology* **15,** 1201–1213.

Leakey A.D.B., Xu F.X., Gillespie K.M., McGrath J.M., Ainsworth E.A. & Ort D.R. (2009b) Genomic basis for stimulated respiration by plants growing under elevated carbon dioxide. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 3597–3602.

Littel R.C., Milliken G.A., Stroup W.W., Wolfinger R.D. & Schabenberger O. (2006) *SAS for Mixed Models* 2nd edn, SAS Institute Inc., Cary, NC, USA.

Logan B.A., Kornyeyev D., Hardison J. & Holaday A.S. (2006) The role of antioxidant enzymes in photoprotection. *Photosynthesis Research* **88,** 119–132.

Ludwikow A. & Sadowski J. (2008) Gene networks in plant ozone stress response and tolerance. *Journal of Integrative Plant Biology* **50,** 1256–1267.

Mauzerall D.L. & Wang X.P. (2001) Protecting agricultural crops from the effects of tropospheric ozone exposure: reconciling science and standard setting in the United States, Europe, and Asia. *Annual Review of Energy and the Environment* **26,** 237–268.

McKee I.F., Farage P.K. & Long S.P. (1995) The interactive effects of elevated CO_2 and O_3 concentration on photosynthesis in spring wheat. *Photosynthesis Research* **45,** 111–119.

McKee I.F., Eiblmeier M. & Polle A. (1997) Enhanced ozone-tolerance in wheat grown at an elevated CO_2 concentration: ozone exclusion and detoxification. *The New Phytologist* **137,** 275–284.

McKee I.F., Mulholland B.J., Craigon J., Black C.R. & Long S.P. (2000) Elevated concentrations of atmospheric CO_2 protect against and compensate for O_3 damage to photosynthetic tissues of field-grown wheat. *The New Phytologist* **146,** 427–435.

Meehl G.A., Stocker T.F., Collins W.D., *et al*. (2007) Global climate projections. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (eds S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor & H.L. Miller), pp. 747–845. Cambridge University Press, New York, NY, USA.

Mittler R. & Blumwald E. (2010) Genetic engineering for modern agriculture: challenges and perspectives. *Annual Review of Plant Biology* **61,** 443–462.

Miyazaki S., Fredricksen M., Hollis K.C., Poroyko V., Shepley D., Galbraith D.W., Long S.P. & Bohnert H.J. (2004) Transcript expression profiles of *Arabidopsis thaliana* grown under controlled conditions and open-air elevated concentrations of CO_2 and of O_3. *Field Crops Research* **90,** 47–59.

Morgan P.B., Ainsworth E.A. & Long S.P. (2003) How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. *Plant, Cell & Environment* **26,** 1317–1328.

Morgan P.B., Bernacchi C.J., Ort D.R. & Long S.P. (2004) An *in vivo* analysis of the effect of season-long open-air elevation of ozone to anticipated 2050 levels on photosynthesis in soybean. *Plant Physiology* **135,** 2348–2357.

Nakamura Y., Koizumi R., Shui G., Shimojima M., Wenk M.R., Ito T. & Ohta H. (2009) *Arabidopsis* lipins mediate eukaryotic pathway of lipid metabolism and cope critically with phosphate starvation. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 20978–20983.

O'Malley R.C. & Ecker J.R. (2010) Linking genotype to phenotype using the *Arabidopsis* unimutant collection. *The Plant Journal* **61,** 928–940.

Overmyer K., Tuominen H., Kettunen R., Betz C., Langebartels C., Sandermann H. & Kangasjärvi J. (2000) Ozone-sensitive *Arabidopsis* rcd1 mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. *The Plant Cell* **12,** 1849–1862.

Overmyer K., Brosché M. & Kangasjärvi J. (2003) Reactive oxygen species and hormonal control of cell death. *Trends in Plant Science* **8,** 335–342.

Overmyer K., Kollist H., Tuominen H., Betz C., Langebartels C., Wingsle G., Kangasjärvi S., Brader G., Mullineaux P. & Kangasjärvi J. (2008) Complex phenotypic profiles leading to ozone sensitivity in *Arabidopsis thaliana* mutants. *Plant, Cell & Environment* **31,** 1237–1249.

Paoletti E. & Grulke N.E. (2005) Does living in elevated CO_2 ameliorate tree response to ozone? A review on stomatal responses. *Environmental Pollution* **137,** 483–493.

Pasquer F., Isidore E., Zarn J. & Keller B. (2005) Specific patterns of changes in wheat gene expression after treatment with three antifungal compounds. *Plant Molecular Biology* **57,** 693–707.

Pell E.J., Schlaghaufer C.D. & Arteca R.N. (1997) Ozone-induced oxidative stress: mechanisms of action and reaction. *Physiologia Plantarum* **100,** 264–273.

Peters C., Li M., Narasimhan R., Roth M., Welti R. & Wang X. (2010) Nonspecific phospholipase C NPC4 promotes responses to abscisic acid and tolerance to hyperosmotic stress in *Arabidopsis*. *The Plant Cell* **22,** 2642–2659.

Plaxton W.C. & Podesta F.E. (2006) The functional organization and control of plant respiration. *Critical Reviews in Plant Sciences* **25,** 159–198.

Prentice I.C., Farquhar G.D., Fasham M.J.R., Goulden M.L., Heimann M., Jaramillo V.J., Kheshgi H.S., Le Quere C., Scholes R.J. & Wallace D.W.R. (2001) The carbon cycle and atmospheric carbon dioxide. In *Climate Change 2001: The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change* (eds J.T. Houghton, Y. Ding, D.J. Griggs, M. Noguer, P.J. van der Linden & D. Xiaosu), pp. 183–239. Cambridge University Press, New York, NY, USA.

Rao M.V., Hale B.A. & Ormrod D.P. (1995) Amelioration of ozone-induced oxidative damage in wheat plants grown under high-carbon dioxide – role of antioxidant enzymes. *Plant Physiology* **109,** 421–432.

Rao M.V., Lee H., Creelman R.A., Mullet J.E. & Davis K.R. (2000) Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. *The Plant Cell* **12,** 1633–1646.

Rogers A., Allen D.J., Davey P.B., *et al*. (2004) Leaf photosynthesis and carbohydrate dynamics of soybeans grown throughout their life-cycle under free-air carbon dioxide enrichment. *Plant, Cell & Environment* **27,** 449–458.

Royal Society (2008) Ground-level ozone in the 21st century: future trends, impacts and policy implications. Science Policy Report 15/08.

Sandermann H., Ernst D., Heller W. & Langebartels C. (1998) Ozone: an abiotic elicitor of plant defence reactions. *Trends in Plant Science* **3,** 47–50.

Schmidt M., Dehne S. & Feierabend J. (2002) Post-transcriptional mechanisms control catalase synthesis during its light-induced turnover in rye leaves through the availability of the hemin cofactor and reversible changes of the translation efficiency of mRNA. *The Plant Journal* **31,** 601–613.

Sharma Y.K., Leon J., Raskin I. & Davis K.R. (1996) Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 5099–5104.

Shen W., Reyes M.I. & Hanley-Bowdoin L. (2009) *Arabidopsis* protein kinases GRIK1 and GRIK2 specifically activate SnRK1 by phosphorylating its activation loop. *Plant Physiology* **150,** 996–1005.

Skarby L., Troeng E. & Bostrom C.A. (1987) Ozone uptake and effects on transpiration, net photosynthesis, and dark respiration in Scots pine. *Forest Science* **33,** 801–808.

Stitt M., Sulpice R. & Keurentjes J. (2010) Metabolic networks: how to identify key components in the regulation of metabolism and growth. *Plant Physiology* **152,** 428–444.

Street N.R., James T.M., James T., Brosche M., Kangasjärvi J., Broadmeadow M.B. & Taylor G. (2011) The physiological, transcriptional and genetic responses of an ozone-sensitive and an ozone-tolerant poplar and selected extremes of their F2 progeny. *Environmental Pollution* **159,** 45–54.

Tallis M.J., Lin Y., Rogers A., Zhang J., Street N.R., Miglietta F., Karnosky D.F., De Angelis P., Calfapietra C. & Taylor G. (2010) The transcriptome of populus in elevated CO_2 reveals increased anthocyanin biosynthesis during delayed autumnal senescence. *The New Phytologist* **186,** 415–428.

Tanaka R. & Tanaka A. (2007) Tetrapyrrole biosynthesis in higher plants. *Annual Review of Plant Biology* **58,** 321–346.

Taylor G., Street N.R., Tricker P.J., Sjodin A., Graham L., Skogstrom O., Calfapietra C., Scarascia-Mugnozza G. & Jansson S. (2005) The transcriptome of populus in elevated CO_2. *The New Phytologist* **167,** 143–154.

Travers S.E., Tang Z.W., Caragea D., *et al*. (2010) Variation in gene expression of *Andropogon gerardii* in response to altered environmental conditions associated with climate change. *Journal of Ecology* **98,** 374–383.

Vapaavuori E., Holopainen J.K., Holopainen T., *et al*. (2009) Rising atmospheric CO_2 concentration partially masks the negative effects of elevated O_3 in silver birch (*Betula pendula* roth). *Ambio* **38,** 418–424.

Volin J.C. & Reich P.B. (1996) Interaction of elevated CO_2 and O_3 on growth, photosynthesis and respiration of three perennial species grown in low and high nitrogen. *Physiologia Plantarum* **97,** 674–684.

Weinl S. & Kudla J. (2009) The CBL-CIPK Ca^{2+}-decoding signaling network: function and perspectives. *The New Phytologist* **184,** 517–524.

Woo H.R., Kim J.H., Nam H.G. & Lim P.O. (2004) The delayed leaf senescence mutants of *Arabidopsis, ore1, ore3*, and *ore9* are tolerant to oxidative stress. *Plant & Cell Physiology* **45,** 923–932.

Wustman B.A., Oksanen E., Karnosky D.F., Noormets A., Isebrands J.G., Pregitzer K.S., Hendrey G.R., Sober J. & Podila G.K. (2001) Effects of elevated CO_2 and O_3 on aspen clones varying in O_3 sensitivity: can CO_2 ameliorate the harmful effects of O_3? *Environmental Pollution* **115,** 473–481.

Zimmermann P. & Zentgraf U. (2005) The correlation between oxidative stress and leaf senescence during plant development. *Cellular & Molecular Biology Letters* **10,** 515–534.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pearson correlations between pairs of environmental variables.

Figure S2. Linear correlation between total antioxidant capacity and ozone (PC1O3).

Figure S3. Transcripts coding for antioxidant recycling components that were significantly affected by $PC1_{O3}$ at elevated [CO_2].

Figure S4. Percentage of transcripts in different functional bins that displayed significant differences in abundance due to environment ($PC2_{ENV}$).

Figure S5. Transcripts coding for components of tetrapyrrole synthesis significantly affected by $PC1_{O3}$ at elevated [CO_2].

Figure S6. Transcripts coding for components of the TCA cycle and mitochondrial electron transport significantly affected by $PC1_{O3}$ at elevated [CO_2].

Figure S7. Scatterplot of nighttime rates of respiratory CO_2 efflux per unit leaf mass from mature leaves grown at [O_3] ranging from 40 to 200 ppb. Data were collected during the first 4-h of after sunset on day of year (DOY) 208 and 222 in 2009 and DOY 195 and 238 in 2010. The line of best fit and statistical results from regression analysis are shown on each panel.

Table S1. List of genes involved in antioxidant metabolism, respiration and tetrapyrrole synthesis that showed significant correlations with $PC1_{O3}$.

Table S2. List of genes that were significantly correlated with $PC1_{O3}$ in both ambient and elevated [CO_2].

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