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Increased protein carbonylation in leaves of *Arabidopsis* and soybean in response to elevated [CO_2]

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Abstract While exposure of C_3 plants to elevated [CO_2] would be expected to reduce production of reactive oxygen species (ROS) in leaves because of reduced photorespiratory metabolism, results obtained in the present study suggest that exposure of plants to elevated [CO_2] can result in increased oxidative stress. First, in *Arabidopsis* and soybean, leaf protein carbonylation, a marker of oxidative stress, was often increased when plants were exposed to elevated [CO_2]. In soybean, increased carbonyl content was often associated with loss of leaf chlorophyll and reduced enhancement of leaf photosynthetic rate (Pn) by elevated [CO_2]. Second, two-dimensional (2-DE) difference gel electrophoresis (DIGE) analysis of proteins extracted from leaves of soybean plants grown at elevated [CO_2] or [O_3] revealed that both treatments altered the abundance of a similar subset of proteins, consistent with the idea that both conditions may involve an oxidative stress. The 2-DE analysis of leaf proteins was facilitated by a novel and simple procedure to remove ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from soluble soybean leaf extracts. Collectively, these findings add a new dimension to our understanding of global change biology and raise the possibility that oxidative signals can be an unexpected component of plant response to elevated [CO_2].

Keywords *Arabidopsis* · Ascorbate peroxidase · 2-Dimensional gel electrophoresis · Protein carbonylation · Soybean

Abbreviations

CBB	Coomassie Brilliant Blue
1-DE	One dimensional
2-DE	Two dimensional
CF	Charcoal filtered
DNPH	2,4-Dinitrophenylhydrazine
FACE	Free air CO_2 enrichment
Pn	Leaf photosynthetic rate
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase/ oxygenase

Introduction

An unavoidable consequence of aerobic metabolism is exposure of cells to reactive oxygen species (ROS), including H_2O_2 (hydrogen peroxide), $^{\bullet}O_2^-$ (superoxide), and the $^{\bullet}OH$ (hydroxyl radical). In plants, low concentrations of ROS function in signal transduction leading to

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activation of defense responses (Mittler 2002), while higher levels lead to oxidative damage of lipids, DNA, and proteins (Ghezzi and Bonetto 2003). We are interested in the impact of oxidative stress on the leaf proteome and in particular, on irreversible oxidative damage to proteins. One consequence of oxidative stress is protein carbonylation, which involves the modification of the side chains of certain amino acids (e.g., Pro, His, Arg, Lys, and Thr) to produce ketone or aldehyde derivatives that are reactive with 2,4-dinitrophenylhydrazine (DNPH) (Dalle-Donne et al. 2003). Derivatization of protein carbonyls with DNPH, followed by immunoblotting with anti-DNP antibodies, is a sensitive and specific method for detection of oxidatively modified proteins and is generally regarded as an indicator of oxidative stress.

A generalization to emerge from studies of reactive protein carbonyls in bacteria and as well as plants and animals is that oxidative damage is selectively targeted rather than a randomly directed process (Johansson et al. 2004; Kristensen et al. 2004; Job et al. 2005). However, the sites and nature of oxidative modifications are largely unknown, as are the consequences, although it is generally assumed that oxidized proteins lose enzymatic activity and may be preferentially degraded (Berlett and Stadtman 1997; Yan and Sohal 1998). In plants, chloroplasts (Davletova et al. 2005), peroxisomes (Foyer and Noctor 2003), and mitochondria (Möller and Kristensen 2004) are recognized to be major points of ROS metabolism. In chloroplasts and mitochondria, electron transport is the major source of ROS and the rate of electron transport, availability of electron acceptors, and the degree of coupling can all influence ROS production. In leaves, photorespiration is a major process resulting in production of ROS and the potential significance of photorespiratory H_2O_2 in signaling and acclimation has been noted (Noctor et al. 2002). Once reactive molecules are produced antioxidant metabolites and ROS-scavenging enzymes function to remove them. Interestingly, while different ROS-scavenging enzymes are distributed in the relevant cellular compartments, the cytosolic ascorbate peroxidase 1 (APX1) plays a unique role and is essential for chloroplast protection during high light stress suggesting a 'cross-compartment' protection (Davletova et al. 2005). Accordingly, induction of APX1 transcription is a marker of oxidative stress.

Leaf protein carbonylation is not, in general, well characterized. In a recent study with Arabidopsis, Johansson et al. (2004) examined developmental factors and reported that protein carbonyl levels increased progressively during vegetative growth and then decreased dramatically just prior to bolting and reproductive development. The large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), as well as other proteins, were identified as major targets of oxidative damage (Johansson et al. 2004). Interestingly, total leaf Rubisco content was constant throughout development, even when protein carbonyl content was decreasing dramatically. The significance of this observation remains to be fully elucidated, but suggests that protein oxidation may be developmentally controlled.

Environmental factors can also affect protein carbonylation in plants and animals. Early work with maize seedlings showed that chilling-induced stress resulted in oxidation of both proteins and lipids (Prasad 1996), and it was subsequently shown that bundle sheath cell proteins had greater oxidative damage (measured as protein carbonylation) compared to mesophyll cell proteins (Kingston-Smith and Foyer 2000). These studies are consistent with the notion that protein oxidation leads to loss of function (in this case, impaired photosynthesis and growth at chilling temperatures). Elevated temperature and heat stress can also increase protein oxidation. In *Drosophila melanogaster*, an increase of 12°C above ambient for 30 days accelerated the accrual of aconitase carbonylation and loss in activity in a mitochondrial matrix extract of flight muscles (Das et al. 2004), raising the possibility that high temperature may be an important factor for plants as well.

One might expect that elevated [CO_2] should decrease oxidative stress by reducing cellular production of ROS in several ways (Pritchard et al. 2000). First, increased CO_2:O_2 ratios within chloroplasts would decrease electron leakage from PSI to O_2, thereby attenuating $^{\bullet}O_2^-$ formation, while decreased photorespiration would reduce cellular H_2O_2 production associated with glycolate metabolism. Second, growth at elevated [CO_2] often improves plant water status, which would indirectly decrease antioxidant activities that are stimulated by water stress (Long et al. 2004). However, the impact of elevated [CO_2] on leaf protein carbonylation has never been examined. Hence, the overall objective of the present study was to determine the impact of elevated atmospheric [CO_2] on leaf protein carbonylation to test the hypothesis that protein carbonylation would be reduced. Field-grown soybean and chamber-grown Arabidopsis were used so that the effects of the gases could be tested using both an important agronomic crop as well as a model plant system. In addition, the impact of elevated [CO_2] is compared in some experiments with the effect of elevated [O_3], a known oxidative stressor.

Materials and methods

Growth of Arabidopsis

Arabidopsis plants were grown in a commercial soil mixture (Sunshine LC1 MIX; Sun Gro Horticulture Canada Ltd.) in a growth chamber that provided approximately 200 μmol PAR m^{-2} s^{-1} with a combination of fluorescent

and incandescent lights. Photoperiod was 14 h and temperature was 23°C day and 19°C night. Plants received a commercial fertilizer (Expert Gardener™ All Purpose Plant Food, Schultz Company, St. Louis, MO, USA) that was provided weekly at the recommended rate. Elevated [CO_2] was provided as indicated at 1,000 μmol mol^{-1} on a 24 h basis. Three replicate samples were harvested from two independent experiments.

Growth and treatment of soybean 'Essex' in open-top chambers

Soybean 'Essex' was planted in insulated pots containing 21 l of a 2:1:1 mixture of sandy loam soil:sand:Metro Mix 220 (Scotts Sierra Horticultural Products Co., Marysville, OH, USA) (pH 6) at a site 5 km south of Raleigh, NC. Plants were grown and treated in 2.4 m tall × 3 m diameter open-top field chambers from germination to physiological maturity with reciprocal treatments of CO_2 or O_3 as described previously (Booker and Fiscus 2005). The treatment combinations were: (a) charcoal-filtered (CF) air at ambient CO_2 (control); (b) CF air plus 345 μmol CO_2 mol^{-1} (elevated CO_2); (c) CF air plus 1.3 times ambient O_3 and ambient CO_2 (elevated O_3); and (d) CF air plus 1.3 times ambient O_3 and 345 μmol CO_2 mol^{-1} (elevated CO_2 and O_3) (Table 1). In the elevated O_3 treatments, O_3 was added in a prescribed function based on the average hourly concentration of O_3 measured at our location 12 h d^{-1} (08:00–20:00 h EST) from 1993 to 1996 during the months June, July, and August. Carbon dioxide was dispensed 24 h day^{-1}. The experiment consisted of four treatments with three replicate chambers per treatment. Midday temperature of upper canopy leaves on three plants per chamber was measured with a steady-state porometer (Li-Cor Model 1600M). Measurements of abaxial leaf surface temperature were made on 32 occasions between 4 to 14 weeks after planting. Treatment effects and means were estimated using a mixed model analysis of variance (SAS Proc Mixed; (Littell et al. 1996).

Growth of soybean 'P93B15' at SoyFACE and gas exchange

Soybean 'P93B15' was grown in 2005 and 2006 at the SOYbean Free Air [CO_2] Enrichment (SOYFACE; www.soyface.uiuc.edu) facility located at the University of Illinois at Urbana-Champaign. Plots received either ambient [CO_2] of 370 μmol mol^{-1}, elevated [CO_2] of 550 μmol mol^{-1}, or elevated [O_3] of 1.23 × current concentration (in ambient [CO_2]) using the FACE design of Miglietta et al. (2002) with modifications as described in detail (Morgan et al. 2004; Bernacchi et al. 2005). Net photosynthesis of mature leaves at the top of canopy was measured at midday in the field, using a portable gas exchange system (Li-Cor 6400; Li-Cor, Inc., Lincoln, NE, USA) as described in detail (Leakey et al. 2006). Measurements were made under growth conditions of PPFD, [CO_2] and RH from leaves on three separate plants per plot.

Protein carbonyl content of aqueous extracts

For experiments with *Arabidopsis*, either whole shoots or leaves as specified were harvested directly into liquid nitrogen. For the open-top chamber experiment with soybean, tissue samples for the protein carbonyl assay (~0.5 g FW) were obtained from leaf 8 and 14 (counting acropetally) at 11 and 14 weeks after planting, from three plants per chamber. For the experiment at SoyFACE, entire mature leaves at the top of the canopy were excised at midday, wrapped in aluminum foil, immediately plunged into liquid nitrogen, and stored at −80°C until analysis. Samples were pooled by leaf position and chamber, ground to a powder in liquid N_2, and mixed with 50 mg of polyvinylpolypyrrolidone. Each tissue preparation was then

Table 1 Seasonal 12-h (08:00–20:00 h EST) daily average CO_2 and O_3 concentrations in the open-top chamber experiment with soybean

Treatment	CO_2 (μmol mol^{-1})	O_3 (nmol mol^{-1})	T_{max} (°C)	T_{avg} (°C)	PPFD (mol m^{-2} day^{-1})	RH (%)	Leaf T (°C)
Ambient	362 ± 1	59 ± 1	32 ± 1	27 ± 1	42 ± 1	58 ± 1	
Control	362 ± 1	29 ± 1					33.5 ± 0.4[a]
Elevated CO_2	709 ± 1	29 ± 1					34.2 ± 0.4[b]
Elevated O_3	366 ± 1	76 ± 1					33.8 ± 0.4[a,b]
Elevated CO_2 and O_3	704 ± 1	72 ± 1					34.1 ± 0.4[a,b]

Plants were treated with charcoal-filtered air (CF) or CF air plus O_3 in combination with ambient or elevated CO_2 concentrations. The treatments were: (a) charcoal-filtered (CF) air and ambient CO_2 (control); (b) CF-air and elevated CO_2 (elevated CO_2); (c) CF-air plus O_3 and ambient CO_2 (elevated O_3); and (d) CF-air plus O_3 and elevated CO_2 (elevated CO_2 and O_3). Seasonal meteorological conditions [maximum temperature (T_{max}), average temperature (T_{avg}), photosynthetic photon flux density (PPFD), and relative humidity (RH) are daytime averages (PPFD > 50 μmol m^{-2} s^{-1})]. Seasonal average temperatures of upper canopy leaves (abaxial surface) are also shown. Values shown are means ± SE. Statistically significant differences in leaf temperature among treatments are indicated by a different letter ($P \leq 0.05$)

mixed with 4 ml of 100 mM Tris–HCl (pH 7) containing 1 mM EDTA and 0.25% (w/v) ascorbic acid. Samples were centrifuged (15,000*g*) for 10 min, and the supernatants were filtered through 0.45 μm nylon filters. Filtered supernatants (2.5 ml) were desalted using gel-filtration (Sephadex G-25, PD-10 columns, Sigma Chemical Co.) and 100 mM Tris–HCl buffer (pH 7). Protein carbonyl concentration was determined by derivatization with DNPH, as described previously (Levine et al. 1994) with some modifications. Protein in duplicate, desalted plant extracts were precipitated in 85% saturation ammonium sulfate. Samples were incubated on ice for 30 min, centrifuged (15,000*g*) for 10 min, and supernatants were discarded. One pellet of a duplicate sample was dissolved in 0.5 ml of 10 mM DNPH derivatization solution while the second pellet (blank) was dissolved in 0.5 ml of 6 M guanidine–HCl buffer containing 3.3% (v/v) concentrated phosphoric acid (pH 2.5) (pH adjusted using 10 M KOH). The DNPH derivatization solution consisted of 283 mg of DNPH dissolved in 3.3 ml of concentrated phosphoric acid added to a final volume of 100 ml of 6 M guanidine–HCl buffer (pH 2.5). The 0.5 ml samples were applied to PD-10 columns followed by 1.8 ml of guanidine buffer. The eluent was discarded. A 1.5 ml-aliquot of guanidine buffer was added to each column and the eluent collected. Absorbance of the collected solutions was measured at 370 nm. The absorbance value of blank samples was deducted from the absorbance value of derivatized samples, and carbonyl concentration of the sample was computed using an extinction coefficient for hydrazone of 22 mM cm^{-1}. Protein concentration in the blank samples was measured using the BioRad assay with BSA as a standard (Bradford 1976). Absorbance interference in the BioRad assay due to guanidine buffer was deducted from calculations of total protein. Treatment effects and means were estimated using a repeated measures, mixed model analysis of variance (SAS Proc Mixed; (Littell et al. 1996). Data were ln-transformed prior to statistical analysis.

Detection of protein carbonylation by immunoblotting

Powdered leaf tissue was ground in a chilled mortar (0.5 g fresh weight ml^{-1}) in protein extraction buffer (PEB) containing 100 mM Tris–HCl, pH 8.0, 2% (v/v) β-mercaptoethanol, 5 mM EGTA, 10 mM EDTA, 1 mM AEBSF, 10 μg ml^{-1} leupeptin, 1 mM *p*-amino-benzamidine, 5 mM 6-aminohexanoic acid, 2 μM E64, 10 μM MG132, 0.2 μM MCLR, 10 mM NaF, 1 mM DPTA, and 1 mM BHT. The leaf soluble protein extracts were mixed with two volumes of 10 mM DNPH in 2 M HCl at room temperature for 30 min with gentle agitation. A control sample was mixed with two volumes of 2 M HCl. Five volumes of ice-cold phenol (Tris-buffered, pH 7.9) was added to each tube. After vortexing for 1 min, the mixture was centrifuged for 10 min at 10,000*g*. The upper phase was removed and discarded leaving the interface intact, and the phenol phase was re-extracted twice with ice-cold Tris–HCl buffer (50 mM, pH 8.0). Five volumes of cold 0.1 M ammonium acetate in methanol was added to the lower phase and incubated at −20°C overnight. The following day, the mixture was centrifuged at 10,000*g* for 20 min, and the resulting pellets were washed three times with 1 ml 0.1 M ammonium acetate in methanol and once with 1 ml cold ethanol. To each pellet, IEF buffer [containing 7 M urea, 2 M thiourea, 4% v/v CHAPS, 65 mM DTT, 20 μl 0.1% bromophenol, and 20 μl IPG buffer, pH 4–7] was added. The samples were incubated for 2 h at room temperature, sonicated at low power, and incubated for 1 h at room temperature. Insoluble material was removed by centrifugation at 10,000 *g* for 20 min at 4°C and the supernatant was transferred to a fresh tube. For 1-DE analysis, SDS-PAGE was performed with 10% or 12% gels with a loading of 10 μg protein samples in each lane. Resolved proteins were electrophoretically transferred to Immobilon-P (PVDF, Millipore) membranes and oxidized proteins were detected using anti-DNP antibodies (DakoCytomation, Denmark).

Protein fractionation and 2-DE analysis

Frozen soybean leaf tissue was powdered in liquid nitrogen and fractionated following the protocol shown schematically in Figure S1. Approximately 1 g leaf powder was extracted in 2 ml of PEB in a chilled mortar and pestle. The homogenate was transferred to a tube and centrifuged at 2,700*g* for 10 min. The supernatant was transferred to a fresh tube and centrifuged at 35,000*g* for 30 min and the resulting supernatant was transferred to a fresh tube and frozen at −80°C overnight. The initial pellet, which contained organellar membranes, was resuspended in 1 ml PEB and stored at −80°C and is referred to as the "membrane fraction." The following day, the frozen 35,000*g* supernatant was allowed to thaw at room temperature for 1 h. After centrifugation at 15,000*g* for 10 min, the supernatant ("Fraction II") was transferred to a fresh tube and the pellet ("Fraction I") was resuspended with 1 ml PEB (see Figures S1 and S2 and Supporting Online Material for additional details). The fraction II protein samples were used for 2-DE analysis as described below following treatment with the 2-D Clean-Up Kit from GE Healthcare.

For difference gel electrophoresis (DIGE), 50 μg of each sample was labeled with 400 pmol of Cy™ dye as specified by the manufacturer (GE Healthcare). The ambient sample was labeled with Cy3 and the experimental treatments with Cy5. The internal standard was a pooled sample composed of equal amounts of each of the three samples that had been mixed and labeled with Cy2 dye. For

2-DE analysis, the first dimension was conducted with an Amersham Ettan™ IPGphor isoelectric focusing (IEF) system using mixtures of ambient, elevated [CO_2], and internal standard protein for gel 1 and ambient O_3, and internal standard protein for gel 2. For each gel, total protein (500 µg) was actively loaded onto Immobiline™ DryStrip pH 3–11 (24 cm) strips and focused on the IPGphor IEF system for a total of 20 h (68 kVh) at 20°C. After focusing, the strips were equilibrated for 10 min in 10 ml of equilibration buffer containing first DTT followed by buffer containing iodoacetamide. The equilibration buffer contained 50 mM Tris–HCl, pH 8.8, 30% v/v glycerol, 2% w/v SDS, 6 M urea, and 65 mM DTT or 125 mM iodoacetamide, as specified. The strips were then placed onto 12% SDS-PAGE gels and run for 6 h. Fluorescent gel images were obtained using a Typhoon™ 9400 multilaser scanner before post-staining with SyproRuby for total protein. Images were analyzed using the DeCyder Differential Analyzer software (GE Healthcare) and gel plugs were taken using the Ettan™ Spot Handling Workstation.

In-gel trypsin digestion and MALDI-ToF MS analysis

Gel plugs were rinsed with 50 mM ammonium bicarbonate in 50% acetonitrile (ACN) and allowed to air dry. Modified trypsin (Genotech, St. Louise, MO) was added to a final concentration of 0.0125 units μl^{-1} in 50 mM ammonium bicarbonate to the gel plugs and allowed to digest for 4 h. The solution was then removed and an aliquot was mixed with saturated HCCA in 50% ACN containing 0.1% TFA. An aliquot (usually 0.3 µl) was spotted onto a GE Healthcare probe and allowed to air dry. MS analysis was performed with an Amersham Ettan™ MALDI-ToF/Pro spectrometer, and spectra were analyzed using Mascot to search the NCBInr database.

Chlorophyll measurement

Chlorophyll concentration in tissue samples from the open-top chamber experiment was determined following extraction twice with 3 ml of 95% ethanol overnight at 4°C. Extracts were pooled by sample, and absorbance at 649 and 665 nm was measured. Chlorophyll concentration was calculated as previously described (Lichtenthaler and Wellburn 1983).

Results and discussion

Elevated [CO_2] increases leaf protein carbonylation in *Arabidopsis*

We conducted experiments to determine whether growth of plants at elevated [CO_2] would reduce protein carbonylation as expected (see 'Introduction'). Surprisingly, the opposite was observed, but the effect depended on length of exposure. This is illustrated with a 1-DE analysis of changes in protein carbonylation in *Arabidopsis* leaves as a function of time of exposure to elevated [CO_2] (Fig. 1). In plants maintained at ambient [CO_2], protein carbonylation was clearly observed and was specific, as the immunoblot signals did not simply reflect protein abundance and only the samples derivatized with DNPH (lanes marked "D") were immunoreactive; the underivatized samples (lanes marked "H" in Fig. 1) and also the lane of marker proteins were completely free of signal. In contrast to an earlier report (Johansson et al. 2004), protein carbonylation during development of *Arabidopsis* plants in our experiments was rather constant at ambient [CO_2]. However, the most important point to note from the results presented in Fig. 1 is that plants at elevated [CO_2] had greatly increased protein carbonylation levels early in plant growth (e.g., days 9 and 13 samples) whereas later in development (e.g., days 23 and 28) the level of protein carbonylation was slightly lower than in ambient [CO_2] plants. The change in effect of elevated [CO_2] with time of exposure is consistent with the conclusion (Johansson et al. 2004) that developmental factors are crucial in the control of leaf protein carbonylation.

In the experiment presented in Fig. 1, whole shoots were harvested at different stages of development and analyzed for protein carbonylation. To determine how elevated [CO_2] affected expanded versus expanding leaves, *Arabidopsis* plants were grown for 3 weeks at ambient [CO_2] and then transferred for 5 days to elevated [CO_2] or maintained under ambient conditions. At daily intervals, the four oldest rosette leaves were harvested separately from the remaining rapidly expanding leaves. As shown in Fig. 2, young and old leaves of plants at ambient [CO_2] contained numerous carbonylated proteins, with prominent carbonylation of Rubisco large subunit. The same was true for plants transferred to elevated [CO_2], but it is visually apparent from the immunoblot results that protein carbonylation was greatly enhanced in both young (expanding) and old (fully expanded) leaves (Fig. 2a). Quantitation of the immunoblot results by densitometry (Fig. 2b, left panel) confirmed that at ambient [CO_2], protein carbonylation in expanded leaves was low and constant over the 5-day experimental period, whereas young leaves initially had higher carbonylation levels (day 0 sample) that decreased progressively as leaves expanded. Upon transfer of plants to elevated [CO_2], there was a rapid initial increase in protein carbonylation ($\sim$1.6-fold) in old leaves that then remained relatively constant (Fig. 2b, right panel). Young expanding leaves, in contrast, continued to increase in protein carbonyl content throughout the 5-day period. Thus, both young and old

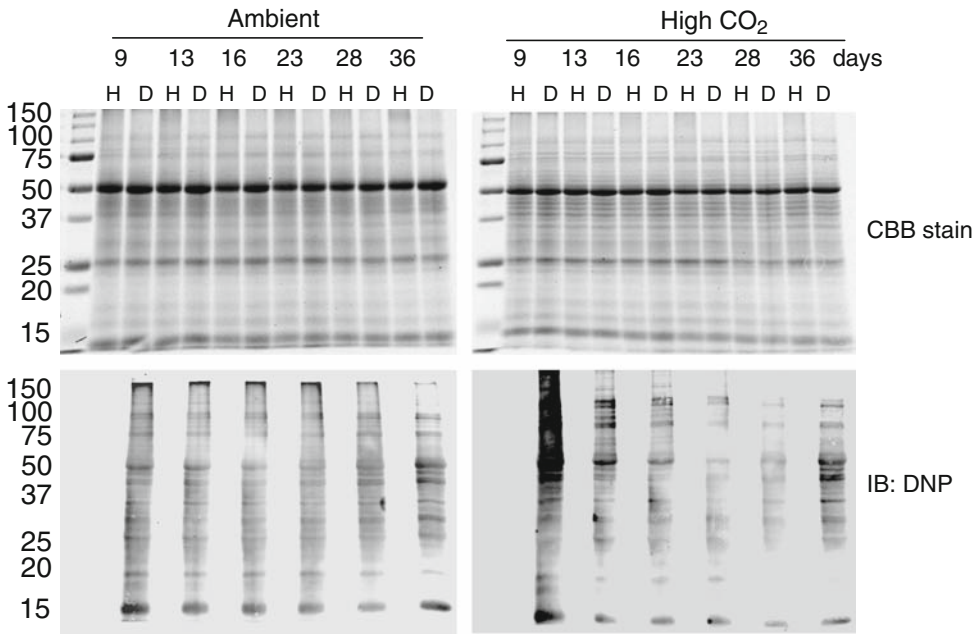

Fig. 1 Elevated [CO_2] increases protein carbonylation detected immunochemically with anti-DNP antibodies following derivatization with DNPH in HCl ('D' lanes). Lanes marked 'H' are controls treated with HCl. *Arabidopsis* plants were grown from sowing at ambient or elevated (1,000 ppm) [CO_2] and shoots were harvested as noted between 9 and 36 days. Upper panels, Coomassie Brilliant Blue (CBB)-stained gels; lower panels, immunoblots probed with anti-DNP antibodies

leaves responded to elevated [CO_2], but relative to ambient [CO_2], the biggest difference was observed in expanding leaves. Collectively, these results suggest that elevated [CO_2] may be associated with an oxidative stress that can be manifested as increased protein carbonylation. The observed decreases in leaf protein carbonyl content during development could reflect changes in antioxidant capacity and/or increased degradation of carbonylated proteins.

Elevated CO_2 and O_3 increase protein carbonylation in soybean

The impact of elevated [CO_2] and [O_3] on soybean 'Essex' plants was assessed in plants grown in open-top field chambers to provide reciprocal treatments of altered [CO_2] and [O_3] (Table 1). The advantage of the open-top chamber is that air can be charcoal filtered to reduce [O_3] substantially below ambient levels, which are often already phytotoxic. Thus, in the present experiment, the control chamber had a seasonal, 12 h daytime average [CO_2] equivalent to ambient [CO_2] (362 μmol mol^{-1}) but [O_3] was about one-half of the ambient level. Elevated [CO_2] was approximately double that of the control and elevated [O_3] was approximately 2.6-fold higher than the control. At 11 and 14 weeks after planting, tissue samples for protein carbonyl content (2-cm diameter leaf disks) and chlorophyll assays (1.3-cm diameter leaf disks) were obtained from mainstem leaf 8 and leaf 14 (counting acropetally). Mid-canopy (leaf 8) and terminal (leaf 14) mainstem leaves were initiated within a 1-week period around 5 and 8 weeks after planting, respectively, in all the treatments. As shown in Table 2, protein carbonyl content was increased by elevated [CO_2] in leaf 8 at 14 weeks and in leaf 14 at 11 weeks, and by elevated [O_3] in both leaves at both sampling times. The combination of the two treatments did not result in greater carbonylation. The most important result from this experiment is that elevated [CO_2] increased protein carbonylation (at least on some sampling dates), similar to O_3, a known pro-oxidant. In contrast to the results with *Arabidopsis*, however, there was no apparent reduction in protein carbonylation after long-term exposure to either treatment. Interestingly, across all treatments, protein carbonyl content was negatively correlated with leaf Chl content (Fig. 3). That the extent of oxidative stress, manifest as protein carbonylation, was associated with loss of Chl from leaves of soybean 'Essex' grown at elevated [CO_2] or [O_3] suggests that both treatments lead to the more visible symptoms of leaf senescence, which is generally considered to be an oxidative process (Pastori and del Rio 1997).

Average midday leaf surface temperature was 0.7°C higher at elevated [CO_2] compared with the ambient CO_2 treatment (Table 1), which is typically attributed to lower stomatal conductance under elevated [CO_2]. Plants grown at elevated [CO_2] at SoyFACE consistently have reduced stomatal conductance and evapotranspiration, and therefore have increased canopy temperatures (Bernacchi et al. 2007). The increased leaf temperature at elevated [CO_2] might impose a mild oxidative stress, although higher leaf temperatures would likely have their most significant impacts at supra-optimal growth temperatures. Nonetheless, a possible role for temperature may be important to consider.

Experiments were also conducted with soybean 'P93B15,' an indeterminate cultivar, grown in the field at

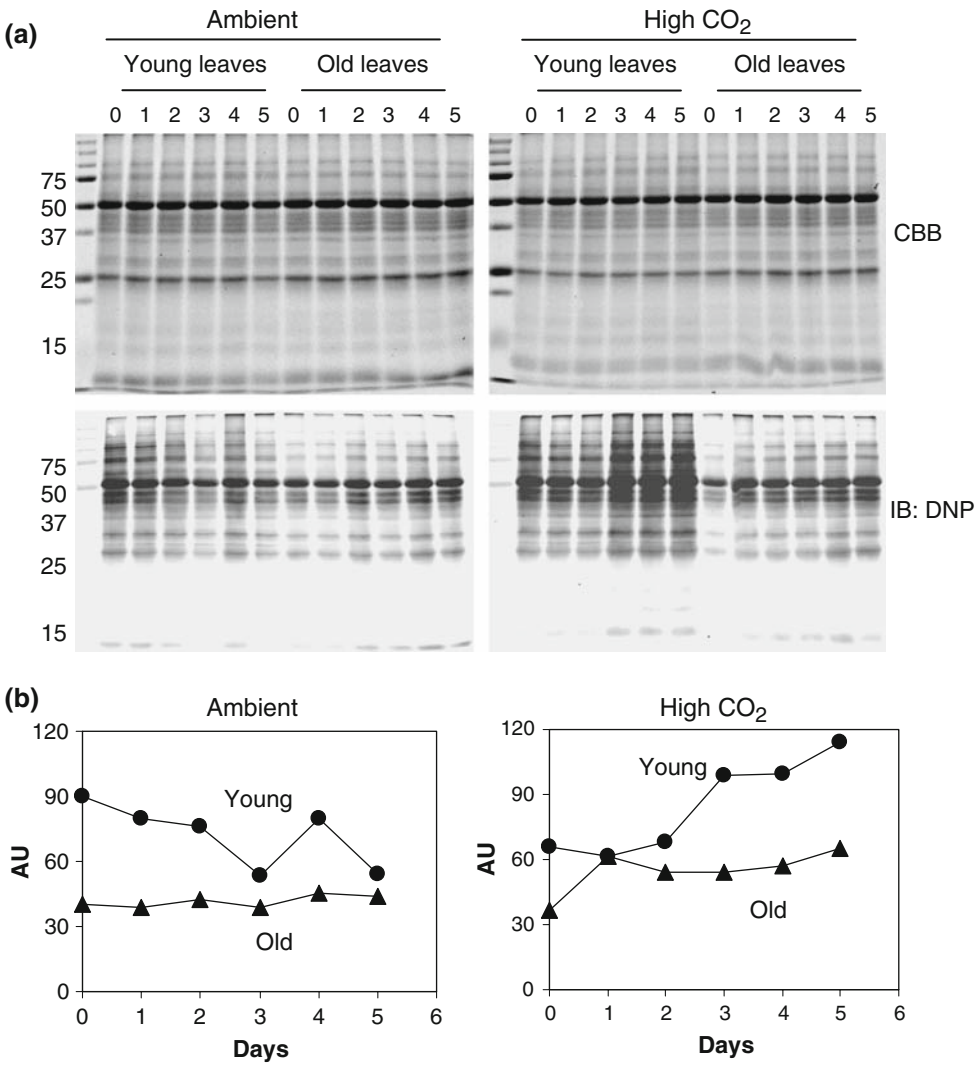

Fig. 2 Elevated CO_2 increases protein carbonylation in expanding and mature *Arabidopsis* leaves following transfer from ambient to elevated [CO_2] (1,000 ppm). (**a**) CBB-stained gels and immunoblot analysis with anti-DNP antibodies following derivatization of soluble proteins with DNPH. Numbers above the gel are the days the leaves were exposed to elevated [CO_2]. (**b**) Quantitation of the immunoblot results by densitometry using the Li-Cor Odyssey (AU, relative absorbance units)

Table 2 Leaf protein carbonyl content in soybean 'Essex' grown in open-top field chambers at low and high concentrations of O_3 and at ambient (361 μmol mol^{-1}) or elevated (709 μmol mol^{-1}) CO_2

Leaf position	Weeks after planting	Charcoal-filtered air		Added O_3	
		Ambient CO_2 (nmol carbonyls mg^{-1} protein)	Elevated CO_2	Ambient CO_2	Elevated CO_2
Leaf 8	11	$16.5 \pm 2.4^{a,A}$	$20.2 \pm 2.9^{a,c,A}$	$25.7 \pm 3.7^{b,c,A}$	$25.2 \pm 3.6^{b,c,A}$
	14	$17.3 \pm 2.5^{a,A}$	$42.9 \pm 6.2^{b,B}$	$42.7 \pm 6.2^{b,B}$	$55.9 \pm 8.1^{b,B}$
Leaf 14	11	$17.5 \pm 2.5^{a,A}$	$26.3 \pm 3.8^{b,A,C}$	$24.9 \pm 3.6^{b,A}$	$28.4 \pm 4.1^{b,A}$
	14	$23.8 \pm 3.4^{a,A}$	$32.8 \pm 4.8^{a,c,C}$	$36.7 \pm 5.3^{b,c,B}$	$36.4 \pm 5.3^{b,c,C}$

Values shown are means ± SE. Statistically significant differences among treatments in each row are indicated by different lower case letters; significant differences among leaf position and sampling period in each column are indicated by different upper case letters ($P \leq 0.05$)

SoyFACE. Fully expanded leaves at the top of the canopy were harvested on two consecutive days in 2005. At stage 4a (pod formation; day 213), there was a 22% stimulation of leaf net assimilation rate by elevated [CO_2] that was statistically significant, whereas on the following day (stage 4b; day 214), assimilation rates were overall reduced and the CO_2 stimulation was not significant (Fig. 4a). The depression of net assimilation rates and the lack of CO_2 enhancement were associated with a 2°C increase in daytime maximum temperature from 30°C (day 213) to 32°C (day 214) and with increased leaf protein carbonylation levels (Fig. 4b). These results suggest

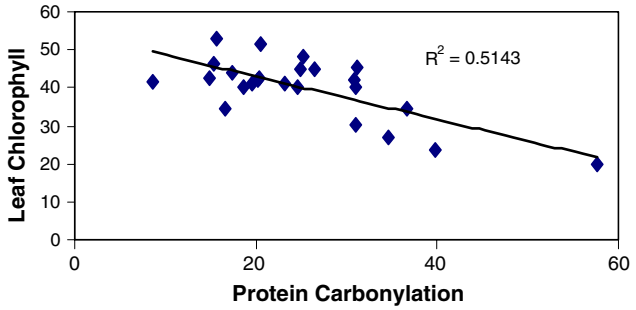

Fig. 3 Protein carbonylation is associated with loss of leaf Chl in soybean 'Essex' plants grown at elevated [CO_2] in open-top chambers in the field. Data from Table 2

that temperature and [CO_2] may be interacting factors affecting the extent of protein carbonylation, and secondly, that carbonylation of leaf proteins may be associated with loss of photosynthetic capacity under some conditions. Furthermore, it appears that protein carbonylation is very dynamic and can increase substantially within a timeframe of hours (i.e., from days 213 to 214).

Based on the results obtained in 2005 (Fig. 4), we monitored leaf protein carbonylation in recently fully expanded leaves of soybean P93B15 at several stages of development in 2006. At stage 2a (full bloom; day 198) and stage 4a (pod formation; day 213), leaves of plants grown at elevated [CO_2] had slightly reduced levels of protein carbonylation relative to ambient [CO_2], whereas at stage 5a (pod fill; day 237), increased protein carbonylation at elevated [CO_2] was observed (Fig. 5a). Increased levels of protein carbonylation at elevated [CO_2] at day 237 were accompanied by reductions in the stimulation of net assimilation by elevated CO_2 (Fig. 5b), and across both experiments in 2005 and 2006, there was an overall negative relationship between protein carbonylation at elevated [CO_2] and CO_2 enhancement of Pn (Fig. 5d).

In the 2006 samples, we also measured the protein content of APX1, which is an important ROS-scavenging enzyme in the cytoplasm (Davletova et al. 2005). At all three sampling dates, leaves of plants grown at elevated [CO_2] had about 20% higher levels of APX1 protein compared to leaves of plants at ambient [CO_2] (Fig. 5c). While changes in APX1 protein content cannot explain the increased carbonylation at stage 5a, the observation that elevated [CO_2] increased APX1 protein suggests that the treatment may involve an unexpected oxidative stress. Consistent with this (Cheeseman 2006), reported that elevated [CO_2] or O_3 increased concentrations of H_2O_2 in leaves of soybean 'P93B15' relative to plants at ambient conditions, and it is well known that APX1 is transcriptionally regulated by ROS. Furthermore, transcript levels of APX1 are elevated 20 to 30% in Arabidopsis leaves by elevated [CO_2], elevated [O_3], or H_2O_2 (http://www.genevestigator.ethz.ch). Two recent studies

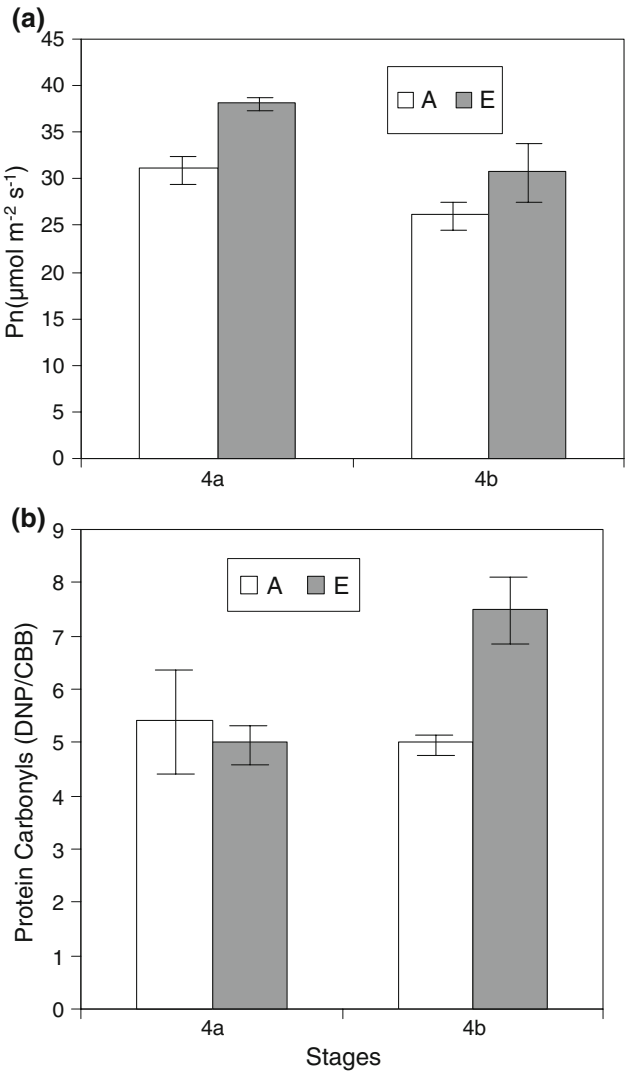

Fig. 4 Photosynthetic parameters in fully expanded soybean 'P93B15' leaves of plants grown at ambient or elevated [CO_2] at SoyFACE. (**a**) Leaf photosynthetic rate and (**b**) leaf protein carbonyl content measured at stages 4a (day 213) and 4b (day 214) in 2005. In this figure, protein carbonyl content was normalized for the measured protein content, by CBB staining, in the samples analyzed. A = ambient [CO_2]; E = elevated [CO_2]

have also shown that elevated [CO_2] upregulated several genes that respond to changes in ROS levels including APX1, NADPH oxidase, glutathione *S*-transferase, as well as other genes that respond to oxidative stress and/or detoxification processes (Miyazaki et al. 2004; Li et al. 2006). Collectively, these reports in the literature and the results obtained in the present study suggest that elevated [CO_2] may result in oxidative stress that is manifest as increased APX1 transcript and protein, and increased protein carbonylation in leaves. Protein carbonylation was associated with reduced stimulation of Pn by CO_2 and, in some cases, loss of chlorophyll. Factors that may influence the oxidative stress imposed by elevated [CO_2] include

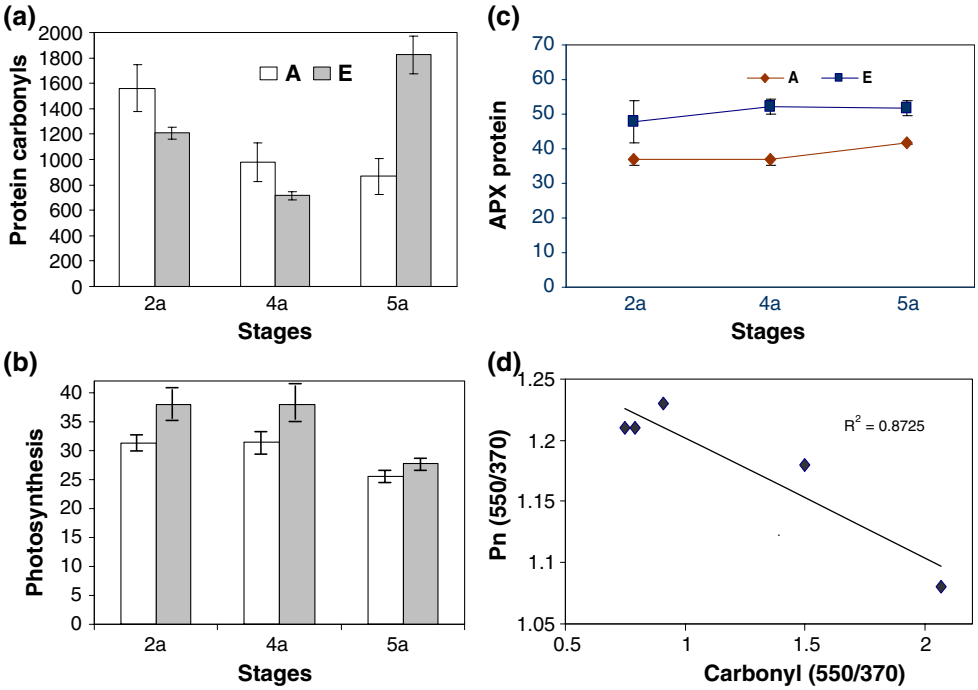

Fig. 5 Effect of elevated [CO_2] and stage of plant development on soybean 'P93B15' leaf (**a**) protein carbonylation, (**b**) leaf photosynthetic rate, and (**c**) content of APX1 protein, measured by immunoblotting. The most recently fully expanded trifoliolate leaf was harvested at stages 2a (full bloom; day 198), 4a (pod formation; day 213), and 5a (pod fill; day 237). A = ambient [CO_2]; E = elevated [CO_2]. (**d**) Inverse relationship between stimulation of Pn at elevated [CO_2] and leaf protein carbonyl content, with both parameters expressed as a ratio of values obtained for plants grown at elevated [CO_2] (550 ppm) versus ambient [CO_2] (370 ppm)

temperature and plant genotype, and will be examined in future studies.

Elevated [CO_2] and [O_3] alter the abundance of a subset of soybean leaf proteins from SoyFACE

If both elevated [CO_2] and O_3 induce oxidative stress, then the two treatments should also affect the steady-state level of a similar subset of leaf proteins. To test this postulate, two-dimensional (2-DE) DIGE (Ettan^{TM} DIGE) was performed. In this procedure, soybean leaf protein samples from different treatments (e.g., ambient and elevated [CO_2], and elevated O_3) can be prepared and individually labeled prior to electrophoresis with one of two spectrally resolved fluorescent tags, usually Cy^{TM}3 or Cy5. The differentially tagged samples are then mixed and electrophoresed on the same 2-DE gel (Alban et al. 2003). The methodology is even more robust as use of a third dye, Cy2, provides a label that can be applied to a common pooled sample to allow for multiple gels to be compared as was done in the present study. For these experiments, we also utilized a simple procedure, described in Figure S1 and characterized further in Figure S2, to remove Rubisco protein from soybean leaf extracts. The removal of the high-abundance Rubisco holoenzyme was a useful approach to enrich and characterize the other, lower abundance leaf proteins by 2-DE. The utility of removing high-abundance proteins prior to 2-DE is well recognized (Chromy et al. 2004). In the experiment depicted in Fig. 6, we compared ambient and elevated [CO_2] samples in one gel while a second gel compared ambient and elevated [O_3] samples; a pooled sample containing equal amounts of the ambient, elevated [CO_2], and elevated [O_3] proteins served as the internal standard that was present in both gels. Shown in Fig. 6 is the SyproRuby-stained gel of the pooled protein sample.

The gels were imaged with the Typhoon Imager and compared and quantified across both gels with the Amersham DeCyder^{TM} software platform using the pooled standard in the analysis. Approximately 800 distinct protein spots were identified by the image analysis, and it was determined that 31 proteins were altered in abundance by *both* elevated [CO_2] and elevated [O_3] ($P < 0.05$; difference in abundance greater than 1.2-fold compared to ambient). We conducted this analysis to determine whether there would be any common changes in abundance of proteins by O_3, known to cause oxidative stress, and elevated [CO_2], which we speculate can also involve an oxidative stress component. The observation that the abundance of a number of proteins is affected similarly by elevated [CO_2] and [O_3] is consistent with the notion that elevated [CO_2] may also involve an oxidative stress. Of the 31 proteins that were altered in abundance by both treatments, four proteins were increased in abundance (open squares in Fig. 6) and the others were decreased in abundance (open circles in Fig. 6). For clarity, spot numbers are only shown for proteins that have been identified at this time; arrows and numbers correspond to proteins that were not altered in abundance relative to the ambient control by 1.2-fold and are identified in Table 3.

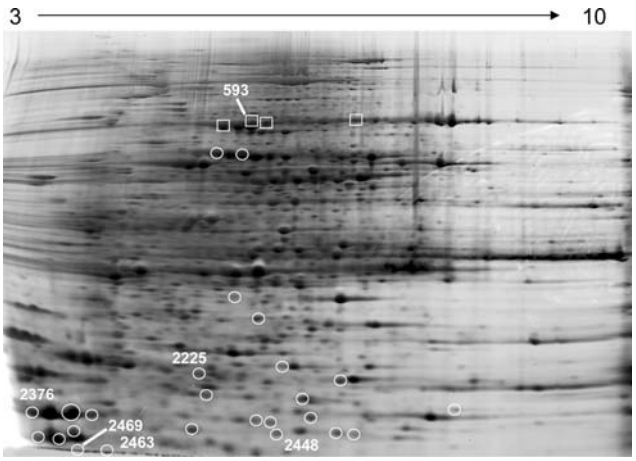

Fig. 6 Growth of soybean 'P93B15' at elevated [CO_2] or elevated [O_3] alters the abundance of a common subset of leaf proteins. Rubisco protein was largely removed from the leaf samples by the procedure described in Figure S1 and the samples were then compared by DIGE analysis as described in Methods. The proteins that were differentially expressed (≥1.2-fold; $P < 0.05$) in response to both elevated [CO_2] and [O_3] treatments are circled (decreased abundance) or enclosed in squares (increased abundance). The spot numbers of those identified by mass spectrometry are shown. Additional proteins that were not differentially affected by the two treatments, but have been identified by mass spectrometry, are listed in Table 3 but for simplicity are not shown in the figure. The gel shown was post-stained with SyproRuby and the pH gradient is indicated at the top of the gel

Of most interest are proteins altered in abundance by both the elevated [CO_2] and [O_3] treatments. Both treatments increased the abundance of the β-subunit of ATP synthase (spot 593) and three additional proteins that could not be identified by MALDI-ToF MS. In addition, APX1 was also identified (spot 1608) and was also increased in abundance by both treatments, but only 1.15-fold relative to ambient, which is similar to the 20% increase determined by immunoblotting. Whether this is a physiologically relevant increase remains to be determined but is certainly consistent with the notion that elevated [CO_2], like elevated [O_3], involves an oxidative stress component.

The majority of the proteins affected by both treatments were reduced in abundance relative to ambient [CO_2], and included ferredoxin-thioredoxin reductase (spot 2225), a hypothetical protein (spot 2469), and Gly-rich protein (spots 2376, 2448, and 2463). The hypothetical protein and the Gly-rich protein were both small proteins and similar in composition, being enriched in Arg, Gly, and Trp residues (data not shown), suggesting some possible common function and regulation by environmental conditions.

Concluding remarks

The original hypothesis that growth at elevated [CO_2] would reduce oxidative stress and protein carbonylation was not supported by the majority of the results obtained in the present study. Rather, the results obtained collectively support the notion that exposure to elevated [CO_2] can result in oxidative stress, as evidenced by: (i) increased protein carbonylation in Arabidopsis and soybean, and (ii) altered abundance of a set of proteins also affected by exposure of plants to elevated [O_3], a known oxidative stressor (Fig. 6). One of the proteins that was increased in abundance was cytosolic APX1, which plays a key role in cross-compartment regulation of ROS removal in plants (Mittler 2002) and is known to be induced at the transcriptional level in response to oxidative stress (Yoshimura et al. 2000; Fourcroy et al. 2004). The present study is purely descriptive and does not provide any information about the mechanism(s) responsible for protein carbonylation in response to elevated [CO_2]. However, we speculate that the unexpected oxidative stress component of elevated [CO_2] may contribute to the leaf chlorosis that has been reported in several studies (Sicher 2001; Booker and Fiscus 2005) and indeed was also observed in the present study with soybean 'Essex' (Fig. 3). Our results are also consistent with recent studies that suggested that elevated [CO_2] can increase ROS production in vivo. For example, the bicarbonate-induced stomatal closure in epidermal peels of *Arabidopsis* was shown to be the result of increased H_2O_2 production in guard cells (Kolla et al. 2006; Kolla et al. 2007). Likewise, Cheeseman (2006) found that leaves of soybean plants grown at elevated [CO_2] had higher levels of H_2O_2 compared to plants at ambient [CO_2], although measurement of tissue H_2O_2 content can be difficult and problematic (Queval et al. 2008). How elevated [CO_2] might promote H_2O_2 production is not clear, but it is possible that bicarbonate may interact directly with iron or heme derivatives to form complexes with an altered redox potential that favors increased generation of ROS (Arai et al. 2005).

While H_2O_2 is not chemically reactive enough to directly result in protein carbonylation, it has been demonstrated in several in vitro systems that various reactive intermediates interact with CO_2/HCO_3^- to form even more reactive compounds that can catalyze the generation of protein carbonyls. For example, the highly reactive carbonate radical anion (CO_3^-) is thought to form when a complex of peroxide with CO_2 is reduced by a protein–Cu(I) complex (Ramirez et al. 2005). Similarly, bicarbonate has been shown to enhance peroxide-catalyzed oxidation of proteins as a result of formation of the reactive peroxymonocarbonate ion, HCO_4^- (Richardson et al. 2003). These are all possibilities that may help explain the paradoxical results obtained in the present study and the observation that elevated [CO_2] upregulates many genes associated with redox control (Miyazaki et al. 2004; Li et al. 2006). Future work will be necessary to understand the impact of protein carbonylation on leaf photosynthetic activity, and the possible significance of elevated [CO_2]-

Table 3 Proteins identified in soybean leaves by mass spectrometry

Spot number	Similar protein	Organism (Gi accession)	MALDI E-value	Q-TOF score
572	Catalase 4	*G. max* (17865456)	0.084	–
595	Catalase 4	*G. max* (17865456)	0.001	–
593	ATP synthase beta subunit	*C. myrtifolia* (5001593)	0.011	–
639	Catalase 4	*G. max* (17865456)	0.001	–
661	ATP synthase beta subunit	*C. myrtifolia* (5001593)	0.000	–
799	Glyceraldehyde-3-phosphate dehydrogenase (NADP)	*N. tabacum* (82167)	0.013	–
832	Glutamine synthetase	*G. max* (13877511)	0.036	–
850	Glutamine synthetase	*G. max* (13877511)	0.042	–
1447	ADP-glucose pyrophosphorylase	*L. esculentum* (1778436)	0.047	–
1608	Cytosolic ascorbate peroxidase 1	*G. max* (37196685)	0.002	–
1626	Cytosolic ascorbate peroxidase 2	*G. max* (37196687)	0.034	–
1686	P21 protein	*G. max* (129320)	0.016	–
2225	Ferredoxin-thioredoxin reductase	*G. max* (3913670)	6×10^{-6}	–
2376	Gly-rich protein (fragment)	*D. carota* (1276971)	–	66.9
2419	Rubisco small subunit	*G. max* (1055368)	0.002	–
2448	Gly-rich protein (fragment)	*D. carota* (1276971)	–	76.8
2449	Rubisco small subunit	*G. max* (1055368)	0.001	–
2463	Gly-rich protein (fragment)	*D. carota* (1276971)	–	70.2
2469	Hypothetical protein	*O. sativa* (37533068)	–	45.0

Spot numbers as in Fig. 6. MALDI, MALDI-TOF MS; Q-TOF, LC-MS/MS

G. max, *Glycine max*; *C. myrtifolia*, *Coriaria myrtifolia*; *L. esculentum*, *Lycopersicon esculentum*; *D. carota*, *Daucus carota*. The *E*-value is the expectation score that the identification is incorrect and thus the lower the number the more confidence in the identification. The Q-TOF score is the Mascot MOWSE score (Matrix Science; www.matrixscience.com) where a score above 33 is considered significant ($P < 0.05$)

induced oxidative stress to photosynthetic adaptation and the response of plant growth to elevated [CO_2]. However, we are speculating that perhaps some of the decline in Pn and Rubisco activity at elevated [CO_2] may be a consequence of carbonylation of the protein in addition to a decline in gene expression for Rubisco (Nie et al. 1995) that is typically thought to be the basis for acclimation.

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