A comparative analysis of transcriptomic, biochemical, and physiological responses to elevated ozone identifies species-specific mechanisms of resilience in legume crops

Craig R. Yendrek¹, Robert P. Koester² and Elizabeth A. Ainsworth¹,²,³

¹ Institute for Genomic Biology, University of Illinois, Urbana, IL 61802, USA
² Department of Plant Biology, University of Illinois, Urbana, IL 61801, USA
³ Global Change and Photosynthesis Research Unit, USDA ARS, Urbana, IL 61801, USA

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

Received 7 April 2015; Revised 17 July 2015; Accepted 4 August 2015

Editor: Nick Smirnoff

Abstract

Current concentrations of tropospheric ozone ([O₃]) pollution negatively impact plant metabolism, which can result in decreased crop yields. Interspecific variation in the physiological response of plants to elevated [O₃] exists; however, the underlying cellular responses explaining species-specific differences are largely unknown. Here, a physiological screen has been performed on multiple varieties of legume species. Three varieties of garden pea (Pisum sativum L.) were resilient to elevated [O₃]. Garden pea showed no change in photosynthetic capacity or leaf longevity when exposed to elevated [O₃], in contrast to varieties of soybean (Glycine max (L.) Merr.) and common bean (Phaseolus vulgaris L.). Global transcriptomic and targeted biochemical analyses were then done to examine the mechanistic differences in legume responses to elevated [O₃]. In all three species, there was an O₃-mediated reduction in specific leaf weight and total non-structural carbohydrate content, as well as increased abundance of respiration-related transcripts. Differences specific to garden pea included a pronounced increase in the abundance of GLUTATHIONE REDUCTASE transcript, as well as greater contents of foliar glutathione, apoplastic ascorbate, and sucrose in elevated [O₃]. These results suggest that garden pea may have had greater capacity for detoxification, which prevented net losses in CO₂ fixation in an elevated [O₃] environment.

Key words: Ascorbate–glutathione cycle, air pollution, Glycine max, Phaseolus vulgaris, photosynthesis, Pisum sativum, RNA-Seq.

Introduction

One of the most harmful air pollutants impacting plant growth today is tropospheric ozone (O₃; Krupa et al., 2001). Over the past 100 years, ground-level O₃ concentrations ([O₃]) have increased four-fold (Seinfeld and Pandis, 2012) and may continue to increase in the absence of precursor gas emission reductions (Jacobson and Streets, 2009). Upon entry of O₃ into leaves, formation of reactive oxygen species (ROS) occurs in the apoplast, triggering enhanced antioxidant metabolism (Sharma and Davis, 1997; Mittler, 2002), which is hypothesized to be driven by an increase in mitochondrial respiration (Amthor 1988; Ainsworth et al., 2012). Exposure to [O₃] that exceed the capabilities of the cellular detoxification
system leads to damage of membranes and proteins, including components of the photosynthetic machinery, resulting in decreased CO₂ assimilation (Ashmore, 2005; Fuscus et al., 2005). Worldwide, the metabolic changes induced by current [O₂] are responsible for reductions in crop productivity estimated to cost US$11–26 billion annually (Van Dingenen et al., 2009; Avnery et al., 2011).

Characterizing the underlying molecular changes that determine the metabolic responses to elevated [O₂] has been an active area of research for decades, especially related to ROS scavenging (Sandermann et al. 1998; Schraudner et al., 1998; Rao et al., 2000; Wohlegemuth et al., 2002). The abundance and redox states of the antioxidants ascorbate and glutathione are important for detoxification of O₂-induced ROS (Foyer and Noctor, 2005), and act to prevent cellular damage and maintain normal metabolic activity. In addition, proper regulation of the suite of enzymes comprising the ascorbate–glutathione cycle is necessary for recycling oxidized ascorbate and glutathione back to the reduced form (Noctor and Foyer, 1998). Global gene expression and proteomics studies have identified antioxidant transcripts and proteins with increased content in response to elevated [O₂] (Agrawal et al., 2002; Baier et al., 2005). Transgenic approaches have also been used to alter the abundance and/or redox state of the ascorbate and/or glutathione pools in order to better understand the mechanisms of cellular O₃ response. These studies have highlighted the complexity of O₂-induced oxidative signaling, which depends on the coordinated expression of specific ascorbate–glutathione cycle isoenzymes in the correct subcellular compartments to maintain redox homeostasis (Noctor and Foyer, 1998; Foyer and Noctor, 2005). For example, tobacco plants overexpressing a manganese SUPEROXIDE DISMUTASE (SOD) gene in the chloroplast showed reduced injury in response to elevated [O₂] (Van Camp et al., 1994), while no protection was afforded by overexpression of a chloroplastic copper or zinc SOD (Pitcher et al., 1991). Complicating the comparison between these two studies are different O₃ exposures (concentration and duration) used to screen the transgenic plants.

Soybean (Glycine max (L.) Merr.) is the world’s most widely grown leguminous crop, and is classified as being highly responsive to [O₂] (Mills et al., 2007). Soybean seed yield is significantly reduced by [O₂] exceeding 40 nL L⁻¹ (Heagle, 1989), and decreased photosynthesis is thought to be one of the major determinants of yield loss (Betzelberger et al., 2012). In addition, antioxidant metabolism and mitochondrial respiration are increased in soybean exposed to elevated [O₂] (Gillespie et al., 2012). While some studies have reported intraspecific variation for the response of soybean to elevated [O₂] (Lee et al., 1984; Burkey and Carter, 2009; Betzelberger et al., 2010), others have found no difference between varieties and that a single response function can accurately describe soybean O₃ responses (Mills et al., 2007; Betzelberger et al., 2012). If most soybean varieties are negatively impacted by elevated [O₂], then a wider examination of species that fail to display typical negative responses to elevated [O₂] might reveal novel cellular strategies that could be used in soybean improvement efforts.

The goal of this study was to characterize cellular changes in leaves of diverse legume species demonstrating a broad range of O₃-induced physiological and growth responses. Previous physiological comparisons have revealed that photosynthesis is not universally affected by growth at elevated [O₂] in some legumes. For example, several photosynthetic parameters in garden pea (Pisum sativum L.) were not significantly reduced by growth in chronic [O₂] of 80 nL L⁻¹ (Farage and Long, 1999). On the other hand, common bean (Phaseolus vulgaris L.) varieties have been developed for use as O₂ bioindicators, and moderate increases in [O₂] (to 50 nL L⁻¹) can decrease yields by up to 50% in some varieties (Burkey et al., 2005). Using a comparative approach, several varieties of soybean, garden pea, and common bean were first screened to identify genotypes exhibiting a broad range of photosynthetic responses to elevated [O₂]. RNA sequencing (RNA-Seq) was then used to compare the global transcriptomic response with changes in antioxidant metabolite content of the selected legumes, and cellular responses associated with the O₂-induced photosynthetic response were identified.

Materials and methods

Plant growth conditions

Seeds of commercially available garden varieties of garden pea (Pisum sativum L.), soybean (Glycine max (L.) Merr.), and common bean (Phaseolus vulgaris L.) were planted in 6L pots (21.6cm tall; 22.9cm diameter) containing sterile soilless media (LC1 Sunshine mix; Sun Gro Horticulture Distribution Inc., Bellevue, WA, USA). All varieties were short-season varieties, with maturity dates ranging from 56 to 62 days after planting for pea, 50 to 53 days for common bean, and 80 to 82 days for soybean. Three pots of each species were placed in each of six growth chambers (Environmental Growth Chamber, Chagrin Falls, OH, USA) set to maintain constant conditions for light (900 µmol m⁻² s⁻¹; 16h d⁻¹), temperature (21°C day; 21°C night), and relative humidity (60%). Plants were watered as needed and fertilized once per week with water-soluble plant food (Miracle-Gro, Scotts Company LLC, Marysville, OH, USA). Three chambers were fumigated with an average [O₂] of 151.2 nL L⁻¹ ± 0.72 nL L⁻¹ for 8h d⁻¹, starting 4h after the start of the light period, throughout the duration of the experiment. O₃ was generated and controlled as described in Yendrek et al. (2013). The other three chambers were maintained at ambient levels of O₂, with an average [O₂] of 12.5 nL L⁻¹ ± 0.96 nL L⁻¹. To determine leaf longevity, the date (approximately 9 d after planting; DAP) at which the third leaf of pea and the first trifolate of soybean and common bean had elongated >0.5cm was subtracted from the date at which leaf abscission was observed. At the conclusion of the experiment (45 DAP), plants were destructively harvested and leaf area was measured with a LI-3100C area meter (LI-COR, Lincoln, NE, USA). Leaves were then dried at 60°C for 48h and weighed.

Gas exchange measurements

For the photosynthesis screen, in situ net assimilation (A) and stomatal conductance (gₛ) were measured on the youngest fully expanded leaf (pea, seventh leaf; soybean, fourth trifoliate; common bean, third trifoliate) of all plants in every chamber 30 DAP at midday, approximately 8h after the lights were turned on in the chambers (4h after the start of O₃ fumigation). For this, an infrared gas analyzer (LI-6400, LI-COR) was set to match the ambient growth conditions, including temperature (block temperature, 24°C), relative humidity (60%), light (900 µmol m⁻² s⁻¹), and [CO₂] (400 µL L⁻¹). In another experiment with varieties selected from the screen (Fig. 1), one plant per species
Quantification of primary metabolites and ROS scavenging molecules

At 34 DAP, one leaflet was collected from the same cohort of leaves used to measure in situ gas exchange, frozen in liquid N, and ground to a fine powder. Tissue from three individual plants per chamber was pooled for each species and approximately 50 mg was used to quantify total non-structural carbohydrate (TNC) content, including glucose, fructose, sucrose, and starch as described in Yendrek et al. (2013). Another 50 mg aliquot of tissue was used to determine total foliar phenolic content. Briefly, phenolic compounds were extracted in 95% methanol at room temperature for 48 h. The leaf extract was then incubated with 10% (v/v) Folin–Ciocalteu solution and 700 mM Na2CO3 at room temperature for 2 h. To calculate total phenolic content, the absorbance of each sample was measured at 765 nm and values were compared to a curve of gallic acid standards (Ainsworth and Gillespie, 2007). To quantify glutathione content, approximately 10 mg of ground leaf tissue was mixed with 1× phosphate buffered saline with 2 mM EDTA (pH 8.0). Total and oxidized glutathione content was assayed using a GSH/GSSG-Glo Assay kit following the manufacturer’s protocol (Promega Corporation, Madison, WI, USA). Quantification of total and reduced ascorbate was determined following the methods of Gillespie and Ainsworth (2007) using approximately 30 mg of ground leaf tissue or 50 μL of apoplastic fluid. Apoplastic fluid was collected from leaves cut in half along the middle vein that was vacuum-infiltrated with 2% metaphosphoric acid with 2 mM EDTA, placed in a conical tube, and centrifuged at 2700 g for 10 min at 4°C (Eller and Sparks, 2006). Apoplastic fluid was immediately frozen in liquid N and stored at −80°C until assayed.

Fig. 1. The response (± SD; n = 3) of net assimilation (A) and stomatal conductance (gs) to elevated [O3] in varieties of garden pea, soybean, and common bean. In situ gas exchange measurements were performed at midday on the youngest, fully expanded leaf after growth in elevated [O3] (150 ppb; 8 h d−1) for one month. Arrows indicate varieties used for additional comparative studies.

Statistical analysis of whole plant, physiological, and biochemical results

An initial analysis using PROC UNIVARIATE (SAS, Version 9.2, Cary, NC, USA) was performed to establish that data were normally distributed. A two-way ANOVA using PROC GLM (SAS) was then done to assess the impact of elevated [O3] on the three legume species. Pairwise comparisons of the least squares means were performed to identify significant differences.

Determination of differential gene expression

Before aligning to the reference transcriptomes, duplicate reads (identical sequences as well as those that mapped to more than one transcriptome location) were removed and the remaining reads were filtered with the FASTQ Quality Filter (FASTX-Toolkit; Gordon and Hannon, unpublished) to keep reads that had a minimum quality score of 20 across at least 90% of the read length. Reads were aligned using the -b2-sensitive option for Tophat2 / Bowtie2 (Langmead and Salzberg, 2012). The reference transcriptome sequence file for pea (Pisum sativum unigene v2) was obtained from the Cool Season Food Legume Genome Database (https://www.coolseasonfoodlegume.org). For soybean (Gmax_189_transcript.fa; Schmutz et al., 2010) and common bean (Pvulgaris_218_transcript.fa; Schmutz et al., 2014), the reference transcriptome files were obtained from Phytozone (http://www.phytozone.net). After sorting and converting the BAM alignment output file with SAMtools (Li et al., 2009), the Python package HTSeq (www-huber.embl.de/users/anders/HTSeq/doc/overview.html) was used to generate read counts for each gene using the intersection-nonempty mode. These counts were then analyzed by the R package edgeR, using the TMM normalization, to generate a list of differentially expressed genes (Robinson et al., 2010). Summary statistics for the bioinformatics analysis are presented in Supplementary Table S1.

Results

Species-specific growth and physiological responses to elevated [O3]

A physiological screen of several garden varieties of pea, soybean, and common bean showed no major reductions in
A and g, in any of the garden pea varieties (Fig. 1). In contrast, each of the soybean and common bean varieties had lower A and g, (Fig. 1). ‘Early Bush Italian’ and ‘Heavyweight II’, both varieties of common bean, were most responsive to growth in elevated [O₃], with reductions in A and g, of ~60% (Fig. 1).

In order to more thoroughly compare leaf responses to elevated [O₃], one representative variety of each legume species, including ‘Sugar Bon’ (garden pea), ‘Be Sweet 292’ (soybean), and ‘Heavyweight II’ (common bean), was investigated further. Garden pea displayed no visual signs of O₃ damage in contrast to soybean and common bean, which both had signs of chlorosis (Fig. 2A). More extensive O₃ damage was observed in common bean, including leaf bronzing and necrosis. No significant change in leaf longevity was detected in garden pea grown in elevated [O₃], while a 12–15 day decrease in soybean and common bean was seen (Fig. 2B). Soybean and common bean also had O₃-induced decreases in photosynthetic capacity, with reductions in V_c,max and J_max exceeding 60% in common bean (Fig. 3). In garden pea, however, estimates of V_c,max and J_max were not decreased by elevated [O₃] (Fig. 3).

Global gene expression changes in legumes exposed to elevated [O₃]

A transcriptome-wide comparison of differentially expressed genes revealed that each legume species had a distinct response to elevated [O₃]. When considering differentially expressed genes with a log₂ fold change >±2.0, garden pea had a greater number of genes (63% of total differentially expressed genes) showing an increase in transcript abundance. In contrast, soybean had a greater number of genes

![Fig. 2](https://academic.oup.com/jxb/article-abstract/66/22/7101/2893272)

![Fig. 3](https://academic.oup.com/jxb/article-abstract/66/22/7101/2893272)
(57% of total differentially expressed genes) with decreased transcript abundance while common bean had a similar number of increased and decreased genes (Supplementary Fig. S2). There was also a difference in the genes expressed in elevated [O₃] that had no detectable transcripts in ambient O₃ conditions. For each species, these genes turned ‘on’ by elevated [O₃] included peroxidases and receptor-like kinases (Supplementary Table S2). Additionally, the expression of numerous stress-related genes and transcription factors was induced by elevated [O₃] in garden pea (Supplementary Table S2).

The distribution of differentially expressed genes across major functional categories revealed species differences in the number of genes related to protein processes (Supplementary Fig. S3A). Because the total number of genes comprising a functional category was different for each species, the percentage of genes that were differentially expressed per functional category is reported. Compared to soybean and common bean, garden pea had a greater percentage of differentially expressed genes involved with protein synthesis, post-translational modification, and protein degradation (Supplementary Fig. S3B). The majority of these genes in garden pea increased in abundance, suggesting enhanced functionality for these processes.

**Elevated [O₃] alters antioxidant transcripts and metabolites**

Markers at key branch points in phenylpropanoid metabolism, including PHENYLAMINOLINE AMMONIA LYASE, CHALCONE SYNTHASE, ISOFLAVONE REDUCTASE, and DIHYDROFLAVONOL 4-REDUCTASE, showed increased transcript abundance in all three legume species, but the log₂ fold changes were greatest in garden pea (Fig. 4A). These transcriptional changes were indicative of a general up-regulation of the entire phenylpropanoid pathway and consistent with the trend of increased total foliar phenolic content (Fig. 4B). Only common bean failed to show a statistically significant increase in phenolic content, which may have been due to the small increase in PAL transcript abundance (Fig. 4A) at the entry point into the pathway and/or the large variation in phenolic content measured at elevated [O₃] (Fig. 4B).

The abundance of transcripts encoding proteins involved in ascorbate–glutathione cycling was also investigated. Multiple genes encoding ASCORBATE OXIDASE (AO), which is localized to the cell wall, were identified in each species. At least one transcript in each species increased in plants grown at elevated [O₃] (Table 1), with pea showing a 5.45-fold increase in expression (Table 1). However, all three species also had AO transcripts that either decreased or did not change in response to elevated [O₃] (Table 1). Transcripts encoding two genes, GLUTATHIONE PEROXIDASE 6 (GPX6) and SOD2 (Fe, chloroplast), were significantly increased in soybean and common bean in response to elevated [O₃], but not in garden pea (Table 1). No evidence of transcript abundance changes [log₂ fold change > 1.0; reads per kilobase of transcript per million reads mapped (RPKM) > 5.0] for several ascorbate–glutathione cycle genes, including ASCORBATE PEROXIDASE, DEHYDROASCORBATE REDUCTASE, MONODEHYDROASCORBATE REDUCTASE, and CATALASE, were observed in any species (Supplementary Table S3). The only ascorbate–glutathione cycle gene that had increased transcript abundance exclusively in garden pea was a cytoplasm-localized GLUTATHIONE-DISULFIDE REDUCTASE (GR; Table 1).
Table 1. Transcript abundance changes of selected ascorbate–glutathione cycle genes demonstrating conserved and distinct species responses to elevated \([\text{O}_3]\).

| Species | Transcript ID | \(\log_2\) fold change | Ambient RPKM | Elevated RPKM |
|---------|---------------|-------------------------|--------------|---------------|
| ASCORBATE OXIDASE [cell wall] | | | | |
| Soy | Glyma05g33470.1 | 2.93 | 3.37 | 25.93 |
| Pea | ID296830_p.sativum_wa1_contig18841 | 5.45 | 1.99 | 84.39 |
| Soy | Glyma13g03650.1 | -0.31 (ns) | 18.32 | 15.14 |
| Soy | Glyma20g12150.1 | -0.99 | 21.01 | 10.70 |
| Bean | Phvul.006G011600.1 | 1.11 | 22.53 | 46.41 |
| Bean | Phvul.006G011700.1 | -1.92 | 19.68 | 4.94 |
| GLUTATHIONE REDUCTASE [cytoplasm] | | | | |
| Soy | Glyma16g27210.1 | -0.11 (ns) | 1.08 | 1.00 |
| GLUTATHIONE PEROXIDASE 6 | | | | |
| Soy | Glyma01g42840.1 | 0.84 | 71.66 | 131.13 |
| Soy | Glyma05g37900.1 | 0.35 (ns) | 13.37 | 17.33 |
| Pea | ID_Pisum_sativum_v2_Contig4699 | -0.12 (ns) | 145.03 | 129.09 |
| GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH) | | | | |
| Soy | Glyma06g01700.1 | 0.37 (ns) | 47.88 | 63.38 |
| Soy | Glyma10g02630.1 | 0.26 (ns) | 52.80 | 64.44 |
| SUPEROXIDE DISMUTASE 2 [Fe; chloroplast] | | | | |
| Soy | Glyma10g33710.1 | 0.53 | 346.98 | 512.39 |
| Soy | Glyma10g33880.2 | 0.53 | 346.98 | 512.39 |
| Bean | Phvul.007G135400.2 | 0.74 | 2.32 | 3.72 |
| Soy | Glyma10g33710.1 | 0.26 (ns) | 52.80 | 64.44 |

Because GR requires NADPH as a co-factor to regenerate reduced glutathione (Halliwell and Foyer, 1978), changes in abundance of transcripts involved in the oxidative branch of the pentose phosphate pathway were also examined. Garden pea transcript ID262852_p.sativum_wa1_contig18926, encoding a cytosolic GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH) was significantly increased (log2FC of 1.91; ambient RPKM, 23.28; elevated RPKM, 85.77), whereas no G6PDH transcripts were differentially expressed in soybean or common bean. Increased transcript abundance of \(\gamma\)-GLUTAMYL TRANSPEPTIDASE (GGT), a gene that mediates glutathione degradation (Storozhenko et al., 2002), was also observed in garden pea (Supplementary Table S4). Genes involved with glutathione biosynthesis were not differentially expressed in any species (Supplementary Table S4).

In garden pea, the content of total foliar glutathione was 2.9- to 4.2-fold higher in ambient \([\text{O}_3]\) and 1.7- to 6.1-fold higher in elevated \([\text{O}_3]\) compared to soybean and common bean (Table 2). Only soybean increased glutathione content in response to elevated \([\text{O}_3]\). Soybean was also the only species that showed a global decrease in the extent to which proteins were glutathionylated (Supplementary Table S5), a post-translational modification that protects proteins from oxidation (Zechmann, 2014). The redox state of the glutathione pool varied among species, with garden pea having significantly less reduced glutathione as a proportion of total glutathione in elevated \([\text{O}_3]\) compared to soybean and common bean (Table 2). Of the three species, common bean had the greatest percentage of reduced glutathione in elevated \([\text{O}_3]\), as well as the lowest total glutathione content (Table 2).

Soybean also increased foliar ascorbate content in elevated \([\text{O}_3]\), in contrast to pea and common bean (Table 2). In addition, no change in total apoplastic ascorbate content was seen in garden pea. However, common bean showed a pronounced decrease in apoplastic ascorbate content in response to elevated \([\text{O}_3]\) (Table 2). Common bean also showed an \(\text{O}_3\)-mediated decrease in the proportion of reduced ascorbate in both foliar and apoplastic pools, whereas the pools of ascorbate in garden pea and soybean were maintained nearly completely in the reduced state (Table 2).

Elevated \([\text{O}_3]\) alters transcription of respiratory genes and reduces TNC content

In each species, many respiration-related genes showed increased transcript abundance in response to elevated \([\text{O}_3]\) (Fig. 5A). Estimates of mitochondrial respiration in the light (\(R_d\)) also indicated a stimulatory effect due to elevated \([\text{O}_3]\) (Fig. 5B). A closer examination revealed that, compared...
to soybean and common bean, garden pea had a marked increase in mitochondrial electron transport-related transcripts (Fig. 5A), including several NADH-dehydrogenase genes (Supplementary Table S6). In contrast, soybean and common bean had larger O$_3$-induced decreases in several glycolysis- and tricarboxylic acid (TCA) cycle-related transcripts (Fig. 5A; Supplementary Table S6). In all three species, a large increase in the abundance of transcripts encoding ALDEHYDE DEHYDROGENASE (ALDH) was observed (Fig. 5A, other; Supplementary Table S6). Only in common bean were several genes in the glycolytic cycle, including ISOCITRATE LYASE and MALATE SYNTHASE, shown to have increased transcript abundance in response to elevated O$_3$ (Fig. 5A, other; Supplementary Table S6).

Midday TNC content was significantly decreased in all three species by elevated O$_3$, as was specific leaf weight (SLW) (Table 3). Foliar starch content, the most abundant TNC constituent, showed a response similar to TNC and SLW (Table 3). Foliar sucrose content did not change in response to elevated O$_3$ in any species and was most abundant in garden pea (Table 3). Foliar glucose content was low in all three species but showed a significant O$_3$-mediated increase in common bean (Table 3).

**Discussion**

Across the three legume species, there were distinct transcriptional, biochemical, and physiological responses to elevated O$_3$. The reductions in photosynthetic parameters (Figs 1 and 3) and leaf longevity (Fig. 2) observed in soybean and common bean are typical of herbaceous annuals (Ainsworth et al., 2012) and similar to previous reports in which the response to elevated O$_3$ was greater in common bean than soybean (Feng and Kobayashi, 2009). Because garden pea did not show these symptoms of O$_3$ damage, we hypothesized that it employed a more effective strategy to detoxify O$_3$-induced ROS. While a previous study using a single variety of garden pea reported no O$_3$-induced reductions in photosynthetic capacity (Farage and Long, 1999), here it was demonstrated that this may be a more general response for the species because none of the garden pea varieties investigated exhibited a negative response to O$_3$ (Fig. 1). The differences in physiological and visual symptoms of O$_3$ exposure between the legumes were unlikely related to flux of the gas into the leaf. g, was not significantly different among the three legume species in ambient O$_3$ (data not shown), and, in contrast to soybean and common bean, there was no change in g in response to O$_3$ exposure in garden pea (Fig. 1). Thus, a reduction in O$_3$ flux into leaves cannot explain the lack of responsiveness in garden pea; rather, the inherent capacity to activate a cellular response to elevated O$_3$ is a more likely mechanism.

Many genes in each species were turned on by elevated O$_3$, including peroxidases and receptor-like kinases (Supplementary Table S2), both of which are known to be transcriptionally regulated by abiotic stress (Noctor and Foyer, 1998, Osakabe et al., 2013). The induction of MYB and WRKY family genes, which mediate transcriptional reprogramming in response to abiotic stresses including elevated O$_3$ (Tosti et al., 2006; Rizzo et al., 2007; Chen et al., 2012; Iyer et al., 2013; Li et al., 2014), suggests that garden pea may have activated signalling networks involved with stress perception to a greater extent than soybean and common bean. In addition, a broad functional analysis revealed that garden pea had a greater stimulation of genes involved with protein processes (Supplementary Fig. S3). While it is known that growth in elevated O$_3$ can damage proteins and trigger proteolysis and protein biosynthesis (Pell et al., 1997), the implication of the species comparison is that garden pea may have removed and replaced damaged proteins more efficiently. Considering that each species had a large number of significantly expressed genes involved with post-translational modifications, the extent of glutathionylation, which can protect proteins from irreversible oxidative damage and be stimulated by ROS (Foyer and Noctor, 2005; Rouhier et al., 2008; Zechmann, 2014) was also examined. While no specific proteins were investigated, the O$_3$-mediated decrease in total glutathionylated proteins in soybean (Supplementary Table S5) suggests limited protective benefit and could possibly be a contributing factor in the responsiveness of soybean to elevated O$_3$.

**Table 2.** Midday foliar and apoplastic antioxidant content of the youngest fully expanded leaf after plants were grown in elevated [O$_3$] (150 ppb; 8h d$^{-1}$) for one month

| Foliar          | Garden pea                  | Soybean                  | Common bean   |
|-----------------|-----------------------------|--------------------------|---------------|
|                 | Ambient                     | Elevated                 | Ambient       | Elevated     |
| Total glutathione [nmol (g FW)$^{-1}$] | 947 ± 242.5$^a$ | 905 ± 131.1$^a$ | 295 ± 9.9$^d$ | 531 ± 21.0$^d$ | 193 ± 22.6$^a$ | 96 ± 12.6$^a$ |
| Reduced glutathione (%) | 78 ± 0.5$^c$   | 69 ± 0.6$^c$    | 76 ± 4.6$^c$ | 86 ± 8.3$^a$ | 99 ± 1.1$^a$ | 91 ± 8.6$^a$ |
| Foliar          | Total ascorbate [µmol (g FW)$^{-1}$] | 7.1 ± 0.25$^d$ | 6.5 ± 1.75$^d$ | 6.3 ± 0.97$^d$ | 10.0 ± 0.90$^a$ | 7.2 ± 1.12$^a$ | 7.0 ± 1.60$^a$ |
| Reduced ascorbate (%) | 100 ± 0.0$^a$ | 100 ± 0.0$^a$ | 100 ± 0.0$^a$ | 92 ± 8.5$^a$ | 84 ± 8.7$^a$ | 60 ± 1.0$^c$ |
| Apoplastic      | Total ascorbate [µmol (g FW)$^{-1}$] | 1.6 ± 0.32$^a$ | 1.8 ± 0.58$^c$ | NA           | NA           | 1.8 ± 0.37$^a$ | 0.2 ± 0.15$^a$ |
| Reduced ascorbate (%) | 100 ± 0.0$^a$ | 100 ± 0.0$^a$ | NA           | NA           | 95 ± 6.3$^a$ | 70 ± 11.7$^c$ |
Generally, the expression and abundance of phenolic metabolites are induced by exposure to elevated [O$_3$]; both have been used as indicators of stress perception (Castagna and Ranieri, 2009). Greater foliar phenolic content was measured in pea and soybean, but not in common bean (Fig. 4B). Common bean also had the greatest O$_3$-mediated decrease in photosynthetic capacity (Figs 1 and 3), which is consistent with the notion that phenolic metabolites may directly scavenge ROS (Rice-Evans et al., 1997; Grace and Logan, 2000). Evidence from garden pea, which had an increase in the abundance of phenolic metabolites (Fig. 4B) and no change in photosynthesis (Figs 1 and 3), also supports this idea. However, photosynthesis was negatively affected by elevated [O$_3$] in soybean (Figs 1 and 3) despite a doubling of phenolic content (Fig. 4B). Previous work with another soybean variety did not report any differences in phenolic content with exposure to either chronic or acute O$_3$ stress (Gillespie et al., 2011). Such inconsistency between phenolic content and responsiveness of other phenotypes has been previously observed in other species (Catagna and Ranieri, 2009) and questions the reliability of using phenolics-related transcripts and metabolites as biomarkers to predict O$_3$ sensitivity. The results presented here do show, however, that transcript abundance changes of phenolics-related biosynthesis genes (Fig. 4A) can be used as general indicators of elevated [O$_3$] perception.

As the first line of defence, extracellular antioxidants including ascorbate are thought to contribute to detoxification of O$_3$ (Chameides, 1989; Luwe et al., 1993; Burkey et al., 2003; Conklin and Barth, 2004; Cheng et al., 2007). Garden pea maintained the size and redox status of the apoplastic ascorbate pool when exposed to elevated [O$_3$], in contrast to common bean, which showed a significant reduction in both the size and redox state of the apoplastic ascorbate pool at elevated [O$_3$] (Table 2). Reduced ascorbate in the apoplast needs to be regenerated from oxidized ascorbate (dehydroascorbate; DHA), which is typically performed by glutathione-dependent dehydroascorbate reductase (DHAR) after transport of DHA across the plasma membrane into the cytoplasm (Conklin and Barth, 2004; Foyer and Noctor, 2005). While no change in transcript abundance was seen for DHAR homologues (Supplementary Table S3), the pool size of glutathione was greatest in garden pea (Table 2). Significant variation in glutathione pools among dicot species has been previously reported, and in healthy leaves most of the glutathione pool is localized to the mitochondria (Zechmann and Müller, 2010). There, glutathione is important for the maintenance of the redox status to avoid or repair oxidative damage (Mari et al., 2009). Pea had greater rates of respiration in both ambient and elevated [O$_3$] than soybean or common bean (Fig. 5), which may in part explain the greater foliar glutathione content. Increased transcript abundance of a cytosolic GR was observed in response to elevated [O$_3$] (Table 1), which along with glutathione availability is important for the recycling of reduced ascorbate (Noctor and Foyer, 1998) and would support DHAR activity. Previous studies have demonstrated that GR content is positively correlated with protection from O$_3$-induced damage (Tanaka et al., 1990; Chernikova et al.,

![Fig. 5. Species comparison of respiration-related transcript and metabolic changes in response to elevated [O$_3$]. (A) Heat map of transcript abundance changes for genes comprising the major respiratory pathways in leaf tissue collected from the youngest fully expanded leaf at midday after plants were grown in elevated [O$_3$] (150 ppb; 8 h d$^{-1}$) for one month. Each coloured block represents the log$_2$ fold change of a separate homologue that was determined to be differentially expressed. (B) The rate of mitochondrial respiration in the light ($R_o$) estimated from A/C$_i$ curves. The mean (± SD; n = 3) is presented with letters representing significant differences (P < 0.05).](https://academic.oup.com/jxb/article-abstract/66/22/7101/2893272)
Table 3. Midday SLW and foliar content of TNC and constituents of the youngest fully expanded leaf after plants were grown in elevated [O₃] (150 ppb; 8 h d⁻¹) for one month

|                     | Garden pea | Soy | Common bean |
|---------------------|------------|-----|-------------|
|                     | Ambient    | Elevated | Ambient | Elevated | Ambien | Elevated | Common bean | Elevated |
| SLW (g m⁻²)         | 81.0 ± 1.90 | 54.8 ± 4.56 | 64.7 ± 1.40 | 38.3 ± 1.78 | 88.0 ± 7.39 | 51.6 ± 4.14 |
| TNC (µmol cm⁻²)     | 53.5 ± 13.23 | 32.3 ± 10.10 | 60.1 ± 10.32 | 19.9 ± 10.63 | 25.6 ± 9.55 | 11.1 ± 6.17 |
| Glucose (µmol cm⁻²) | 1.5 ± 0.83 | 1.7 ± 0.79 | 0.9 ± 0.34 | 1.2 ± 0.32 | 0.1 ± 0.01 | 0.7 ± 0.22 |
| Sucrose (µmol cm⁻²) | 8.4 ± 1.25 | 9.2 ± 4.33 | 2.1 ± 0.88 | 2.1 ± 0.39 | 0.8 ± 0.01 | 3.2 ± 2.29 |
| Starch (µmol cm⁻²)  | 42.8 ± 10.02 | 21.3 ± 5.41 | 57.1 ± 10.54 | 16.8 ± 10.29 | 24.7 ± 0.94 | 7.0 ± 3.82 |

Absolute values represent glucose equivalents based on a comparison to a standard curve and were normalized to the amount of tissue per unit leaf area. The mean (± SD; n = 3) is presented with letters representing significant differences (P < 0.05).

2000). Other O₃-responsive genes also increased transcript abundance specifically in garden pea that would enable GR function and help regulate adequate levels of reduced glutathione, such as G6PDH and GGT (Okama-Ohtsu et al., 2007; Wang et al., 2008; Yang et al., 2014). Collectively, this evidence suggests that garden pea both harnessed the scavenging potential of the apoplastic ascorbate pool in response to elevated [O₃], and regenerated the reduced ascorbate needed for efficient detoxification or signalling. The fact that no increase in abundance of chloroplastic GPX6 and SOD2 transcripts was seen in garden pea further supports this inference. The response of GPX6 and SOD2 transcripts to elevated [O₃] in soybean and common bean (Table 1) indicates that penetration of ROS into chloroplasts was likely, resulting in damage to photosynthetic proteins and decreased photosynthetic rates (Figs 1 and 3).

In response to elevated [O₃], increased rates of respiration have been observed in soybean (Gillespie et al., 2012), bean (Todd, 1958; Amthor, 1988), and other plant species (Volin and Reich, 1996; Kellomaki and Wang, 1998; Biswas et al., 2008). Increased respiration is hypothesized to supply the energy demands associated with antioxidant scavenging and cellular repair mechanisms (Amthor, 1988). All three of the legume species investigated in this study showed increased mitochondrial respiration in the light (R₉), estimated from A/Cᵢ curve analysis (Fig. 5B). All three species also decreased TNC content at elevated [O₃] (Table 3), a result in soybean and common bean that may be driven both by decreased photosynthesis and increased respiration. However, in garden pea, photosynthetic capacity was similar in ambient and elevated [O₃], while respiration rates increased and TNCs decreased, which may be interpreted as further evidence for the hypothesis that increased respiration drives increased antioxidant and defence metabolism. Enhanced expression of transcripts involved in glycolysis, the TCA cycle, and mitochondrial electron transport was observed in all three species (Fig. 5A). In elevated [O₃], garden pea increased the transcript abundance of NADH dehydrogenase, which is a component of complex I in the mitochondrial electron transport system (Fig. 5A; Supplementary Table S6) and plays an important role in regulating ATP synthesis (Rasmusson et al., 1998). Considering that mitochondria are responsible for maintaining redox homeostasis in response to elevated [O₃] (Dutilleul et al., 2003), the increased abundance of NADH dehydrogenase transcripts may represent a control point in the transcriptional crosstalk involved in regulating the energy demands of enhanced ROS detoxification. Such a response would facilitate the suite of changes observed in antioxidant metabolism that were described previously for garden pea. However, the change in respiration at elevated [O₃] may not be solely attributed to greater demand for energy for antioxidant metabolism. All three species also showed decreased SLW in elevated [O₃] (Table 3). Across a very broad range of functional types and species, SLW is negatively correlated with dark respiration rates (Reich et al., 1988). It is possible that the effect of elevated [O₃] on R₉ is driven by the decrease in SLW at elevated [O₃]. Amthor (1988) suggested that there are mechanisms which would both enhance and decrease respiration rates in plants exposed to elevated [O₃], and at the transcriptional level there is evidence in the three legume species studied here that genes associated with respiration both increase and decrease (Fig. 5).

While several mechanisms of O₃ response were identified that were different among the three legume species, there were also common responses. For example, all three species increased ALDH transcript abundance at elevated [O₃] (Supplementary Table S6). Aldehydes are potentially toxic intermediates generated by several metabolic pathways and can accumulate in response to abiotic stress (Kirch et al., 2004). In Arabidopsis, overexpression of ALDH improved oxidative stress tolerance owing to its role in reactive aldehyde detoxification, an oxidative process generating NADPH that can be then used in respiratory ATP synthesis (Kotchoni et al., 2006). The fact that all three species increased ALDH transcript abundance suggests that aldehyde detoxification may be a universal response to counter the negative effects of growth in elevated [O₃].

In conclusion, a broad range of O₃-mediated growth, injury, and physiological responses were observed among the three legume species, from the typical O₃-mediated reductions in leaf-level photosynthesis and leaf longevity in soybean and common bean to no change in garden pea. Comparing global transcriptomic changes with leaf antioxidant content and redox state identified the induction of transcripts encoding phenolic compounds and a greater quantity of phenolic compounds in pea, as well a reduced proportion of...
apoplastic ascorbate. Pea also had greater foliar glutathione content than soybean and common bean, greater respiration rates, and enhanced NADH dehydrogenase transcript abundance. These general responses of garden pea could be used to develop screens for more tolerant varieties of soybean and common bean, or used in biotechnology applications to improve their response to elevated \([O_3]\).

**Supplementary data**

Supplementary material are available at *JXB* online.

**Supplementary Fig. S1** Quality assurance of RNA used for library preparation.

**Supplementary Fig. S2** Volcano plot of \(P\)-values against the expression ratio between elevated and ambient \([O_3]\).

**Supplementary Fig. S3** The distribution of differentially expressed genes in each of the major functional categories and protein processes sub-categories.

**Supplementary Table S1** RNA-Seq summary statistics.

**Supplementary Table S2** Transcripts with no detectable reads in ambient \([O_3]\) that were induced by elevated \([O_3]\).

**Supplementary Table S3** Transcript abundance changes of all ascorbate–glutathione cycle genes.

**Supplementary Table S4** Transcript abundance changes of glutathione biosynthesis and catabolism genes.

**Supplementary Table S5** Global abundance of glutathionylated proteins.

**Supplementary Table S6** Transcript abundance changes of respiration-related genes presented in Fig. 5.

**Acknowledgements**

The authors would like to thank Sara Kammlade and Chris Montes for technical assistance and Kiran Donthu, Jothi Thimmapuram, and Courtney Leinser for advice with bioinformatics analysis. We thank Steve Huber for assistance with analysis of glutathionylated proteins. This work was funded by the National Soybean Research Laboratory’s Soybean Disease Biotechnology Center and the United States Department of Agriculture Agricultural Research Service.

**References**

Agrawal GK, Rakwal R, Yonekura M, Kubo A, Saji H. 2002. Proteome analysis of differentially displayed proteins as a tool for investigating ozone stress in rice (Oriza sativa L.) seedlings. Proteomics 2, 947–959.

Ainsworth EA, Gillespie KM. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nature Protocols 2, 875–877.

Ainsworth EA, Yendrek CR, Sitch S, Collins WJ, Emberson LD. 2012. The effects of tropospheric ozone on net primary productivity and implication for climate change. Annual Review of Plant Biology 63, 637–661.

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

Ashmore MR. 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell and Environment 28, 949–964.

Avnery S, Mauzerall DL, Liu J, Horowitz LW. 2011. Global crop yield reductions due to surface ozone exposure: 1. Year 2000 crop production losses and economic damage. Atmospheric Environment 45, 2284–2296.

Baier M, Kandibinder A, Golldack, D, Dietz KJ. 2005. Oxidative stress and ozone: perception, signalling and response. Plant Cell and Environment 28, 1012–1020.

Betzberger AM, Gillespie KM, McGrath JM, Koester RP, Nelson RL, Ainsworth EA. 2010. Effects of chronic elevated ozone concentration on antioxidant capacity, photosynthesis and seed yield of 10 soybean cultivars. Plant Cell and Environment 33, 1569–1581.

Betzberger AM, Yendrek CR, Sun JD, Leinser CP, Nelson RL, Ort DR, Ainsworth EA. 2012. Ozone exposure response for U.S. soybean cultivars: linear reductions in photosynthetic potential, biomass, and yield. Plant Physiology 160, 1827–1839.

Bilgin DD, DeLucia EH, Clough SJ. 2009. A robust RNA isolation method suitable for Affymetrix GeneChip analysis and quantitative real-time RT-PCR. Nature Protocols 4, 333–340.

Biswas DK, Xu H, Li YG, Sun JZ, Wang ZX, Han XG, Jiang GM. 2008. Genotypic differences in leaf biochemical, physiological and growth responses to ozone in 20 winter wheat cultivar released over the past 60 years. Global Change Biology 14, 46–59.

Burkey KO, Eason G, Fiscus EL. 2003. Factors that affect leaf extracellular ascorbic acid content and redox status. Physiologia Plantarum 117, 51–57.

Burkey KO, Miller JE, Fiscus EL. 2005. Assessment of ambient ozone effects on vegetation using snap bean as a bioindicator species. Journal of Environmental Quality 34, 1081–1086.

Burkey KO, Carter TE. 2009. Foliar resistance to ozone injury in the genetic base of U.S. and Canadian soybean and prediction of resistance in descendent cultivars using coefficient of parentage. Field Crops Research 111, 207–217.

Castagna A, Ranieri A. 2009. Detoxification and repair process of ozone injury: from \(O_3\) uptake to gene expression adjustment. Environmental Pollution 157, 1461–1469.

Chameides WL. 1989. The chemistry of ozone deposition to plant leaves: role of ascorbic acid. Environmental Science and Technology 23, 595–600.

Chen L, Song Y, Li S, Zhang L, Zou C, Yu D. 2012. The role of WPKY transcription factors in plant abiotic stresses. BBA-Gene Regulatory Mechanisms 1819, 120–128.

Cheng F-Y, Burkey KO, Robinson JM, Booker FL. 2007. Leaf extracellular ascorbate in relation to \(O_3\) tolerance of two soybean cultivars. Environmental Pollution 150, 355–362.

Chernikova T, Robinson JM, Lee EH, Mulchi CL. 2000. Ozone tolerance and antioxidant enzyme activity in soybean cultivars. Photosynthesis Research 64, 15–26.

Conklin PL, 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 and Environment 27, 959–970.

Dixon RA, Paiva NL. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097.

Dutilleul C, Garmier M, Noctor G, Mathieu C, Chetrit P, Foyer CH, dePapee R. 2003. Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. Plant Cell 15, 1212–1226.

Eller ASD, Sparks JP. 2006. Predicting leaf-level fluxes of \(O_3\) and \(NO_2\): the relative roles of diffusion and biochemical processes. Plant Cell and Environment 29, 1742–1750.

Farge PK, Long SP. 1999. The effects of \(O_3\) fumigation during leaf development on photosynthesis of wheat and pea: an *in vivo* analysis. Photosynthesis Research 59, 1–7.

Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO2 assimilation in leaves of C3 species. Planta 151, 70–79.

Feng Z, Kobayashi K. 2009. Assessing the impacts of current and future concentrations of surface ozone on crop yield with meta-analysis. Atmospheric Environment 43, 1510–1519.

Fiscus EL, Booker FL, Burkey KO. 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant Cell and Environment 28, 997–1011.

Foyer CH, Noctor G. 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell and Environment 28, 1056–1071.

Gillespie KM, Ainsworth EA. 2007. Measurement of reduced, oxidized and total ascorbate content in plants. Nature Protocols 2, 871–874.

Gillespie KM, Rogers A, Ainsworth EA. 2011. Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity...
and response to acute oxidative stress in soybean (Glycine max). Journal of Experimental Botany 62, 2667–2678.

Gillespie KM, Xu F, Richter KT, McGrath JM, Markelz RJ, Ort DR, Leakey ADB, Ainsworth EA. 2012. Greater antioxidant and respiratory metabolism in field-grown soybean exposed to elevated O3 under both ambient and elevated CO2. Plant Cell and Environment 35, 169–184.

Grace SC, Logan BA. 2000 Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Philosophical Transactions of the Royal Society of London-Biological Sciences, 355, 1499–1510.

Halliwell B, Foyer CH. 1978. Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta 149, 5–17.

Heaige AS. 1989. Ozone and crop yield. Annual Review of Phytopathology 27, 397–423.

Iyer NJ, Tang Y, Mahalingham R. 2013. Physiological, biochemical and molecular responses to a combination of drought and ozone in Medicago truncatula. Plant Cell and Environment 36, 706–720.

Jacobson MZ, Streets DG. 1998. Relationships of leaf dark respiration to leaf nitrogen, specific leaf area and leaf life-span: a test across biomes and functional groups. Oecologia 114, 471–482.

Kellomaki S, Wang KY. 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.

Kirch HH, Bartels D, Wei Y, Schnabl PS, Wood AJ. 2004. The ALDH gene superfamily of Arabidopsis. Trends in Plant Science 9, 371–377.

Kotcheno SO, Kuhns C, Ditzer A, Kirch HH, Bartels D. 2006. Over-expression of different aldehyde dehydrogenase genes in Arabidopsis thaliana confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress. Plant Cell and Environment 29, 1033–1048.

Krupa S, McGrath MT, Andersen CP, Booker FL, Burkey KO, Chappelka AH, Chevone BI, Pell EJ, Zilinskas BA. 2001. Ambient ozone and plant health. Plant Disease 85, 4–12.

Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology 10, R25.

Dornsife N, Wang KY. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9, 357–359.

Lee EH, Jersey JA, Gilford C, Bennett J. 1984. Differential ozone tolerance in soybean and snapbean: analysis of ascorbic acid in O3 susceptible and O3-resistant cultivars by high-performance liquid chromatography. Environmental and Experimental Botany 24, 331–341.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Purcell EK, McVicker G, D Lieu S, He L, Durbin R. 2009. The sequence alignment/map (SAM) format and SAMtools. Bioinformatics 25, 2078–2079.

Li C, Ng CKY, Fan LM. 2014. MYB transcription factors, active players in abiotic stress signaling. Environmental and Experimental Botany 114, 80–91.

Long SP, Bernacchi C J. 2003. Gas exchange measurements, what can they tell us about the underlying limitation to photosynthesis? Procedures and sources of error. Journal of Experimental Biology 204, 2393–2401.

Luwe MWF, Takahama U, Heber U. 1993. Role of ascorbate in detoxifying oxygen in the apoplast of spinach (Spinacia oleracea L.) leaves. Plant Physiology 101, 969–976.

Marí M, Morales A, Calell A, García-Ruiz C, Fernández-Checa JC. 2009. Mitochondrial glutathione, a key survival antioxidant. Antioxidants & Redox Signaling 11, 2685–2700.

Mills G, Buse A, Gimeno B, Bermejo V, Holland M, Emmerson L, Pleijel H. 2007. A synthesis of AOT4-based response functions and critical levels of ozone for agricultural and horticultural crops. Atmospheric Environment 41, 2630–2643.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7, 405–410.

Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annual Review of Plant Physiology and Plant Molecular Biology 49, 249–279.

Okhama-Ohtsu N, Radwan S, Peterson A, Zhao P, Badr AF, Xiang C, Oliver DJ. 2007. Characterization of the extracellular γ-glutamyl transpeptidase, GGT1 and GGT2, in Arabidopsis. Plant Journal 49, 865–877.

Osakabe Y, Yamaguchi-Shinozaki K, Shinozaki, K, Tran LSP. 2013. Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. Journal of Experimental Biology 64, 445–458.

Pell EJ, Schlagnhaufer CD, Artega RN. 1997. Ozone-induced oxidative stress: mechanisms of action and reaction. Physiologia Plantarum 100, 264–273.

Pitcher LH, Brennan E, Hurley A, Dunsmuir P, Tepperman JM, Zilinskas BA. 1991. Overproduction of petunia chloroplastic copper/zinc superoxide dismutase does not confer ozone tolerance in transgenic tobacco. Plant Physiology 97, 452–455.

Rao MV, Koch JR, Davis KR. 2000. Ozone: a tool for probing programmed cell death in plants. Plant Molecular Biology 44, 345–358.

Rasmussen AG, Heiser V, Zabaleta E, Brennicke A, Grohmann L. 1998. Physiological, biochemical and molecular aspects of mitochondrial complex I in plants. BBA-Bioenergetics 1364, 101–111.

Reich PB, Walters MB, Eilsworth DS, Vose JM, Volin JC, Gresham C, Bowman WD. 1998. Relationships of leaf dark respiration to leaf nitrogen, specific leaf area and leaf life-span: a test across biomes and functional groups. Oecologia 114, 471–482.

Rice-Evans CA, Miller NJ, Paganga G. 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science 2, 152–159.

Rizzo M, Bernardi R, Salvini M, Nali C, Lorenzini G, Durante M. 2007. Identification of differentially expressed genes induced by ozone stress is sensitive and tolerant poplar hybrids. Journal of Plant Physiology 164, 945–954.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140.

Rohwer N, Lemaire SD, Jacquot JP. 2008. The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annual Review of Plant Biology 59, 143–166.

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

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J et al. 2010. Genome sequence of the palaeopolyploid soybean. Nature 463, 179–183.

Schmutz J, McClean PE, Mamidi S, Wu GA, Cannon SB, Grimood J, Jenkins J, Shu S, Song Q, Chavarro C et al. 2014. A reference genome for common bean and genome-wide analysis of dual domestications. Nature Genetics 46, 707–713.

Schrautner M, Moeder W, Wiese C, Van Camp W, Inzé D, Langebartels C, Sandermann H Jr. 1998. Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. Plant Journal 16, 235–245.

Seinfeld JH, Pandis SN. 2012. Atmospheric chemistry and physics: from air pollution to climate change . New York: John Wiley and Sons.

Sharma YK, Davis KR. 1997. The effects of ozone on antioxidant responses in plants. Free Radical Biology and Medicine 23, 480–488.

Storozhenko S, Bellès-Boix E, Babychuk E, Herouart D, Davey MW, Slooten L, Van Montagu M, Inze D, Hushnir S. 2002. β-glutamyl transpeptidase in transgenic tobacco plant. Cellular localization, processing, and biochemical properties. Plant Physiology 128, 1109–1119.

Tanaka K, Machida T, Sugimoto T. 1990. Ozone tolerance and glutathione reductase in tobacco cultivars. Agricultural and Biological Chemistry 54, 1061–1062.

Todd GW. 1958. Effect of ozone on ozonated 1-hexene on respiration and photosynthesis of leaves. Plant Physiology 33, 416–420.

Tosti N, Pasqualini S, Borgogni A, Ederli L, Falistocco E, Crispi S, Paolocci F, Tosti N, Pasqualini S, Borgogni A, Ederli L, Falistocco E, Crispi S, Paolocci F. 2006. The ALDH gene superfamily of Arabidopsis. Trends in Plant Science 11, 331–341.

Van Camp W, Willekens H, Bowler C, Van Montagu M, Inze D, Reupold-Popp P, Sandermann H, Langebartels C. 1994. Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. Nature Biotechnology 12, 165–168.
Van Dingenen R, Dentener FJ, Raes F, Krol MC, Emberson L, Cofala J. 2009. The global impact of ozone on agriculture crop yields under current and future air quality legislation. Atmospheric Environment 43, 604–618.

Volin JC, Reich PB. 1996. Interaction of elevated CO₂ and O₃ on growth, photosynthesis and respiration of three perennial species grown in low and high nitrogen. Physiologia Plantarum 97, 674–684.

Wang X, Ma Y, Huang C, Li N, Bi Y. 2008. Glucose-6-phosphate dehydrogenase plays a central role in modulating reduced glutathione levels in reed callus under salt stress. Planta 227, 611–623.

Wohlgemuth H, Mittelstrass K, Kschieschan S, Bender J, Weigel H-J, Overmyer K, Kangasjärvi J, Sandermann HJ, Langebartels C. 2002. Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Plant, Cell and Environment 25, 717–726.

Yang Y, Fu Z, Su Y, Zhang X, Li G, Guo J, Que Y, Xu L. 2014. A cytosolic glucose-6-phosphate dehydrogenase gene, ScG6PDH, plays a positive role in response to various abiotic stresses in sugarcane. Science Reports 4, 7090.

Yendrek CR, Leisner CP, Ainsworth EA. 2013. Chronic ozone exacerbates the reduction in photosynthesis and acceleration of senescence caused by limited N availability in Nicotiana sylvestris. Global Change Biology 19, 3155–3166.

Zechmann B. 2014. Compartment-specific importance of glutathione during abiotic and biotic stress. Frontiers in Plant Science 5, 566.

Zechmann B, Müller M. 2010. Subcellular compartmentation of glutathione in dicotyledonous plants. Protoplasma 246, 15–24.