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Changes of soluble protein expression and leaf metabolite levels in *Arabidopsis thaliana* grown in elevated atmospheric carbon dioxide

Hanhong Bae*, Richard Sicher

USDA-ARS, Plant Sciences Institute, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, USA

Abstract

Plant responses to elevated atmospheric CO_2 vary with species and with environmental conditions. Rates of dry matter formation were initially enhanced in response to CO_2 enrichment but these accelerated growth rates typically were not maintained over long periods of time. The objective of this study was to better understand the basis for this acclimation process. Changes of metabolite levels and of total protein expression in response to CO_2 enrichment were studied using biochemical assays and two-dimensional gel electrophoresis. *Arabidopsis thaliana* (L.) Henyh. (Columbia ecotype) plants were grown for 2–6 weeks in controlled environment chambers providing 36 (ambient) or 100 (elevated) Pa CO_2. Averaged over all harvest dates above-ground biomass was greater ($P < 0.05$) in the elevated than in the ambient CO_2 treatment but shoot biomass did not differ between treatments on the final harvest. Flowering was delayed by CO_2 enrichment. One or more flowers were observed for 52% and 100% of the elevated and ambient CO_2 grown plants, respectively, after 4-weeks growth. Starch and sucrose levels were increased 132 and 43%, respectively, in leaves of 6-week-old plants in response to CO_2 enrichment. Nitrate varied with plant age, although mean nitrate levels in rosettes were decreased 31% by CO_2 enrichment when averaged over all harvest dates. Chlorophyll, the chlorophyll *a*/*b* ratio, carotenoids and total soluble protein did not differ between CO_2 treatments. Total Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity decreased with plant age and was lower ($P < 0.01$) in the elevated compared to the ambient CO_2 treatment. The above results suggested that acclimation to elevated CO_2 occurred in Arabidopsis without developing symptoms of N-deficiency. A total of 400 major proteins were separated and compared by two-dimensional gel electrophoresis. No proteins appeared de novo or disappeared in response to CO_2 enrichment, although pixel densities for 13 protein spots differed significantly between CO_2 treatments on at least one harvest date. Six of these proteins were identified by mass spectrometry. Three of these identified proteins were involved in plant growth and development or were associated with stress. Two other proteins were encoded by genes with putative functions. Only one protein, the 23 kDa subunit

Abbreviations: OEC23, 23-kDa protein of the oxygen evolving complex; Chl, chlorophyll; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RBCS, rubisco small subunit transcript; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; DTT, dithiothreitol; TBP, tributyl phosphine; IPG, immobilized pH gradient.

* Corresponding author. Tel.: +1 301 504 6632; fax: +1 301 504 5823.
E-mail address: rbae@asrr.arsusda.gov (H. Bae).

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of the oxygen evolving complex (OEC23), was involved in photosynthesis. It was concluded that long-term plant growth in elevated CO_2 caused only small changes in the Arabidopsis proteome.

Keywords: Proteomics; Photosynthetic acclimation; Leaf carbohydrates; CO_2 enrichment

1. Introduction

The photosynthetic capacity of a number of plant species was diminished during prolonged plant growth in CO_2 enriched atmospheres and this acclimation response was attributed to increased carbohydrates and to reduced foliar levels of inorganic and organic N (Sage, 1994; Stitt, 1991; Griffin and Seemann, 1996; Moore et al., 1999). Whole plant development and the presence of C sinks were a critical factor in the onset of photosynthetic acclimation (Stitt, 1991; Sage, 1994). Photosynthetic rates of younger leaves typically did not acclimate to elevated CO_2, possibly because of active sucrose metabolism and altered hormonal conditions (Nie et al., 1995; Van Oosten and Besford, 1995). A critical interaction between nutrient availability and the responses of photosynthesis and growth to elevated CO_2 also has been identified (Stitt and Krapp, 1999). Decreased photosynthetic capacity during CO_2 enrichment was greatest when the N supply was limiting (Farage et al., 1998). Conversely, when the N supply was sufficient photosynthetic acclimation was minimal and there were no major changes in the internal levels of either inorganic or organic N containing leaf constituents.

In most studies total chlorophyll [Chl (a + b)] and photosynthetic proteins such as ribulse 1,5-bisphosphate carboxylase/oxygenase (Rubisco) were the principal N-constituents in leaves modified by CO_2 enrichment. Specifically, growth in elevated CO_2 markedly increased non-structural carbohydrates and concomitantly decreased both transcripts and polypeptides corresponding to the large and small subunits of Rubisco in several species of CO_2-enriched plants including Arabidopsis (Cheng et al., 1998); wheat (Nie et al., 1995), tomato (Van Oosten and Besford, 1995), and pea (Majeau and Coleman, 1996). A diurnal relationship was observed between Rubisco small subunit (RBCS) mRNA levels and leaf carbohydrates in Arabidopsis and wheat (Nie et al., 1995; Cheng et al., 1998). Increased RBCS levels in the dark were correlated with decreased leaf sugars. However, plant responses to CO_2 enrichment were not always consistent. For example, Moore et al. (1999) using corn, parsley, pea and spinach, reported that Rubisco protein was unchanged and transcript levels for the large and small subunits of Rubisco were increased slightly in response to elevated CO_2.

Several other genes involved in photosynthesis were altered by plant growth in elevated CO_2. Nuclear transcripts or enzyme activities for Rubisco activase (Van Oosten and Besford, 1995), carbonic anhydrase (Peet et al., 1986; Majeau and Coleman, 1996) and the Chl a/b binding protein (Van Oosten et al., 1995; Moore et al., 1998) were decreased in elevated compared to ambient CO_2 grown plants. Idso et al. (2001) observed seasonal changes in major 33, 31 and 21 kDa polypeptides in leaves of various citrus species including sour orange trees. These proteins were identified as vacuolar storage proteins and seasonal variations, as well as CO_2 treatment differences, were correlated with periods of rapid tree growth. However, comparatively little is known about the responses of nonphotosynthetic proteins to CO_2 enrichment.

Transcriptional and/or post-transcriptional processes may regulate protein levels during plant growth in elevated CO_2. One suggestion was that soluble carbohydrates and hexoses in particular, repressed gene expression by an intracellular signaling mechanism that was likely mediated by hexokinase (Jang and Sheen, 1997). Alternative carbohydrate dependent signaling mechanisms that are independent of hexokinase also have been reported (Jang et al., 1997). Moore et al. (1998) used the activity of soluble acid invertase in leaves to predict photosynthetic acclimation to elevated CO_2. Species with high acid invertase activities typically exhibited decreased Rubisco content during plant growth in elevated CO_2. These authors concluded that metabolic sucrose cycling may have an important role in carbohydrate signaling and in photosynthetic acclimation to elevated CO_2.

To date, studies of plant gene expression under CO_2 enrichment have primarily employed RNA and protein blots and typically have been limited to a small number of transcripts or proteins primarily associated with photosynthesis. We utilized a proteomics approach, which enabled us to examine four hundred major proteins. *Arabidopsis thaliana* was used because the entire genome was mapped and sequenced (Wortman et al., 2003), which greatly aided protein identification. While Arabidopsis is typically grown at low to moderate light levels, high fluences promote the acclimation responses to CO_2 enrichment described above (Sicher and Kremer, 1994). Therefore, we measured leaf constituents that responded to elevated CO_2 in Arabidopsis before proceeding with protein expression analyses. The objective was to observe acclimation responses in Arabidopsis to CO_2 enrichment under N sufficient conditions. Large plants grown in pots often develop symptoms of N-limited growth in response to CO_2 enrichment (Arp, 1991). Since Arabidopsis is a very small flowering plant, it was not obvious how N availability would be impacted by CO_2 enrichment. We report that 13 proteins responded to CO_2 enrichment on at least one measurement date. Six of the 13 proteins of interest were identified using peptide sequences obtained by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and only one was involved directly in photosynthesis.

2. Materials and methods

2.1. Plant materials

Arabidopsis thaliana (Columbia ecotype) was grown from seed in controlled environment chambers (model M-2, EGC Corp., Chagrin Falls, OH). Seeds were sown in 90 cm square pots filled with a pre-wetted potting medium (Jiffy Mix, Hummert International, Earth City, MI). Up to 24 pots were maintained in rectangular plastic trays that were used for sub-irrigation. Pots were incubated in the dark for 48–72 h at 6–9 °C to stratify the seeds and improve germination. Plants were grown continuously for 6 weeks at either 36 Pa (ambient) or 100 Pa (elevated) CO_2. Pots were thinned to single plants and only vigorously growing seedlings were retained. A 16 h light/8 h dark photoperiod and a 22 °C/18 °C day/night temperature were provided. The irradiance was 350 ± 20 μmol m^{-2} s^{-1} photosynthetically active radiation. Relative humidity was not controlled in this experiment but it was monitored continuously and never fell below 50%. Chamber air CO_2 was controlled by an infrared analyzer equipped with a set point controller (model WMA-3, PP Systems, Haverhill, MA). Week 0 refers to the period of cold treatment and subsequent harvest dates were calculated from the day pots were transferred to controlled environment chambers. Plants were watered twice per week with tap water. A complete mineral solution containing 12 mM NO_3^- and 2.5 mM NH_4^+ (Robinson, 1984) was applied on Weeks 0, 2 and 4 in lieu of water. Rosettes were harvested during the middle of the photoperiod. Above-ground plant parts were dried in a forced-air oven at 70 °C for 2–3 days before determining dry matter. Freshly harvested rosettes (i.e., shoots with flower stalks removed) were placed in sample bags and immediately transferred to liquid N_2 to stop metabolism. Rosettes were either used immediately for laboratory analyses or were stored at −80 °C.

2.2. Biochemical analyses

Frozen leaf material was ground to a powder with liquid N_2 in a mortar and pestle. Samples (0.25 g fresh weight) were extracted with 2 ml of methanol:chloroform:water (63:26:11 containing 52 mM $Tris^+$ base) at 4 °C in a ground glass tissue homogenizer (Sicher, 2001). Homogenates were centrifuged at 5000 × *g* for 10 min and the pellets were washed with 1 ml of 80% methanol and centrifuged as above. Supernatants were combined and partitioned with chloroform:water (1:2). The aqueous alcohol fractions were dried under a stream of N_2 gas at 37 °C and were reconstituted in 1 ml deionized H_2O. Total Chl (*a* + *b*) was determined spectrophotometrically using the organic fraction (Lichtenthaler, 1987). Soluble carbohydrates, NO_3^- and amino acids were quantified using the aqueous fraction according to Sicher (2001). Nitrate was measured by high-pressure liquid chromatography (HPLC) using a 10 mm × 250 mm analytical anion exchange column (Whatman Partisil-10 SAX, Clifton, NJ) according to Thayer and Huffaker (1980). The pellet fraction was gelatinized in 2 ml boiling water and starch was measured as glucose following

hydrolysis with α-amylase and amyloglucosidase (Bergmeyer et al., 1974).

Total Rubisco activity was measured using a coupled enzyme procedure according to Sharkey et al. (1991). Samples were ground to a fine powder as described above and 0.1 g FW was homogenized on ice with a ground glass tissue homogenizer in 1 ml of extraction buffer containing 50 mM Bicine–NaOH (pH 8.1), 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v), glycerol, 1% PVP-40, 0.01% Triton X-100, and 5 mM dithiothreitol (DTT). Extracts were transferred to 1.5 ml microcentrifuge tubes and spun for 3 min at 14,000 × *g*. Supernatants (0.5 ml) were transferred to new tubes and immediately frozen in liquid N_2. Prior to assay Rubisco was activated for 10 min on ice by adding 5 μl each of 0.5 M $NaHCO_3$ and 0.5 M $MgCl_2$ to 0.25 ml thawed extract. Total Rubisco activity was measured at 25 °C by adding 20 μl of the activated extract to 0.98 ml of assay buffer containing 50 mM Bicine–NaOH (pH 8.1), 1 mM EDTA, 15 mM $MgCl_2$, 20 mM NaCl, 10 mM DTT, 10 mM $NaHCO_3$, 2.5 mM phosphocreatine, 0.5 mM ATP, 0.31 mM NADH, 0.6 mM ribulose 1,5-bisphosphate, 2 IU phosphocreatine kinase, 50 IU 3-phosphoglycerate kinase and 50 IU glyceraldehyde 3-phosphate dehydrogenase. Decreased absorbance was measured spectrophotometrically at 340 nm.

2.3. Two-dimensional gel electrophoresis

Total protein extracts were prepared from a liquid N_2 powder of Arabidopsis rosettes essentially as described by H. Theillement and co-workers at the University of Geneva. (http://www.pierroton.inra.fr/genetics/2D/Proteomevert/Protocoles/protocole.geneve. html). Tissue samples (0.5 g FW) were extracted with 5 ml of ice-cold precipitation solution [10% (w/v) trichloroacetic acid (TCA) in acetone containing 0.07% (v/v) 2-mercaptoethanol)] using a ground glass tissue homogenizer. The glass homogenizer was rinsed with 5 ml precipitation solution and the extracts were combined and transferred to 35 ml centrifuge tubes. The extracts were incubated at −20 °C for 1 h and then centrifuged at 27,000 × *g* for 15 min at −20 °C. The supernatants were discarded and the pellets were resuspended in 10 ml of ice-cold rinse solution (0.07% 2-mercaptoethanol in acetone). The resuspened pellets were incubated at −20 °C for 1 h and then centrifuged as described above. The preceding wash steps were repeated two–three times until the supernatants were clear. The washed pellets were dried in a vacuum desiccator for 30 min and proteins were dissolved in 1 ml of resolubilization solution [9 M urea, 1% (w/v) CHAPS, 1% (w/v) DTT and 1% (v/v) Biolytes pH 3–10 (Bio-Rad Corp., Hercules, CA)] at room temperature. The resuspended pellets were agitated in a sonicator bath at room temperature for 5 min. Samples were centrifuged for 5 min at 27,000 × *g* at room temperature and supernatants were stored at −20°C. Prior to use, an aliquot of total protein was precipitated with an equal volume of 10% aqueous TCA, centrifuged and the pellets were dissolved in 1 N NaOH. Total protein concentrations were then measured using a dye binding method (Bradford, 1976).

Except where indicated two-dimensional electrophoresis was performed using equipment and supplies from Bio-Rad Corp., Hercules, CA. Total solubilized protein (0.1 mg for silver-stained gels or 1 mg for Coomassie Brilliant Blue G-250 stained gels) was added to sufficient rehydration buffer [8.3 M urea, 2% CHAPS, 2 mM tributyl phosphine, 0.5% (w/v) Biolytes pH 3–10] to obtain a final volume of 250 μl. Note that isoelectric focusing was performed using equal amounts of protein from the two CO_2 treatments. Samples in rehydration buffer were applied to dry immobilized pH gradient (IPG) strips [Immobiline DryStrip, 180 mm × 3 mm × 0.5 mm, pH 4–7 (Amersham Pharmacia Biotech, Piscataway, NJ)] and were placed gel-side down in an 18 cm focusing tray. The IPG strips were covered with mineral oil to prevent dehydration and were allowed to rehydrate passively for 12 h. Isoelectric focusing was performed in a Protean IEF cell using a preset method with a maximum limit of 50 μA per IPG strip. The focusing temperature was 20 °C and initially 250 V was applied for 15 min. The voltage was increased linearly to 10,000 V over 30 min and then 10,000 V was maintained for a total of 60,000 V h. If necessary the IPG strips were maintained at 500 V until focusing was stopped. The focusing strips were used for electrophoresis immediately or were stored at −80 °C. Prior to electrophoresis focused IPG strips were incubated for 15 min each in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris–HCl, pH 8.80, 20% glycerol, 2% DTT and a trace of Bromophenol Blue)

and equilibration buffer II (6 M urea, 2% SDS, 0.375 M Tris–HCl, pH 8.8, 20% glycerol, 2.75% iodoacetamide and a trace of Bromophenol Blue). Equilibrated strips were placed directly on top of a 20 cm × 20 cm × 1 cm 12% (w/v) separating gel prepared according to Laemmli (1970) and the strips were bonded to the gel with 0.5% agarose in electrode buffer. Electrophoresis was performed in a PROTEAN II apparatus at 6 °C for 14–16 h. Up to six gels were run at one time using 15–30 mA per gel. Gels initially were stained with Silver Stain Plus and were scanned with a FluorS MAX multimager (Bio-Rad Corp., Hercules, CA). Spot intensities and treatment differences were obtained from digitized gel images that were analyzed using PDQuest software (Bio-Rad Corp., Hercules, CA). Essentially, the black and white contrast for a single template gel image was optimized manually and then the background density was subtracted. The software then automatically applied these parameters from the template to all of the other digital images in the dataset. The computer software then assigned pixel densities to all designated spots on each image. Treatment effects on protein expression were then compared as differences in pixel densities.

For peptide identification a second set of gels was stained with Commassie Brilliant Blue G-250. Appropriate protein spots were excised (1–2 mm diameter) and after destaining were digested in solution with trypsin according to the protocol recommended by the University of Minnesota, Mass Spectrometry Facility, (http://www.cbs.umn.edu/mass_spec). Peptide sequences were determined by MALDI-TOF mass spectrometry at the same facility. Arabidopsis proteins were identified via deduced nucleotide sequences using a web-based software program (MASCOT, http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html). The molecular mass (M_r) and isoelectric point (pI) obtained from the protein database and from the two-dimensional gel were then used to confirm protein identity.

3. Results

3.1. Plant growth and dry weight

By Week 2, shoot dry mass was 36% greater in the elevated compared to the ambient CO_2 treatment. This difference was maintained through Week 5, but by Week 6, above ground biomass was similar for both treatments. Thus, after Week 2, plants at ambient conditions actually grew more rapidly than those in the elevated CO_2 treatment. The overall increase in dry matter between 2 and 6 weeks growth was about 600- and 440-fold for the ambient and elevated CO_2-grown plants.

Flowering was delayed in the elevated compared to the ambient CO_2-grown plants. All plants had at least one open flower after 4 weeks growth in the ambient CO_2 treatment. In comparison, only 52% of plants had a visible flower in the elevated CO_2 treatment on this date (data not shown).

There was no visual indication of leaf yellowing during the later stages of plant growth in either treatment. In agreement with this observation, total Chl (a + b) did not differ ($P > 0.05$) between treatments (Table 1). Significant differences between CO_2 treatments also were not detected in the Chl a/b ratio or in the carotenoid (x + c) content ($P > 0.05$) when means for all harvests were compared. Total Chl (a + b) decreased by about one-half between 2 and 6 weeks growth when averaged over both the ambient and elevated CO_2 treatments (Fig. 1).

3.2. Non-structural carbohydrates

Mean foliar starch concentrations were 105% greater ($P < 0.01$) in the elevated than in the ambient CO_2-grown plants when combined over all harvests (Fig. 2A). Although starch levels fluctuated with age ($P < 0.01$) treatment effects were detected throughout the experiment. Leaf sucrose concentrations also differed ($P < 0.01$) between CO_2 treatments (Fig. 2B). Sucrose levels were similar at Week 2 and increased with plant age in both treatments. Between Weeks 3 and 6 mean sucrose concentrations were 1.87 and 2.65 μmol glucose equivalents g^{-1} fresh weight (FW) in the ambient and elevated CO_2 treatments, respectively. There was a substantial age-dependent buildup ($P < 0.05$) of glucose and fructose in both CO_2 treatments (Fig. 2C and D) and maximal hexose levels were detected at Week 6 in both treatments. Averaged over all harvests, mean glucose and fructose levels were unaffected ($P > 0.05$) by CO_2 enrichment.

Table 1
Changes of leaf constituents and Rubisco activity in arabidopsis rosettes in response to plant age and CO_2 treatment

Constituent	Plant age (week)								Probability		
	2		3		4		5	6	Treatment	Age	Interaction
	36[a]	100[a]	36[a]	100[a]	36[a]	100[a]	36[a]	36[a] 100[a]			
Chlorophyll (mg g^{-1} FW)	0.73	0.70	0.63	0.66	0.57	0.53	0.58	0.36 0.52	ns	**	ns
Chlorophyll *a*/*b* ratio	3.58	3.56	3.69	3.79	3.47	3.40	3.48	3.19 3.48	ns	**	ns
Soluble protein (mg g^{-1} FW)	11.44	12.66	17.25	14.26	11.63	10.89	10.62	6.57 10.75	ns	**	**
Nitrate (μmol g^{-1} FW)	200.22	94.45	239.21	225.34	36.08	45.58	170.60	221.12 67.82	**	**	**
Glutamine (μmol g^{-1} FW)	0.06	0.07	1.07	1.51	0.40	0.37	0.31	0.43 0.31	ns	**	**
Glutamate (μmol g^{-1} FW)	1.86	1.88	2.14	2.01	1.38	1.30	0.98	0.93 1.23	ns	**	*
Total Rubisco activity (μmol min^{-1} mg^{-1} protein)	4.66	4.28	3.55	3.53	3.68	3.28	3.7	3.27 3.19	**	**	ns

Values are means of $n = 12$. Significant differences were for $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P > 0.05$ (ns), respectively.
[a] CO_2 treatment (Pa).

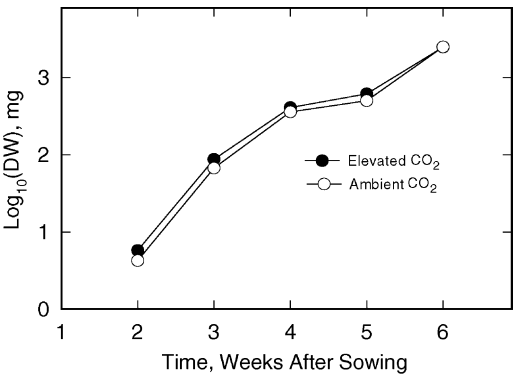

Fig. 1. Effect of CO_2 enrichment on above-ground dry matter formation. Arabidopsis plants were grown from seed for 6 weeks in controlled environment chambers at either 36 Pa CO_2 and 100 Pa CO_2. Rosettes were harvested at indicated times and natural logs of shoot dry mass are given as means ± S.E. (n = 12–24).

3.3. Nitrate, Rubisco activity and leaf N constituents

Total soluble protein did not differ ($P > 0.05$) between the two treatments when compared over all harvests (Table 1). Soluble protein was maximal in 3 week old plants from both CO_2 treatments, and decreased thereafter. Total Rubisco activity was similar between the two treatments on the first harvest (Table 1). However, total Rubisco activity decreased 45% and 31%, respectively, between Weeks 2 and 6 in the elevated and the ambient CO_2-grown plants. Consequently, Rubisco activity was 24% lower ($P < 0.01$) in the elevated compared to the ambient CO_2 treatment on Week 6.

Nitrate levels fluctuated in both treatments largely in association with the biweekly application of nutrient solution. Significant differences in NO_3^- concentrations between CO_2 treatments were detected ($P < 0.01$) when results were combined over all harvest dates (Table 1). Decreased NO_3^- levels were observed in the elevated compared to ambient CO_2 grown plants on Weeks 2, 5 and 6. Arabidopsis rosettes contained maximal concentrations of NO_3^- on Weeks 3 and 6 (> 200 μmol g^{-1} FW). Minimal NO_3^- levels in the ambient and elevated CO_2 treatments were observed at Week 4 in both treatments and these were 36.1–45.6 μmol g^{-1} FW, respectively. The greatest difference in foliar NO_3^- levels between CO_2 treatments was observed at Week 5 when NO_3^- levels were 60% lower in the elevated than in the ambient CO_2 treatment. The

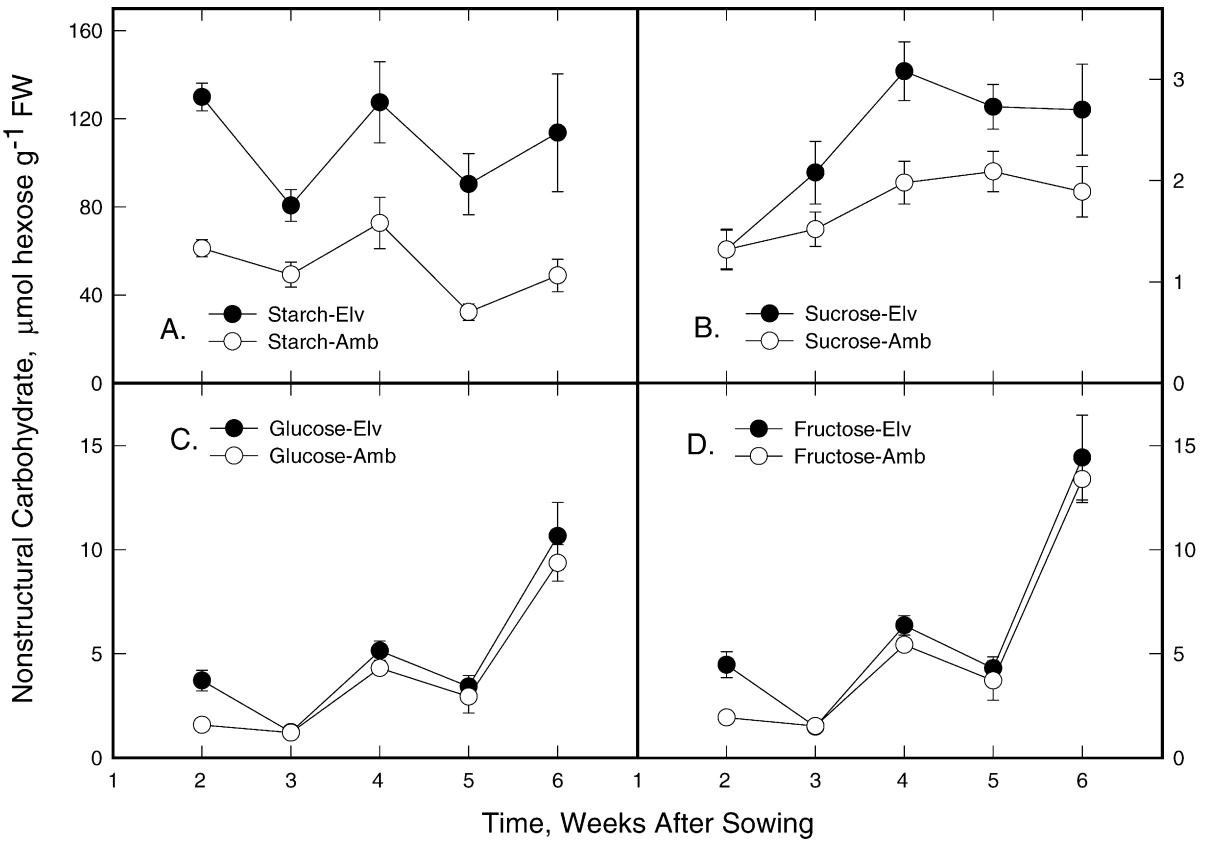

Fig. 2. Effect of CO_2 enrichment on non-structural carbohydrate levels in arabidopsis rosettes. Starch (A), sucrose (B), glucose (C) and fructose (D) concentrations were measured at indicated times.

final NO_3^- level was 22% lower for elevated compared to ambient CO_2-grown plants.

Glutamate and glutamine usually are the principal amino acids present in foliar tissue. Glutamate did not differ ($P > 0.05$) between CO_2 treatments (Table 1). When compared over all harvest dates, glutamate decreased 47% on average between Weeks 3 and 5 in both CO_2 treatments. Glutamine also did not differ ($P > 0.05$) between the two CO_2 treatments except at Week 3 when glutamine was 41% higher in the elevated than in the ambient CO_2-grown plants (Table 1). Glutamine was unaffected by CO_2 enrichment in older plants in both treatments.

3.4. Two-dimensional gel electrophoresis

Total soluble proteins were extracted from rosettes of 4-, 5- and 6- week old arabidopsis plants and were analyzed by two-dimensional gel electrophoresis. Approximately 400 major protein spots were compared between CO_2 treatments on these three dates. A representative silver-stained gel of proteins using 4-week-old plants from the ambient CO_2 treatment is shown in Fig. 3. As indicated on the gel 14 spots were detected that were either increased or decreased by CO_2 enrichment at one or more time points. We did not observe any spots that were consistently present in one treatment and not the other. Three spots (450, 508 and 509) were both increased and decreased in response to CO_2 enrichment depending on plant age. Spot number 1 designates a known protein, the Rubisco large subunit. The remaining thirteen unknown spots were selected for sequence analysis using MALDI-TOF mass spectrometry based on mean differences obtained from three replicate experiments. Six polypeptides were successfully identified by

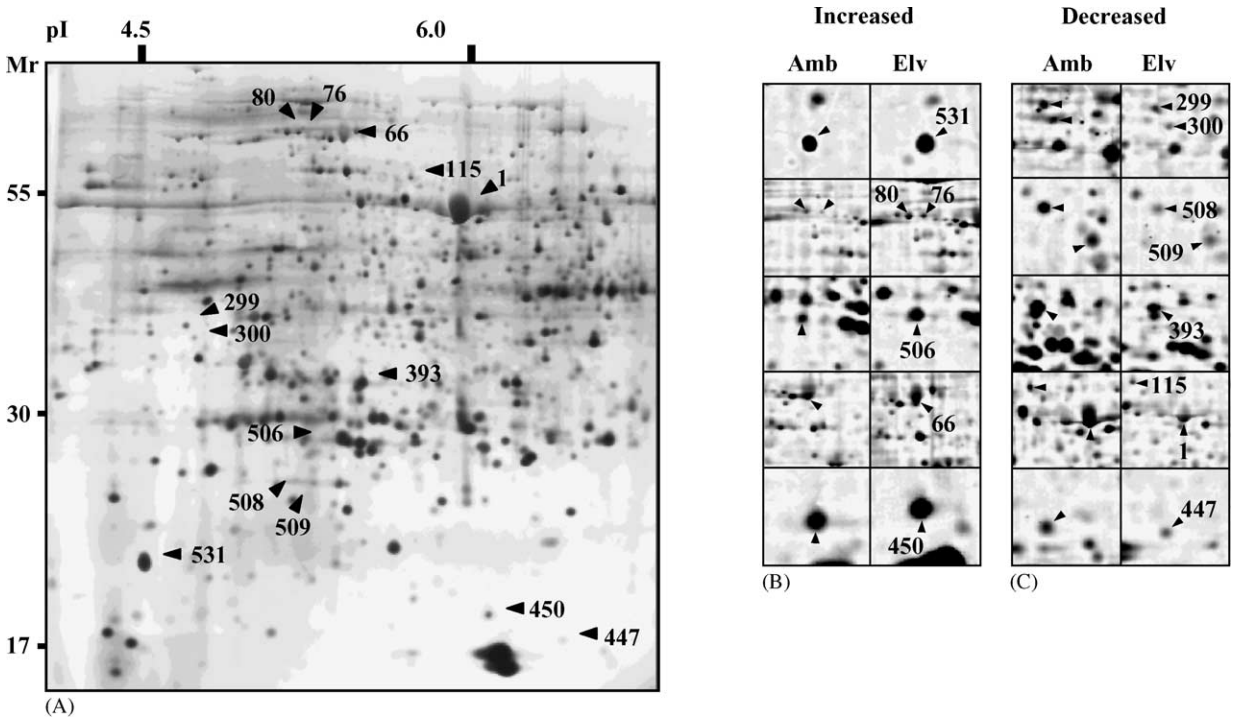

Fig. 3. Silver-stained two-dimensional gel electrophoresis images of leaf proteins from *arabidopsis thaliana*. (A) A representative gel image of total soluble proteins obtained from 4-week-old *arabidopsis* rosettes from the ambient CO_2treatment. Numbered spots denote proteins altered during growth in elevated CO_2. Isoelectric point (p*I*) and molecular mass (kDa)) are indicated in the horizontal and vertical dimensions, respectively. Boxes represent enlarged gel regions and numbered spots that were increased (B) or decreased (C) by the elevated CO_2 treatment are paired with the corresponding image for that date from the ambient CO_2 treatment.

Table 2
Identified proteins from arabidopsis rosettes that were altered by CO_2 enrichment

No.	Spot no.	Mass (kDa)	p*I*	Identity	Accession no.	Mean pixel density ratios
1	66	63.0	5.26	Myrosinase precursor	At5g26000	225 ± 129 (4 week)
2	80	67.8	5.05	Luminal binding protein 2 (BiP2)	At5g42020	123 ± 83 (5 week)
3	393	30.3	5.28	Putative 3-beta hydroxysteroiddehydrogenase/ isomerase protein	AY085201	−36 ± 1.85 (6 week)
4	447	20.0	6.19	Nucleoside diphosphate kinase I	At4g09320	89.6 ± 53(4 week) −42 ± 18.4 (5 week)
5	450	17.5	5.91	Major latex protein-related	At4g23670.1	38 ± 3.9 (4 week) −56 ± 16 (5 week) 40 ± 9.2 (6 week)
6	506	25.0	5.24	Photosystem II oxygen-evolving complex 23 (OEC23)	At1g06680.1	101 ± 68 (6 week)

Appropriate polypeptides were excised from Coomassie brilliant blue G-250 stained gels, destained, digested with trypsin and sequence information was obtained by MALDI-TOF mass spectrometry. Gene accession numbers were obtained from public databases. Mean pixel densities were obtained for digitized images of three normalized gels from each treatment. Negative numbers indicated that pixel densities were decreased for the elevated compared to the ambient CO_2 treatment. Harvest dates where CO_2 treatment differences were observed are shown in parenthesis. (kDa, kiloDalton; p*I*, isoelectric point).

sequence analysis (Table 2) and these were the following: #66) myrosinase precursor (63 kDa, #80) luminal binding protein 2 (BIP2, 67.8 kDa, #393) putative 3-β hydroxysteroid dehydrogenase/isomerase protein (30.3 kDa, #447) nucleoside dikinase II (20.0 kDa, #450) major latex protein-related (17.5 kDa, and #506) photosystem-II oxygen evolving complex 23 (OEC23, 23 kDa). Note that the available genome data for two of the above protein spots were insufficient to positively identify functionality. Based on mean pixel densities from three replicate experiments myrosinase precursor was increased 365% by CO_2 enrichment between Weeks 4 and 6. It should also be pointed out that protein spots corresponding to the large and small subunits of Rubisco visually were lower in the elevated compared to the ambient CO_2 treatment. However, pixel densities for these spots were saturated and it was not possible to quantify differences in Rubisco proteins by this method.

4. Discussion

The physiological and biochemical responses of plants to atmospheric CO_2 enrichment have been well studied (Griffin and Seemann, 1996; Stitt and Krapp, 1999; Bunce, 2001; Woodward, 2002). In the majority of these earlier investigations, the initial enhancement of photosynthesis was diminished and in some instances was completely abolished after a few days to weeks of plant growth in CO_2 enriched atmospheres. This decreased photosynthetic capacity in response to elevated CO_2 often was accompanied by an increase in the foliar C/N ratio, a buildup of nonstructural carbohydrates, decreased total Chl (a + b) and Chl a/b protein levels, decreased inorganic NO_3^-, losses of Rubisco activity and/or Rubisco protein and decreased levels of α-amino N (Wong, 1979; Stitt, 1991; Sicher and Bunce, 1997; Stitt and Krapp, 1999). It has been suggested that many of these physiological changes during plant growth in elevated CO_2 resulted in part from accelerated plant development and premature senescence (Miller et al., 1997; Sicher and Bunce, 2001). Decreased soluble proteins and Chl (a + b) levels in response to elevated CO_2 also have been attributed to a negative regulation of gene expression by increased concentrations of nonstructural carbohydrates (Jang and Sheen, 1997) or to an insufficient N supply (Ferrario-Méry et al., 1997; Stitt and Krapp, 1999). However, acclimation to elevated CO_2 varies between plant species and is affected by growth conditions or by sink development (Stitt, 1991; Sage, 1994; Idso and Kimball, 1997). Note that acclimation responses of photosynthesis or of growth rates to enhanced CO_2 were not always observed (Jones et al., 1995; Norby et al., 1995; Idso and Kimball, 1997).

Atmospheric CO_2 effects on biomass accumulation also may be sensitive to N nutrition. Elevated CO_2 generally increased growth rates when plants were well fertilized but enhancement of growth was diminished when the N supply was limiting (Fonseca et al., 1997). In the current study, shoot biomass was greater ($P < 0.01$) in the elevated compared to the ambient CO_2 treatment on Weeks 2–5 but above ground dry matter was similar between treatments on Week 6. Since most of the dry mass in Arabidopsis was attained after Week 3 differences in biomass between treatments were small.

Organic and inorganic N constituents also were monitored here to insure that protein changes were not due to insufficient N fertility as discussed above. Increased rates of plant growth in response to CO_2 enrichment could induce N insufficiency and alter the physiological status of the plant (reviewed in Stitt and Krapp, 1999). Moreover, signals derived from nitrate or N metabolites, such as glutamine or glutamate, control the expression of genes involved in nitrate and ammonium uptake or assimilation as well as starch accumulation and organic acid synthesis. Gene regulation by N signaling affected photosynthesis, shoot-root allocation, root architecture, carbohydrate accumulation and flowering (Paul and Driscoll, 1997; Scheible et al., 1997). Foliar nitrate levels are a simple estimate of N availability in CO_2 enrichment studies. Previous investigators (Ferrario-Méry et al., 1997; Geiger et al., 1998) observed that leaf nitrate concentrations often were lower in the elevated compared to the ambient CO_2 treatment. In the present study, foliar nitrate also was reduced by CO_2 enrichment when mean values for all harvests were combined. The nitrate levels in this study varied by date largely because nutrient was applied biweekly. However, the maximum leaf nitrate concentrations in this study (>32 mmol m^{-2}) were high and the minimum concentrations observed in this study were likely sufficient

for rapid growth. Note also that the nutrient solution used here contained 2.5 mM ammonium.

Glutamine and glutamate responded differently to elevated CO_2 in Arabidopsis leaves. Glutamine did not differ in ambient and elevated CO_2-grown Arabidopsis leaves except at Week 3 when glutamine was 41% higher in the elevated compared to the ambient CO_2-grown plants. This was in agreement with earlier findings that glutamine levels were increased in young tobacco leaves grown in elevated CO_2 (Geiger et al., 1998). According to these authors a buildup of glutamine in younger leaves in response to CO_2 enrichment is evidence of N sufficiency. In contrast to glutamine, glutamate levels in Arabidopsis rosettes were essentially unaffected by CO_2 enrichment.

The Arabidopsis plants used here displayed many of the conventional acclimation responses to CO_2 enrichment observed in previous studies. Cheng et al. (1998) reported large changes of Rubisco activity, Rubisco protein and Rubisco subunit mRNA levels in Arabidopsis in response to CO_2 enrichment. These authors also noted a buildup of non-structural carbohydrates in response to the continuously elevated CO_2 treatment. In the present study Rubisco activity was lower and starch and sucrose levels were greater in the elevated compared to the ambient CO_2 treatment. However, soluble proteins and total Chl ($a + b$) were largely unaffected by the elevated CO_2 treatment in the current study, suggesting that a major N-insufficiency did not occur. Based on the above discussion, it was concluded that the changes of protein expression reported here were not due to insufficient N supply.

In the current study, we also observed that flowering was delayed in the elevated compared to the ambient CO_2 treatment. In contrast to these results, Cheng et al. (1998) reported that Arabidopsis (Columbia ecotype) bolted prematurely in response to CO_2 enrichment. Although not specifically stated it was likely that this early bolting resulted in early flowering. The variable effects of CO_2 enrichment on flowering between these two studies may reflect the fact that Cheng et al. (1998) used a 10 h photoperiod. This was 6 h shorter than the photoperiod used in the present study. As noted previously (Lee et al., 2000; Samach et al., 2000), physiological factors such as age, day length, nutrient level and temperature are important in controlling the expression of genes involved in the floral initiation and meristem identity. The promotion of floral initiation by sugar levels has been reported in several plant species (King and Bagnall, 1996). However, soluble sugars also were shown to delay flowering (Zhou et al., 1998). Ohto et al. (2001) suggested that the promotive and inhibiting effects of sugars on floral transition depended on genotype, sugar concentration and time of sugar addition. Furthermore, it was not clear whether starch accumulation was also a factor in delayed flowering. Eimert et al. (1995) noted that some late-flowering mutants of Arabidopsis (e.g., *gi*, *cam1*) accumulated foliar starch upon bolting. These authors suggested that starch accumulation and floral initiation may share a common regulatory pathway.

Six proteins were identified in Arabidopsis rosettes that differed in abundance on Weeks 4, 5 or 6 in the ambient and elevated CO_2 treatments. Four of the six genes that were identified using peptide sequences obtained via MALDI-TOF have known functions.

(1) *Myrosinase precursor (β-thioglucoside glucohydrolase)*. Myrosinase hydrolyzes a special class of S containing compounds in *Brassica* spp. known as glucosinolates. The enzyme and substrate are physically separated and react in response to stress resulting in the release of glucose and sulfate (Rask et al., 2000; Wittstock and Halkier, 2002). In addition, amines, epithionitrile, isothiocyanate, nitrile, thiocyanate, oxazolidine-2-thione and other less prevalent products are formed. These degradation products are toxic to herbivores and pathogens and have antitumerogenic properties in humans (Rask et al., 2000). The transcript for this protein contains a transit peptide, hence the designation myrosinase precursor. Recent research suggested that the myrosinase-glucosinolate system also may play an important role in plant growth and development (Reintanz et al., 2001). There are two myrosinase genes in Arabidopsis and these are highly expressed in developing tissue, the flower bud and the flower (Rask et al., 2000).

(2) *Luminal binding protein (BiP)*. BiP controls the translocation and folding of newly synthesized proteins targeted to the secretory pathway and functions as a heat shock protein (i.e., HSP 70) localized in the endoplasmic reticulum (Forward et al., 2002). The expression of BiP in plants is dependent on developmental, seasonal, hormonal, stress-induced, and diurnal regulation (Forward and Misra, 2000). BiP is highly expressed in actively dividing, secretory and

seed storage tissues and is induced by wounding or heat stress (Forward et al., 2002).

(3) *Nuleoside diphosphate (NDP) kinase*. NDP kinase transfers a γ-PO_4^- from ATP to NDP via autophosphorylation and is involved in maintaining stable GTP levels in various metabolic pathways (Parks and Agarwal, 1973). NDP kinase is essential for cell elongation processes in rice (Pan et al., 2000). In Arabidopsis, NDP kinases, especially NDP kinase 2, have an important role in oxidative stress. The overexpressing transformant of Arabidopsis, AtNDPK2, exhibited enhanced tolerance to abiotic stresses, such as cold, salt and H_2O_2 (Moon et al., 2003). The AtNDPK2 transformant also induced the expression of genes involved in oxidative stress, including peroxidase, glutathione reductase, glutathione transferase, thioredoxin reductase and peroxiredoxin, as well as heat-shock proteins.

(4) *Oxygen evolving complex (OEC) 23*. There are three proteins with molecular masses of 18, 23 and 33 kDa that bind to the donor side of photosystem II forming an OEC in higher plants and green algae (Hankamer and Barber, 1997). However, the exact function of this protein in photosynthesis remains unknown (Ifuku and Sato, 2001). OEC23 is involved in both Ca^{2+} and Cl^- retention, ions that are essential cofactors for photosynthetic O_2 evolution.

Two other Arabidopsis proteins were identified in this study that responded to CO_2 enrichment but the molecular functions of these two proteins is unclear. Putative 3-hydroxysteroid dehydrogenase/isomerase had sequence similarity to human and mouse proteins involved in the synthesis of androgens, progestins and estrogens and may be involved in the synthesis of brassinosteroids. The second protein had low sequence similarity to a major latex protein from *Papaver somniferum* and presumably is involved in alkaloid biosynthesis.

In summary, growth in elevated CO_2 increased foliar carbohydrate levels and total above-ground biomass of Arabidopsis. Flowering also was delayed by the elevated CO_2 treatment and this was possibly due to altered sucrose concentrations. Rubisco activity was lower in elevated compared to ambient CO_2 grown Arabidopsis plants. We identified six of 13 proteins that responded to elevated CO_2 by using two-dimensional gel electrophoresis. The role of these six proteins in the acclimation of plants to atmospheres enriched with CO_2 is uncertain at this time and this matter will require further study. The observation that NDP kinase responded to CO_2 treatment is certainly interesting because this enzyme may function in GTP signaling pathways that control a number of plant responses to various stresses and other external stimuli. At the present time up to 27,000 proteins are encoded by the Arabidopsis genome (Wortman et al., 2003). Because of post-translational modifications, such as phosphorylation and transit peptide removal, there can be as many as four spots on a two-dimensional gel for each encoded protein. Consequently, the entire Arabidopsis proteome potentially can generate as many as 100,000 spots on a two-dimensional gel. The small number of polypeptides we identified in the current study that responded to CO_2 enrichment is partly due to the fact that two-dimensional gel techniques only resolve the most abundant proteins. The techniques used here also do not adequately resolve hydrophobic proteins and other proteins that are difficult to solubilize. As all of the proteins examined in this study were present in both treatments, it was concluded that growth in elevated CO_2 did not have a major impact on protein expression in Arabidopsis. The function of CO_2 responsive proteins that were identified in this study by two-dimensional gel electrophoresis can be examined further using techniques employing reverse genetics, such as T-DNA insertion knock-out mutants.

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