Plant growth in elevated CO_2 alters mitochondrial number and chloroplast fine structure

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With increasing interest in the effects of elevated atmospheric CO_2 on plant growth and the global carbon balance, there is a need for greater understanding of how plants respond to variations in atmospheric partial pressure of CO_2. Our research shows that elevated CO_2 produces significant fine structural changes in major cellular organelles that appear to be an important component of the metabolic responses of plants to this global change. Nine species (representing seven plant families) in several experimental facilities with different CO_2-dosing technologies were examined. Growth in elevated CO_2 increased numbers of mitochondria per unit cell area by 1.3–2.4 times the number in control plants grown in lower CO_2 and produced a statistically significant increase in the amount of chloroplast stroma (nonappressed) thylakoid membranes compared with those in lower CO_2 treatments. There was no observable change in size of the mitochondria. However, in contrast to the CO_2 effect on mitochondrial number, elevated CO_2 promoted a decrease in the rate of mass-based dark respiration. These changes may reflect a major shift in plant metabolism and energy balance that may help to explain enhanced plant productivity in response to elevated atmospheric CO_2 concentrations.

Carbon dioxide enrichment of the global atmosphere is one of the most significant ecological changes produced by industrialization during the past two centuries, increasing the partial pressure of CO_2 in the atmosphere by 30% (1). Within the global carbon cycle, photosynthesis and respiration by plants are the primary biological processes linking inorganic carbon in the atmosphere (CO_2) with organic carbon in the biosphere. This knowledge has led to widespread interest in understanding more fully how these physiological responses are coupled to changes in atmospheric CO_2 partial pressures. Furthermore, the magnitude of carbon fluxes between the atmosphere and the biosphere resulting from both photosynthesis and autotrophic respiration is very large (approximately 120 and 60 billion metric tons per year, respectively) (2). The magnitudes of these carbon fluxes are so large that a change of only a few percent could account for the discrepancy between the known sources and sinks for carbon (2, 3). As atmospheric CO_2 partial pressures continue to rise as the result of human activities such as fossil fuel combustion and land clearing (3), it is critical to identify and quantify the biochemical, physiological, and ecological processes that are sensitive to atmospheric CO_2. Clearly these processes can impact significantly the rate and/or extent of changes in the CO_2 content of the Earth's atmosphere, as well as our conclusions concerning appropriate future policies regarding greenhouse gas emissions.

An integrative understanding of the responses of plants to increased atmospheric CO_2 partial pressure is important because both short- and long-term photosynthetic and respiratory responses to elevated CO_2 have been demonstrated (recently reviewed in refs. 4–9). Although information from the ecosystem, community, population, plant, leaf, physiological, biochemical, and molecular scales exists (e.g., refs. 9–12), there is very little information at the scale of cellular fine structure (e.g., ref. 13). There is persuasive evidence that elevated CO_2 may affect chloroplast functional microanatomy. For example, Norby *et al.* (9) report that field-grown trees maintained at atmospheric CO_2 partial pressures that are 30 Pa higher than ambient have 60% higher leaf-level photosynthetic rates. Despite an abundance of physiological data, very little information exists on the effects of elevated CO_2 partial pressure on the ultrastructure of the photosynthetic apparatus. What little information does exist suggests that growth in elevated CO_2 can change chloroplast structure primarily through the accumulation of large starch grains (refs. 14–18, but see ref. 19). However, we have only limited information concerning possible changes in the photosynthetic apparatus, particularly the grana and intergrana chlorophyll-containing membranes.

Similarly, respiratory carbon flux rates have been shown to be sensitive to ambient CO_2 partial pressure, both increasing and decreasing, depending on experimental conditions and plant species (8). Respiration from higher plants accounts for half of the global respiratory carbon flux, and mass-based respiration rates in C_3 plants (95% of all higher plant species) tend to decrease by an average of 15% when plants are grown in a doubled atmospheric CO_2 partial pressure (6, 20). Although the activities of several respiratory enzymes are inhibited directly by exposure to elevated CO_2, neither the precise mechanisms controlling these responses nor their physiological and ecological implications are fully known (6, 8, 17, 21). Surprisingly, despite decreases in respiratory carbon flux with elevated CO_2, the number of mitochondria in the cells of young (7-day-old) wheat leaves has been found to dramatically increase in the first few hours of CO_2 exposure (22).

To examine the long-term ultrastructural changes of mitochondria and chloroplasts and the physiological responses to growth in elevated CO_2, we collected fully expanded leaves from a wide variety of plant types (C_3, herbaceous and woody plants) from several research sites representing four substantially different CO_2 dosing technologies. Two herbaceous C_3 plants, soybean [*Glycine max* (L.) Merr.] and velvet-leaf (*Abutilon theophrasti* Medic.) were grown in controlled environment chambers in two separate experiments. Leaves of radiata pine [*Pinus radiata* (D.) Don], a nonnative conifer, were sampled from open-top chambers in New Zealand (23). *Piper auritum* (Kunth) and *Epipremnum pinnatum* [(L.) Engl.] were sampled from a tropical rainforest mesocosm inside the Biosphere 2 research facility (24). Four tree species were sampled from two different free-air CO_2 enrichment (FACE) facilities. At the Duke Uni-

Abbreviation: FACE, free-air CO_2 enrichment.

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versity FACE facility (Durham, NC), a dominant canopy tree, loblolly pine (*Pinus taeda* L.), and three understory trees, red maple (*Acer rubrum* L.), sweetgum (*Liquidambar styraciflua* L.), and redbud (*Cercis canadensis* L.) were sampled (25). *L. styraciflua* also was sampled from the Oak Ridge National Laboratory FACE facility, where it is the dominant canopy tree.

Materials and Methods

Growth Chambers. Environmental conditions within the chambers were 28°C daytime, 17°C nighttime, approximately 60% relative humidity, a 14-h photoperiod with approximately 500 μmol m^{-2} s^{-1} of photosynthetically active radiation at the upper leaf surface. All plants were watered each day with deionized water, and each pot contained 17 g of slow-release fertilizer (8.2% ammoniacal nitrogen/5.8% nitrate nitrogen/14% P_2O_5/14% K_2O, Osmocote, Scotts–Sierra Horticultural Products Company, Marysville, OH). Two herbaceous C_3 annual plants, soybean [*G. max* (L.) Merr.] and velvet-leaf (*A. theophrasti* Medic.) were grown in controlled environment chambers in two separate experiments. *A. theophrasti* was grown at either 36 Pa CO_2 (current ambient) or 73 Pa CO_2 (elevated), and *G. max* was grown at a preindustrial CO_2 partial pressure (25 Pa) and a greatly elevated CO_2 (100 Pa).

Open-Top Chambers. Our experimental site was established at Christchurch, New Zealand (43°32′ S, 172°42′ E) and is more fully described elsewhere (26). Sixteen circular open-top chambers (3.6 m tall, 4.6 m diameter) were established on a recently stabilized free-draining Kairaki dune sand. The CO_2 supplied to the chambers was separated from biogas by using a three-stage filtration process at a nearby wastewater treatment facility (27). Experimental CO_2 partial pressure treatments were monitored and controlled automatically and supplied 24 h d^{-1} for the entire tree life. Each chamber had three *P. radiata* trees.

Biosphere 2. The 2,000 m^2 rainforest mesocosm of Biosphere 2 is 35,000 m^2 in volume (maximum height, 28 m). Designed to simulate an equatorial rainforest climate, it is a functional model of a humid tropical forest, including new and old world species (28, 29). In 1996, during the 6 months preceding the first leaf sampling, the average CO_2 concentration was 83 ± 5.2 Pa, and during the previous 5 years, concentrations were well above 36 Pa. The peak light intensity varied seasonally from 800 to 1,300 μmol m^{-2} s^{-1}, air temperature was 24.6 ± 0.1°C, and the relative humidity was 79 ± 0.3%.

Leaf samples were taken in duplicate during the period of elevated CO_2 concentrations in 1996. Subsequently, as a means of comparison, recently grown leaves from the same species and at the same location on the plants were taken in 1999. During the 6 months before sampling in 1999 (the period of leaf development for the new samples), the atmospheric CO_2 partial pressure averaged 48 ± 1.1 Pa. Outside ambient air, drawn in by large fans, produced this reduction. Photosynthetically active radiation in the rainforest was constant, and during the second sampling period, the peak intensity varied from 800 to 1,300 μmol m^{-2} s^{-1} seasonally. Air temperature in the rainforest averaged 25 ± 0.4°C, and daily relative humidity was 87.4 ± 0.5%. Thus, each plant in Biosphere 2 served as its own control in this time-series study of elevated CO_2 concentrations on leaf fine structure.

Of the two species collected, *P. auritum* transitions from an understory plant to a small tree, and *Epipremnum* is strictly an understory plant. We chose *P. auritum* from among the available trees because *Piper* is of economic importance and has a positive canopy-growth response to experimentally elevated atmospheric CO_2 concentrations (30). The vine, *E. pinnatum*, represented a contrasting understory herbaceous plant with considerable shoot growth.

Duke Forest FACE Site. The FACE facility is a well-established experimental system, described more fully elsewhere (25, 31, 32), in a 13-year-old loblolly pine (*P. taeda*) plantation in the Piedmont region of North Carolina (35°97′ N 79°09′ W). The atmospheric CO_2 partial pressure is increased by 20 Pa above ambient (≈56 Pa) within a 30-m-diameter experimental plot by using a system of vertical pipes (with regularly spaced exit ports along the length) connected to a circular plenum supplied with CO_2 gas by a highly regulated computer-based control system. Nearby control sites of equivalent design and plant composition are at ambient atmospheric CO_2 levels. Leaves were sampled in early summer 1999, 2 years after the onset of the CO_2 fumigation. Leaf samples in duplicate were taken at the same height from each plant in the experimental and control rings. Plants were sampled at a location of comparable light intensity, as measured by a quantum sensor (Model LI-1776, Li-Cor, Lincoln, NE). Understory tree species were among the more abundant saplings at the sampling site and represent genera in diverse families (Aceraceae, Leguminosae, and Hamamelidaceae) that occur widely in temperate broadleaf forests.

Oak Ridge FACE Site. This site was established in an 11-year-old sweetgum plantation in the Oak Ridge National Environmental Research Park, Oak Ridge, TN. One-year-old sweetgum seedlings were planted in 1988 at a spacing of 2.3 m × 1.2 m in a total area of 1.7 ha. At the time of sampling, the sweetgum trees were an average height of 15 m in a closed canopy with a leaf area index of 6. The live canopy began at 8 m from the ground. The atmospheric CO_2 partial pressure was increased by 20 Pa above ambient (≈57 Pa daytime/65 Pa nighttime) within two 25-m-diameter experimental plots, enclosing about 120 trees each, by using essentially the same FACE technology as the Duke FACE site (25). CO_2 enrichment was maintained for 24 h d^{-1} during the growing season. Two nearby control rings of similar size used blowers to maintain an ambient CO_2 partial pressure (≈37 Pa daytime/45 Pa nighttime). Leaves were sampled from each of the four rings in late summer 1999, in the second growing season of CO_2 fumigation. Leaf samples were taken at the same height (near the top of the canopy in full sun) from eight trees in each of the two CO_2 treatments (ambient and elevated).

Electron Microscopy. Duplicate samples of leaves, taken at similar heights and positions on the control (lower CO_2) and treatment (elevated CO_2) plants, were fixed immediately in glutaraldehyde [2% (wt/vol) in 0.05 M potassium phosphate buffer, pH = 7.2]. The sample was postfixed in phosphate-buffered 2% (wt/vol) osmium tetroxide for 2 hours at 5°C, dehydrated in a graded acetone series, embedded in catalyzed epon (TAAB resin, Energy Beam Sciences, Agawan, MA), polymerized at 65°C, and sectioned with a Porter–Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, CT) fitted with a diamond knife. The sections were collected on copper grids, post-stained with Reynolds lead citrate, and observed with a Philips 201 transmission electron microscope (Einthoven, the Netherlands).

A random sample of transmission electron micrographs (10 per treatment) was taken from at least two different leaf samples for each species in each of the experimental and control environments by using a computer-generated table of random numbers. The proportion of the total chloroplast volume occupied by stroma and grana thylakoids was determined by superimposing a grid of points on the electron micrograph of each plastid and determining what proportion of the points fell on stroma and grana thylakoids, respectively, compared with all points falling within the boundaries of the chloroplasts according to standard stereological methods (33). The mean ratios of stroma thylakoids to grana thylakoids, stroma thylakoids to total chloroplast area, and grana thylakoids to total chloroplast area were determined for combined data from replicate samples of leaves. Similarly, a

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Table 1. Chloroplast fine structure of plants grown in elevated and ambient atmospheric CO_2 partial pressures

	Stroma/Grana thylakoids		Stroma thylakoids/Total area		Grana thylakoids/Total area	
Species	Elevated CO_2	Ambient CO_2	Elevated CO_2	Ambient CO_2	Elevated CO_2	Ambient CO_2
A. rubrum	0.87 ± 0.09	0.49 ± 0.05	0.31 ± 0.02	0.19 ± 0.01	0.37 ± 0.02	0.39 ± 0.02
	(1.8, $t = 3.8$, $P < 0.01$)		(1.6, $t = 5.1$, $P < 0.01$)		(0.9, $t = -0.5$, ns)	
C. canadensis	0.82 ± 0.04	0.52 ± 0.02	0.30 ± 0.02	0.20 ± 0.01	0.37 ± 0.02	0.38 ± 0.02
	(1.6, $t = 6.6$, $P < 0.01$)		(1.6, $t = 5.0$, $P < 0.01$)		(0.9, $t = -0.2$, ns)	
L. styraciflua	1.30 ± 0.18	0.43 ± 0.04	0.30 ± 0.03	0.20 ± 0.02	0.25 ± 0.02	0.48 ± 0.03
	(3.0, $t = 4.7$, $P < 0.01$)		(1.6, $t = 3.5$, $P < 0.01$)		(0.5, $t = -5.9$, $P < 0.01$)	
P. auritum	1.19 ± 0.13	0.67 ± 0.06	0.31 ± 0.03	0.17 ± 0.02	0.27 ± 0.04	0.26 ± 0.02
	(1.8, $t = 3.6$, $P < 0.01$)		(1.8, $t = 3.5$, $P < 0.01$)		(1.0, $t = 0.3$, ns)	
E. pinnatum	0.89 ± 0.09	0.71 ± 0.03	0.29 ± 0.02	0.32 ± 0.01	0.34 ± 0.02	0.45 ± 0.01
	(1.3, $t = 1.7$, $P < 0.05$)		(0.9, $t = -1.1$, ns)		(0.8, $t = -4.3$, $P < 0.01$)	

Results are means ± 1 SEM ratios of proportions (column headings). Entries in parentheses are elevated to ambient CO_2 ratios and statistical significance level.

random sample of leaf tissue sections was examined in transmission electron micrographs (10 per treatment) from at least two different leaf samples for each species in each of the experimental treatments to assess the number of mitochondria per cell. For each pair of control and elevated CO_2 treatments, mitochondria were enumerated and expressed per 100 μm^2 of cell area, on the basis of the quantification of 50–250 cells per species. Because comparative mitochondrial counts per unit cell area are meaningful only if the mitochondria are of equivalent size, we examined the sizes of mitochondria in the low CO_2 and high CO_2 treatment conditions for each of the experimental regimes. At least 20 random samples of mitochondria per treatment were measured with the transmission electron microscope. The species examined were *G. max* and *A. theophrasti* (controlled climate chamber experiments), *P. auritum* (Biosphere 2 experiments), and *P. radiata*, *P. taeda*, and *L. styraciflua* (FACE ring experiments). The major and minor axes of the mitochondrial profiles in ultrathin sections were measured and converted to square micrometer area units. One-tailed *t* tests of mean differences were obtained with a STATVIEW SE + graphics computer application (Abacus Concepts, Berkeley, CA).

Oxygen Electrode Measurements. Respiration measurements were made before fixation in *P. radiata* by using a Clark-type liquid-phase oxygen electrode (Rank Brothers, Cambridge, U.K.). In *G. max* and *A. theophrasti*, oxygen electrode measurements were made on different parts of the same leaf before the collection of the transmission electron microscopy samples. Oxygen consumption was assayed in the dark at 25°C in 20 mM Mes buffer (pH 6.0) that had been equilibrated in ambient air. Intact leaf punches and/or sliced needles were placed in the electrode cuvette, and the depletion of oxygen was recorded. All measurements were terminated before a 50% depletion of oxygen to avoid O_2 limitation.

Gas-Exchange Measurements. Gas-exchange measurements of carbon exchange were made on the canopy dominant *L. styraciflua* samples from the Oak Ridge National Laboratory FACE facility before transmission electron microscopy sample collection. Dark respiration measurements were done at night in the field by using two portable photosynthesis systems (Model Li-6400, Li-Cor). Leaves were placed into a sealed cuvette (nighttime growth CO_2 level of 45 or 65 Pa at 25°C and ambient humidity) for 20 min before the rate of CO_2 evolution was measured. Nineteen leaves were measured from each of the two CO_2 treatments (ambient and elevated) over a consecutive 3-night period. No measurements were made at the Duke FACE facility or at Biosphere 2.

Results

Chloroplasts. A statistically significant greater proportion of stroma (nonappressed) to grana (appressed) thylakoids was found for all tree species (*A. rubrum, C. canadensis, L. styraciflua,* and *P. auritum*), grown in high atmospheric CO_2 compared with ambient CO_2 concentrations (Table 1) but not significantly greater for the understory vine *E. pinnatum*. The ratio of grana thylakoid to total chloroplast area varied among the species examined. There were no significant differences for *A. rubrum, P. auritum,* or *C. canadensis*. However, the ratio of grana thylakoids to total chloroplast area in *L. styraciflua* and *E. pinnatum* was lower in samples from elevated CO_2 environments compared with ambient CO_2 environments, i.e., there were proportionately more grana stacks in leaf samples from ambient CO_2-grown plants than in those from plants grown at elevated CO_2 partial pressure. This observation is consistent with an increased proportion of stroma thylakoids that occupy more of the chloroplast stroma in some species and a concomitant reduction in the amount of grana thylakoids.

It is important to note that the samples from Biosphere 2 were obtained from the same plants in a time series study. Thus, the differences in chloroplast fine structure of leaves between elevated and near ambient CO_2 atmospheric partial pressures reflect changes in the leaves of the same plants over time. These data indicate that after a 6-month exposure to elevated atmospheric CO_2 partial pressure, the rainforest plants adapted by changing leaf ultrastructure as new leaves subsequently developed in an atmosphere of lower CO_2 partial pressure. Furthermore, the observed chloroplast responses to growth in elevated CO_2 were consistent, regardless of the CO_2 dosing technology.

The comparative fine structure of *P. auritum* plastids (containing starch grains) grown in elevated and ambient CO_2 partial pressure (Fig. 1) illustrates the increased amounts of stroma thylakoids in the leaves of plants in elevated CO_2. Overall, the elevated CO_2 partial pressure in the Biosphere 2 and FACE ring environments produced increased amounts of stroma thylakoids but no consistent effect on the amount of grana membranes or the number of starch grains. In some cases, very little or no starch was stored in the plastids of the plants investigated in this study.

Mitochondria. In all cases, growth in elevated CO_2 partial pressure resulted in an increase in the number of mitochondria in all plant cells measured. This increase was always highly significant and ranged between 1.3 times in *A. rubrum* to 2.4 times in *P. auritum* and *L. styraciflua*. Although the effect of growth in elevated CO_2 was consistent, the absolute number of mitochondria varied significantly among species, from 0.3 per 100 μm^2 of total cell area in low CO_2-grown *A. theophrasti* and *A. rubrum* to 2.9 per 100 μm^2 of cell area in high CO_2-grown *L. styraciflua* (Table 2). The cells of *P. radiata* were more polygonal than other species, making it difficult to assess total cell area by using geometric formulas. Hence, the mitochondrial densities were expressed as number per unit area of cytoplasm lining the walls of the cell as

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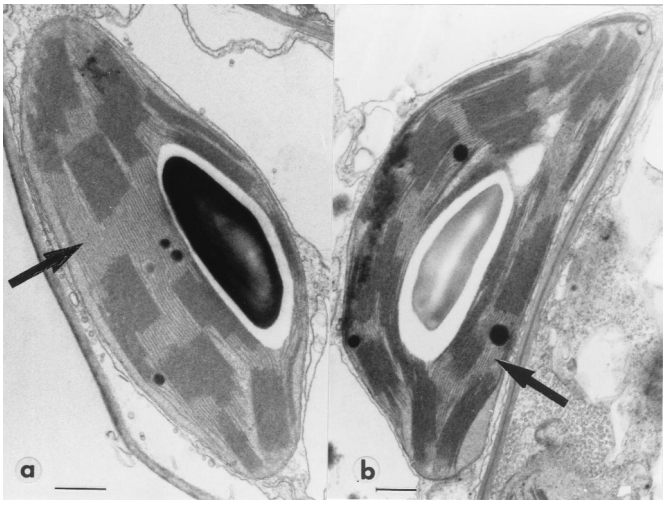

Fig. 1. Chloroplasts in *P. auritum* showing stroma thylakoids (arrows) when grown at elevated CO_2 atmospheric concentrations (*a*) and at ambient CO_2 concentrations (*b*). (Bar = 0.5 μm.)

measured on a random sample of electron micrograph negatives. Similarly, the elevated CO_2 grown *G. max* cells had very large starch grains, contorting the cell structure, thus these results also are expressed per unit living cytoplasm to factor out the influence of the starch accumulation. It is important to note that the size of individual mitochondria from the low- and high-CO_2 treatments was not significantly different [*G. max* ($t = 0.14$, $P = 0.9$, df = 45), *A. theophrasti* ($t = 0.47$, $P = 0.6$, df = 66), *P. auritum* ($t = 1.6$, $P = 0.1$, df = 54), *P. radiata* ($t = 1.1$, $P = 0.3$, df = 60), *P. taeda* ($t = 1.3$, $P = 0.19$, df = 50), *L. styraciflua* ($t = 0.32$, $P = 0.8$, df = 51)]. Therefore, relative counts of the number of mitochondria per unit cell area represent differences in the amount of mitochondria per unit cell area.

Dark Respiration. Dark respiration rates were generally lower in elevated CO_2-grown plants compared with ambient CO_2-grown plants (Table 3). Leaf mass-based respiration rates were significantly reduced between 14 and 32% in elevated CO_2-grown plants. Area-based measurements were quite variable, and only *P. radiata* and *L. styraciflua* had significantly lower respiration rates, decreasing by 14 and 12%, respectively, in elevated CO_2.

Table 2. Mitochondrial counts of plants grown in elevated and ambient atmospheric CO_2 partial pressures

	Mitochondria per 100 μm^{-2} cell area			
Species	Ambient CO_2	Elevated CO_2	Elevated/ ambient	*t* test $P <$
G. max	1.0 ± 0.1	1.6 ± 0.1	1.6	0.001
A. theophrasti	0.3 ± 0.03	0.4 ± 0.03	1.3	0.01
*P. radiata**	14.0 ± 1.4	30.0 ± 1.8	2.1	0.001
P. auritum	0.4 ± 0.05	0.9 ± 0.08	2.4	0.0001
P. taeda	0.5 ± 0.07	0.9 ± 0.10	1.9	0.001
A. rubrum	0.3 ± 0.03	0.6 ± 0.06	1.7	0.001
L. styraciflua (Duke)	0.7 ± 0.06	1.5 ± 0.22	2.2	0.001
C. canadensis	1.3 ± 0.15	2.4 ± 0.22	1.8	0.001
L. styraciflua (Oak Ridge)	1.2 ± 0.76	2.9 ± 1.37	2.4	0.0001

Mitochondrial sizes in low and high CO_2 treatments are statistically equivalent, as reported in the text. Relative counts of mitochondria per unit cell area represent differences in mitochondrial area. Results are means ± 1 SEM.
*Expressed as mitochondria per 100 μm^{-2} of living cytoplasm.

In all other species, the trend was for reduced respiration in elevated CO_2-grown trees.

Discussion

In the nine species we examined, representing seven plant families, collected from several different growth facilities with alternative CO_2-dosing technologies, growth in elevated CO_2 consistently increased numbers of mitochondria per unit cell area by 1.3–2.4 times the number in control plants grown in lower CO_2. Although the precise control over the determination of cellular mitochondrial density is not known (34), it is generally recognized that cells with higher energy demands have larger numbers of mitochondria, because this organelle provides the majority of the ATP required by cells via oxidative phosphorylation (35, 36). In animal systems, the symmorphosis hypothesis states that mitochondrial structure, including mitochondrial volume, is optimally designed to meet the functional requirements of the system (5, 35, 36). No similar hypothesis exists for plant systems, yet plants grown at elevated CO_2 tend to have higher rates of photosynthesis and grow faster (5), suggesting an increased energy demand correlating with the observed increase in mitochondrial number. Furthermore, these results indicate that the mitochondrial response to growth in elevated CO_2 can be sustained over longer time periods but with a magnitude similar to the short-term (7-day) response previously reported (22).

Despite the observed increase in mitochondrial number, nighttime respiration tended to decrease both in our plants and in those measured elsewhere (5, 6, 20), suggesting the rate of ATP production via oxidative phosphorylation decreased. The respiratory finding may appear inconsistent with the implied increase in energy demand associated with the observed increase in mitochondrial number. However, Wang *et al.* (37) find that nighttime respiration in elevated CO_2-grown *Xanthium strumarium* decreased only after 69 days of growth in elevated CO_2, whereas previous measurements made at 38 and 47 days found respiration was either greater than or equal to ambient CO_2-grown plants. Accordingly, it is possible that the number of mitochondria is related to a previous period of high nighttime energy demand. We counted only intact structurally complete mitochondria, excluding any that were enclosed within autophagosomes, which would indicate they were senescent and being digested or nonfunctional. In addition, Robertson *et al.* (22) report correlations among elevated CO_2, mitochondrial number and the 2-oxoglutarate dehydrogenase complex and pyruvate dehydrogenase complex of young wheat leaves, indicating functional mitochondria. Our observation of decreased rates of nighttime respiration in elevated CO_2 is most likely the result of a direct inhibition of the activity of two mitochondrial enzymes, succinate dehydrogenase and cytochrome *c* oxidase (7, 21).

Growth in elevated atmospheric CO_2 partial pressures also increased the proportion of stroma thylakoid membranes relative to grana membranes in leaves of all plants in our investigation. Likewise, the proportion of stroma thylakoid membranes relative to total chloroplast volume for the tree species measured increased when grown in elevated CO_2. As with altered mitochondrial numbers, these changes in the chloroplast structure could have significant implications for leaf-level energy balance, because the concentration of photosystems I and II varies spatially within the membranes. The grana thylakoids contain largely photosystem II and an oxygen-evolving system, whereas the intergrana or stroma thylakoids are enriched in photosystem I centers where NADPH is produced for reduction of CO_2 and its incorporation into carbon intermediates of the Calvin cycle (e.g., refs. 38–40). Elevated CO_2 partial pressure leads to higher net photosynthetic rates, increasing the demand for reductant to be used in carbon fixation. Without a concomitant increase in available light energy, biochemical and physiological adjust-

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Table 3. Leaf respiration rates of plants grown in elevated and ambient atmospheric CO_2 partial pressures

Species	*n*	Mass-based respiration, μmol kg^{-1} s^{-1}				Area-based respiration, μmol m^{-2} s^{-1}			
		Ambient CO_2	Elevated CO_2	Elevated/ambient	*t* test $P <$	Ambient CO_2	Elevated CO_2	Elevated/ambient	*t* test $P <$
G. max	4	52.06 ± 5.88	35.43 ± 3.03	0.68	0.05	2.34 ± 0.20	1.99 ± 0.22	0.85	ns
A. theophrasti	9	—	—	—	—	1.47 ± 0.13	1.23 ± 0.12	0.83	ns
P. radiata	8	4.10 ± 0.25	3.05 ± 0.26	0.73	0.01	0.35 ± 0.03	0.30 ± 0.03	0.86	0.01
L. styraciflua	19	9.34 ± 0.28	8.04 ± 0.28	0.86	0.01	1.12 ± 0.05	0.99 ± 0.06	0.88	0.01

Results are means ± 1 SEM.

ments would be necessary to continue to meet the energy demand of photosynthesis specifically and, more generally, overall cellular metabolism. Therefore, the significant increase in the ratio of stroma to grana thylakoids suggests that these C_3 plants may be compensating for the increased availability of CO_2 by elaborating the intergrana thylakoids to more efficiently fix CO_2 into sugar products by increasing energy (reductant) production. The contrasting lack of significant differences in the ratio of stroma thylakoids to total chloroplast area in *E. pinnatum* may reflect in part differences in stroma content. *E. pinnatum* had very little stroma surrounding the thylakoid membrane system, whereas the other species had considerable peripheral stroma in most chloroplasts. Additionally, the arrangement of the thylakoid membranes in *E. pinnatum* may be adapted to the energetic constraints of the low-light conditions present in the understory and may have less plasticity to respond to elevated CO_2.

Qualitatively similar chloroplast fine structure findings were reported for sugar beet (*Beta vulgaris* L.) cultivated in normal and elevated CO_2 partial pressure (41), although the differences were not highly significant. Here we found highly significant differences, which may be attributed in part to long-term CO_2 exposure period and the denser amount of intergrana thylakoids in our species, thus increasing the sample size and statistical power. Our data for plants from Biosphere 2 also indicate leaf plasticity in responding to changes in atmospheric CO_2 concentrations. Plants sampled in the first phase (1996) had grown for many months in elevated atmospheric CO_2 and had increased amounts of stroma thylakoids similar to the high CO_2-grown Duke FACE ring plants. When new leaves from the same plants were sampled in 1999 after growth in near-ambient levels of atmospheric CO_2, the thylakoid organization had reverted, resembling the leaf fine structure of plants in the control FACE rings.

Microscopic and molecular evidence indicates that leaf structure is modified in response to changes in environmental variables such as light intensity, temperature, and elevated CO_2 partial pressure, although there are variations among species (e.g., refs. 16, 19, 22, 42, and 43). Our findings concerning variations in mitochondrial numbers and chloroplast membranous fine structure add additional evidence that plant growth at elevated CO_2 partial pressures may have a marked effect on the fine structure of C_3 plants. Furthermore, if these structural changes are related to leaf energy demand, they may help explain increased photosynthetic efficiency at the macromolecular level of membrane organization. Increased photosynthetic efficiency arising from changes in Rubisco kinetics and light-trapping efficiency, as evidenced by concomitant elaboration of NADPH-producing membranes, may account for enhanced carbon dioxide fixation and growth of some plants when exposed to elevated atmospheric CO_2 concentrations. Several other lines of evidence support this finding; for example, chlorophyll fluorescence measurements from leaves of the *C. canadensis* and *L. styraciflua* trees grown in the elevated CO_2 Duke FACE rings indicated these trees had increased rates of electron transport to maintain a balance between RuBP carboxylation and regeneration in the Calvin cycle (44).

We further hypothesize that the increased number of mitochondria in elevated CO_2-grown plants also is a response to increased energy demand during the daylight hours (45–48). Increased carbon fixation in plants grown at elevated CO_2 suggests more cellular energy and reductant is used in triosephosphate and starch production, RuBP regeneration, and sugar transport, perhaps leaving other cellular energy demands unmet. Increased mitochondrial respiration during the day could meet the increased daytime energy demands of faster growing, more metabolically active cells and therefore account for the increased number of mitochondria. Wang *et al.* (37) report that leaf respiration in the light (R_L) was significantly higher in *X. strumarium* plants grown at elevated CO_2 compared with ambient CO_2 partial pressure. Furthermore, the ratio of R_L to the light-saturated photosynthetic rate was higher at elevated CO_2, but the ratio of nighttime respiration to photosynthesis was unaffected by CO_2 levels. Light inhibited leaf respiration at both CO_2 concentrations, but to a lesser degree for elevated (17–24%) than for ambient (29–35%) CO_2 treatments, presumably because elevated CO_2-grown plants had a higher demand for energy and carbon skeletons than ambient CO_2-grown plants. Other lines of evidence support the link between daytime energy demand and mitochondrial respiration. For example, by using oligomycin to inhibit mitochondrial ATP synthase, it has been shown that photosynthesis strongly depends on mitochondrial energy production (45, 46). Furthermore, 25–50% of the redox equivalents produced in plant mitochondria are oxidized elsewhere in the cell (49). These uses of ATP and reductant also would influence the cellular adenylate energy charge and ATP/ADP ratio, which is thought to have primary control of respiration (7), and could provide a proximate signal for increased mitochondrial biogenesis. ATP production is not the only function of the mitochondria, and there are several other potential links between mitochondrial numbers, chloroplast structure, and their responses to growth in elevated CO_2 partial pressures (*cf.* ref. 50). Linkages with the glyoxylate cycle, photorespiration, nitrogen assimilation, and stromal redox state also may be involved.

Although many respiratory responses to plant growth in elevated CO_2 are subtle and often variable, the change in mitochondrial number presented here is dramatic and clear. Furthermore, whereas leaf-level studies suggest several mechanisms that regulate general photosynthetic responses, these long-term responses can be modulated by the genetic, physiological, biochemical, and morphological traits of the plants and the environmental variables regulating them (e.g., refs. 4, 13, 51–53). Consequently, a predictive understanding of the long-term photosynthetic response to growth in elevated CO_2 partial pressures has been elusive. Thus it is important to note that the plants studied here, although of many different families, life histories, morphologies, and habitats, all responded similarly ultrastructurally regardless of the CO_2-dosing technology. Although the precise cause of these responses is unknown, there is evidence to suggest that cellular energy demands may increase when plants are grown at elevated CO_2, particularly during the daylight hours. Clearly, a more complete understanding of this observed response and its implications for the 60 gigatonne yr^{-1}

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of carbon that enters the Earth's atmosphere via plant respiration is essential.

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