Plant Physiol. (1995) 108: 469–474

# Mitochondria Increase Three-Fold and Mitochondrial Proteins and Lipid Change Dramatically in Postmeristematic Cells in Young Wheat Leaves Grown in Elevated $CO_2$[1]

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**A dramatic stimulation in mitochondrial biogenesis during the very early stages of leaf development was observed in young wheat plants (*Triticum aestivum* cv Hereward) grown in elevated $CO_2$ (650 $\mu L\ L^{-1}$). An almost 3-fold increase in the number of mitochondria was observed in the very young leaf cells at the base of the first leaf of a 7-d-old wheat plant. In the same cells large increases in the accumulation of a mitochondrial chaperonin protein and the mitochondrial 2-oxoglutarate dehydrogenase complex and pyruvate dehydrogenase complex were detected by immunolabeling. Furthermore, the basal segment also shows a large increase in the rate of radiolabeling of diphosphatidylglycerol, a lipid confined to the inner mitochondrial membrane. This dramatic response in very young leaf cells to elevated $CO_2$ suggests that the numerous documented positive effects of elevated $CO_2$ on wheat leaf development are initiated as early as 12 h postmitosis.**

The effect of elevated levels of $CO_2$ on the developing wheat leaf (*Triticum aestivum* cv Hereward) in young plants is of critical importance to the understanding of the plant's long-term response to rising $CO_2$ levels. In mature leaf tissue, elevated $CO_2$ has been shown to have a positive effect on plant growth and development (Akita and Moss, 1972; Neales and Nicholls, 1978; Havelka et al., 1983; du Cloux et al., 1989; Chaudhuri et al., 1990; Lawlor and Mitchell, 1991; Long and Drake, 1992). Perturbations to the initial stages of leaf differentiation herald future changes and form the foundation of our understanding of the nature of the mature plant's complex reaction to its changing environment.

In the developing wheat leaf after only 7 d of growth, a stimulation in growth rate has been detected by an increase in cell and chloroplast size in elevated $CO_2$-grown tissue (Robertson and Leech, 1995). Other studies have reported a wide variation in the respiratory response of plants to elevated $CO_2$ dependent on species, developmental stage, and growth conditions (Gifford et al., 1985; Hrubec et al., 1985; Amthor, 1991; Bunce and Caulfield, 1991; Farrar and Williams, 1991; Ziska and Teramura, 1992; McKee and Woodward, 1994). Since mitochondrial gene expression in the young wheat leaf is known to be maximal in the very young basal cells (Topping and Leaver, 1990), i.e. during the earliest stages of chloroplast development, we have also analyzed the effects of elevated $CO_2$ on mitochondrial development in the young wheat leaf. In this paper we show that organelle changes stimulated by elevated $CO_2$ are not restricted to chloroplasts, but that there is an accelerated rate of mitochondrial biogenesis after only 12 h of cellular development postmitosis in the basal cells of the leaf.

## MATERIALS AND METHODS

### Plant Material

Wheat plants (*Triticum aestivum* cv Hereward) were grown in controlled environment cabinets (Fi-Tottron 600H; Fisons Ltd., Loughborough, UK) in the Department of Biology, University of Essex. Two seedlings were sown in sand per 0.7-L pot to minimize source-sink restrictions. Nutrient conditions were nonlimiting. The light source consisted of Osram Powerstar HQI-R 250W/NDL lamps with a red/far red ratio of 2.01. A day/night temperature regime was maintained at 20/15°C during a 14-h photoperiod with a PPFD of 750 $\mu mol\ m^{-2}\ s^{-1}$ and RH at 65 to 70%. The cabinets were assigned to either ambient (350 $\mu L\ L^{-1}$) or elevated (650 $\mu L\ L^{-1}$) $CO_2$ conditions. The first leaf after 7 d of growth was harvested and the coleoptile and second leaf were removed prior to embedding. Ten leaves were harvested from 10 individual seedlings for each $CO_2$ level. Slices of tissue (5 mm in thickness) were cut transversely from defined areas of each leaf. Each 5-mm slice of tissue was further dissected into 5 × 1 mm consecutive transverse

[1] Supported by Agricultural and Food Research Council (UK) grants [LR87/524(W)] to R.M.L. and [PG72/524(W)] to J.L.H.

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Abbreviations: OGDC, 2-oxoglutarate dehydrogenase complex; PDC, pyruvate dehydrogenase complex.

**Table I.** *Mitochondrial number per cell wall length*

Mitochondria density per cell wall length (μm) in 5-μm-thick leaf sections of basal cells (0–0.5 cm from leaf base) from elevated (650 μL $L^{-1}$) and ambient (350 μL $L^{-1}$) $CO_2$-grown first leaves of 7-d-old wheat plants of *T. aestivum* cv Hereward. The means and SE values in parentheses were of 30 separate samples. $P < 0.001$ (Student's *t* test). Note: In both ambient and elevated $CO_2$-grown tissue, mesophyll cells contain 40% more mitochondria than vascular cells.

| Cell Type | 350 μL $L^{-1}$ $CO_2$ | 650 μL $L^{-1}$ $CO_2$ |
|---|---|---|
| | *No. of mitochondria per 100 μm of cell wall* | |
| Mesophyll | 27 (2) | 73 (5) |
| Vascular | 19 (1) | 52 (2) |

slices of tissue, giving a total of 50 tissue slices for each defined area. Subsequent analysis was made taking random samples from this pooled tissue. The ultrastructures of at least three slices of tissue taken from the tissue pool were examined for each $CO_2$ level. Mitochondrial antigens were localized within at least five separate tissue slices for each $CO_2$ level.

### Preparation of Leaf Tissue for Transmission EM

Slices (2 to 3 mm in thickness) were cut from the intact leaf tissue from between 0 and 0.5 cm from the leaf base and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at room temperature. The samples were washed three times in 0.1 M phosphate buffer, pH 7.2, before postfixation in 1% (v/v) osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature. After three additional washes in 0.1 M phosphate buffer, the tissue was dehydrated through an acetone series and embedded in Spurr's epoxy resin (TAAB Laboratories Equipment Ltd, Reading, Berkshire, UK; Spurr, 1969). Ultrathin sections were cut for EM using a diamond knife and mounted on 200 square mesh copper grids coated with 0.3% formvar. Double staining in uranyl acetate and lead citrate preceded examination on a Jeol JEM-1200EX electron microscope.

### Preparation of Leaf Tissue for Immunocytology

Slices of leaf tissue were cut from intact leaf tissue as described above. The leaf tissue was cut into 3% (w/v) paraformaldehyde, 50% (v/v) ethanol, and 5% (v/v) acetic acid (Bauwe, 1984). Fixation was carried out overnight at room temperature. Dehydration through an ethanol series preceded embedding in molten PEG 1500 (Fisons Ltd.) at 56°C (Hawes, 1988). Peel-away molds (Polyscientific, Moulton Park, Northampton, UK) were used to facilitate orientation of the samples. Transverse tissue sections (5 μm thick) were cut on a Spencer AO 820 microtome using a disposable steel blade. Sections were adhered to poly-L-Lys (Sigma)-coated slides and left to dry on a hotplate at 35 to 40°C overnight. Poly-L-Lys-coated slides were prepared by cleaning new glass slides in 1% HCl in 70% ethanol for 2 h and soaking in diluted poly-L-Lys solution (1:10 with deionized water) for 5 min at room temperature prior to drying in a 60°C oven for 1 h or at room temperature overnight. Ribbons of six sections were laid onto dampened slides. The sections could be stored dry for several weeks at 4°C without loss of antigenicity.

### Antibody Preparation

For the purification of chaperonin hsp 58 homolog, about 1 g of total mitochondrial protein was isolated from potato tubers as described by Burt and Leaver (1994). Mitochondria were subfractionated and the hsp 58 chaperonin protein was purified essentially as described by McMullin and Hallberg (1987). For antibody production, about 400 μg of purified hsp 58 protein was solubilized in 0.1% (w/v) SDS and injected into rabbits in the presence of Freund's complete adjuvant. Two additional injections, using the same amount of protein, were given at 14-d intervals. The serum was then isolated and tested by western blot analysis using a 1:5000 dilution of the serum against total protein extracted from tubers. Polyclonal antibodies to highly purified PDC and OGDC from bovine heart were raised as described previously (De Marcucci et al., 1985). For immunofluorescence analysis, IgG was purified from these antisera essentially as described by Joplin et al. (1991).

### Immunolocalization

PEG 1500 was removed from the sections by soaking the slides in twice-distilled water from a glass still for 30 s at room temperature and air drying. Prior to staining, the sections were rehydrated through an ethanol series to water and finally PBS consisting of 0.16 M NaCl, 8.0 mM $Na_2HPO_4$, 2.7 mM KCl, and 1.5 mM $KH_2PO_4$. The sections were incubated overnight at 4°C with 100 μL of primary antibody diluted in 0.5% (w/v) BSA (Sigma) in PBS. Antibodies to a mitochondrial chaperonin protein, to the OGDC, and to the PDC were applied to leaf sections.

**Figure 1.** (On facing page). Immunolocalization of mitochondrial chaperonin, PDC, and OGDC in 5-μm-thick, PEG-embedded transverse sections from the very young basal cells (12 h postmitosis) of first leaves of *T. aestivum* cv Hereward. Arrowheads indicate examples of labeled mitochondria. Chaperonin localization is seen within mitochondria in leaf tissue sections a and b and in individual cells c, d, e, and f. a, c, and e are from elevated (650 μL $L^{-1}$) $CO_2$-grown leaf tissue and b, d, and f are from ambient (350 μL $L^{-1}$) $CO_2$-grown leaf tissue. Note: c and d are mesophyll tissue and e and f are vascular tissue. a, Leaf tissue, elevated $CO_2$; b, leaf tissue, ambient $CO_2$; c, mesophyll, elevated $CO_2$; d, mesophyll, ambient $CO_2$; e, vascular, elevated $CO_2$; f, vascular, ambient $CO_2$. PDC localization: g, mesophyll, elevated $CO_2$; h, mesophyll, ambient $CO_2$; i, vascular, elevated $CO_2$; j, vascular, ambient $CO_2$. OGDC localization: k, mesophyll, elevated $CO_2$; l, mesophyll, ambient $CO_2$. Cross-reaction with plastid chaperonin is evident in a through f. Autofluorescence of the cell wall (especially in the vicinity of the vascular bundle) is evident in a and b. Bar = 10 μm. Ab, Abaxial epidermis; Ad, adaxial epidermis; ME, mesophyll cell; P, plastid; VP, vascular parenchyma; VB, vascular bundle.

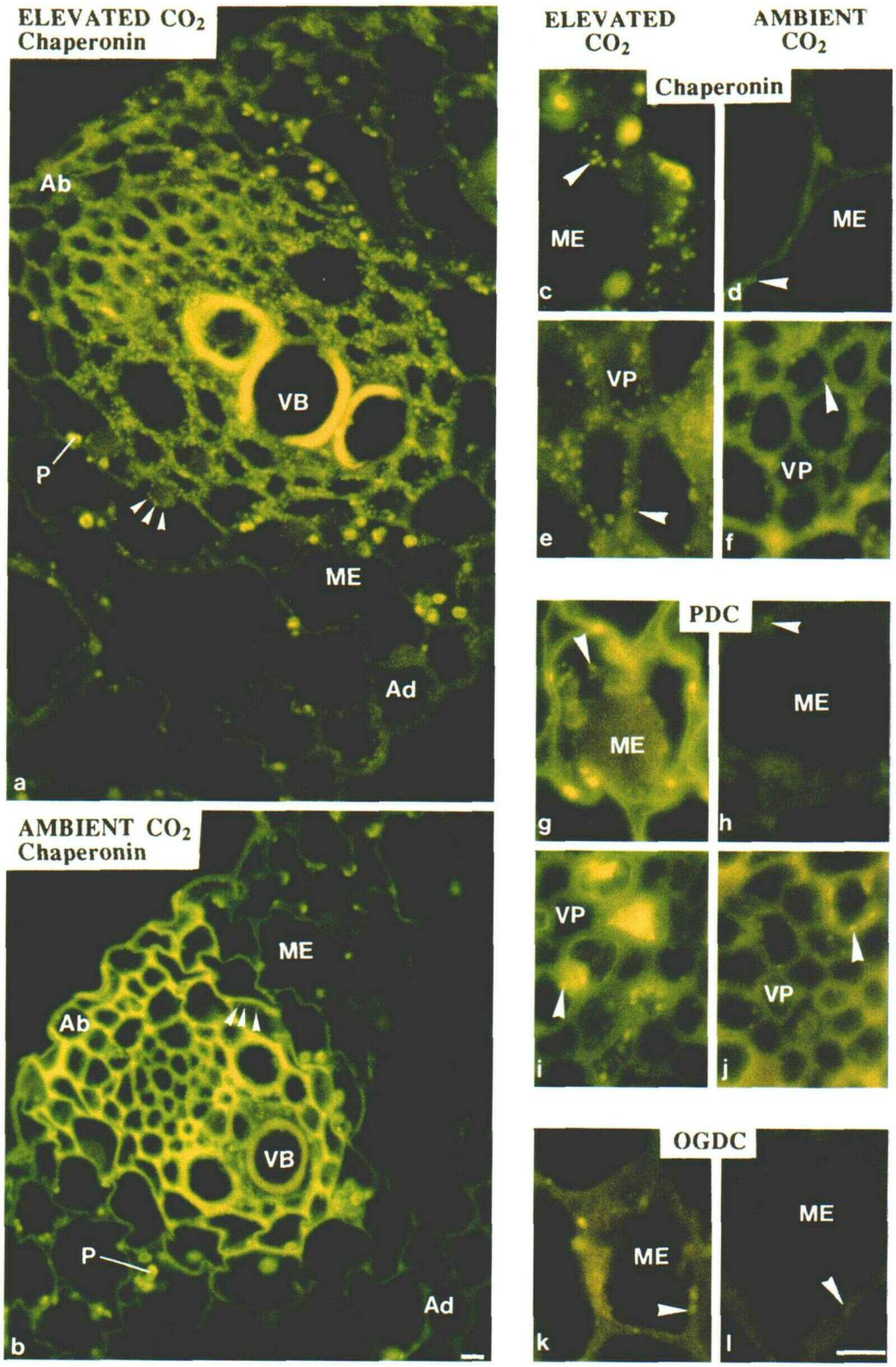


**Figure 1.** Legend appears on facing page.

Antibody dilutions were selected after extensive experimentation to obtain the best labeling conditions. After primary antibody incubation, slides were washed for 15 min with 0.5% BSA in PBS followed by 0.01% Tween 20 in PBS and finally in PBS before incubation for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (whole molecule) antisera (Sigma), diluted as recommended by the manufacturer, and rinsed as above. The slides were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Primary antibody was substituted by 0.5% BSA in PBS in the control experiment to confirm the specificity of the antibodies. Approximately 15 slides (90 sections) taken from at least five different leaf samples for each $CO_2$ level were examined for each antibody treatment.

### Fluorescence Photomicrography

Sections were viewed using a Nikon Microphot-FXA microscope with an epifluorescence attachment and a 100-W high-pressure mercury lamp. Nikon's superior-quality NCF Fluor Epi-Fluorescence oil objectives (×40, ×100; numerical aperture 1.3–0.8) provided an enhanced optical system. The B-2H epi-fluorescence filter combination (dichroic mirror block, 510: excitation filter, 450 to 490: barrier filter, 515IF) was used for the excitation of the fluorochrome, fluorescein isothiocyanate. Photomicrography was fully controlled by a microcomputer system and recorded on Kodak Ektachrome 400 color slide film. Color photomicrographs of immunolabeled mitochondria in leaf sections were used to calculate the number of mitochondria via an image analysis system (Seescan Imaging Ltd., Cambridge, UK) linked to a monochrome charge coupled device camera. Due to the difficulty of determining exactly within which cell a mitochondrion is located within a section, mitochondria were counted on either side of a measured length of cell wall.

### Lipid Labeling and Analysis

Lipids were labeled from [1-$^{14}$C]acetate (1 $\mu$Ci) by incubation of basal leaf segments under conditions described by Walker et al. (1988). Lipids were extracted by a high-salt extraction method (Garbus et al., 1963). Individual lipid classes were separated by TLC using 0.2 M ammonium sulfate-impregnated plates. The solvent system consisted of a chloroform to methanol to 6.5 M ammonia solution (17:7:1, v/v). Detection of lipid classes, identification, and measurement of radioactive incorporation were as described previously (Walker et al., 1988).

## RESULTS AND DISCUSSION

To determine the number of mitochondria in the very young leaf cells at the base of the young wheat leaf, mitochondria were visualized by immunolabeling wheat leaf sections for a mitochondrial chaperonin protein (see "Materials and Methods"). Mitochondria were counted per 100 $\mu$m of cell wall length in mesophyll and in vascular parenchyma cells from elevated and ambient $CO_2$-grown tissue. An almost 3-fold increase in the mitochondrial density was seen in tissue grown in elevated $CO_2$ (Table I). This increase was seen in both the vascular parenchyma and in the mesophyll cells. Mesophyll cells have very large central vacuoles and a thin layer of cytoplasm containing the mitochondria. In contrast, the vascular parenchyma cells are smaller and less vacuolated with a proportionally larger volume of cytoplasm. The dramatic change in mitochondria number was seen in every cell examined in both mesophyll and vascular tissue. The mitochondria number increase and the universal response of every cell to elevated $CO_2$ is clearly visualized in the comparative plates in Figure 1.

To confirm this increase in mitochondrial number in elevated $CO_2$ and compare the fine structure of the mitochondria in elevated and ambient-grown $CO_2$ tissue, resin-embedded sections of basal leaf tissue were examined in the electron microscope. In these 90-nm-thick sections, representative mitochondrial populations were most evident in the vascular tissue where the cells were less vacuolated than in mesophyll tissue. The increase in mitochondrial number in elevated compared to ambient-grown tissue was confirmed as illustrated in the electron micrographs in Figure 2. The internal structure of the mitochondria was not affected by $CO_2$ level, with no obvious difference in the number and size of the cristae. Mitochondria were distributed throughout the cytoplasm in both ambient and elevated $CO_2$-grown tissue (Fig. 2).

The increase in mitochondrial number suggests that a stimulation in mitochondrial biogenesis had occurred under elevated $CO_2$-growth conditions. To establish whether the accumulation of mitochondrial proteins had also been stimulated by the increase in $CO_2$ level, leaf sections were immunolabeled for a mitochondrial chaperonin, the mitochondrial OGDC and the PDC. The application of fluorescently labeled probes to these tissue sections demonstrated clearly the large increase in the mitochondrial population and corresponding increases in protein accumulation in the very young basal cells grown in elevated $CO_2$ (Fig. 1). The increase in fluorescence staining was evident for all three probes in the leaf tissue sections grown at 650 $\mu$L L$^{-1}$ $CO_2$ compared to those taken from leaves grown at 350 $\mu$L L$^{-1}$ $CO_2$, but was significantly more pronounced for chaperonin (Fig. 1, a–f) than for PDC and OGDC (Fig. 1, g–l). As observed in the electron microscope, the distribution of the mitochondria was not affected by increasing the $CO_2$ level. In both elevated and ambient-grown tissue the mitochondria are located throughout the cytoplasm. We conclude that increasing the atmospheric concentration of $CO_2$ stimulates mitochondrial protein and mitochondrial complex accumulation in the very young cells in the base of the first leaf of *T. aestivum* cv Hereward.

Bligny and Douce (1980) showed clearly that in plants, diphosphatidylglycerol was localized specifically within the inner mitochondrial membrane. This distribution has been confirmed for a variety of tissues (see Harwood, 1987). Therefore, we chose to follow radiolabeling of diphosphatidylglycerol as an independent measure of mi-

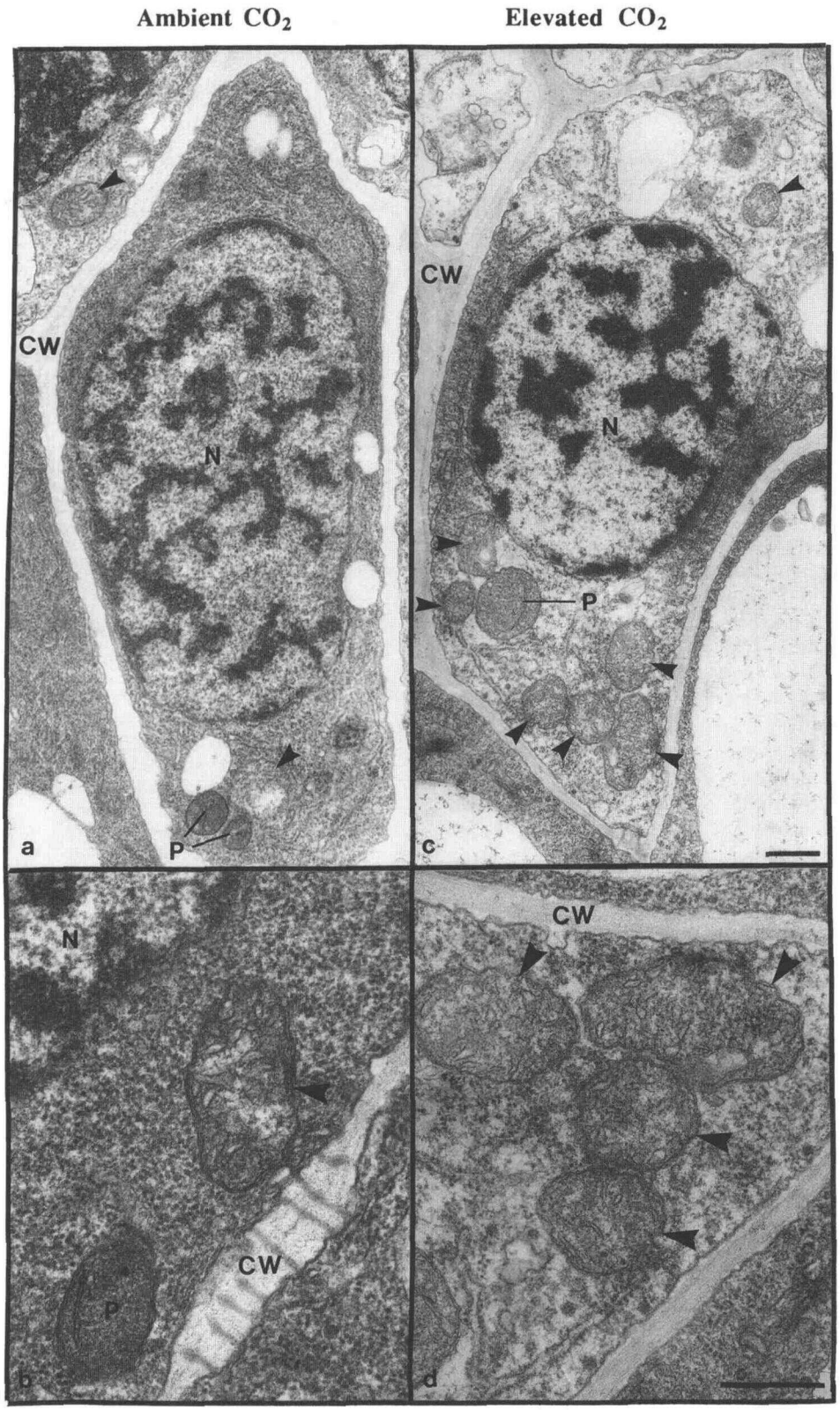


**Figure 2.** Electron micrographs of very young leaf cells (12 h postmitosis) at the base of the first leaf of *T. aestivum* cv Hereward. Ambient (350 $\mu$L $L^{-1}$) (a and b) and elevated (650 $\mu$L $L^{-1}$) (c and d) $CO_2$-grown leaf tissue. Low-power micrographs of single cells show few mitochondrial profiles (a, ambient) and many mitochondrial profiles (c, elevated). High-power micrographs of ambient (b) and elevated (d) cells illustrate the unchanged morphology of the mitochondria. Bar = 500 nm. CW, Cell wall; N, nucleus; P, plastid.

**Table II.** *Labeling rates of total and individual lipids by the basal region of wheat leaves from [1-$^{14}$C]acetate*

First leaves of 7-d-old wheat plants were cut transversely and the basal region from elevated (650 $\mu$L L$^{-1}$) and ambient (350 $\mu$L L$^{-1}$) $CO_2$-grown plants was incubated with [1-$^{14}$C]acetate (1 $\mu$Ci) for 4 h (see "Materials and Methods"). Data represent mean ± SD ($n$ = 3). Numbers in parentheses represent percentages of total lipid incorporation.

| Lipid | 350 $\mu$L L$^{-1}$ $CO_2$ | 650 $\mu$L L$^{-1}$ $CO_2$ |
|---|---|---|
| | *Lipid labeling (dpm × 10$^{-3}$)/0.1 g fresh weight* | |
| Nonpolar fraction | 79.6 ± 5.9 | 51.7 ± 1.9 |
| | (42.9 ± 3.2) | (32.8 ± 1.2) |
| Phosphatidylcholine | 47.3 ± 9.3 | 49.1 ± 4.7 |
| | (26.0 ± 6.0) | (31.3 ± 2.9) |
| Diphosphatidylglycerol | 0.7 ± 0.2 | 4.0 ± 0.3 |
| (Cardiolipin) | (0.4 ± 0.1) | (2.5 ± 0.2) |
| Phosphatidylglycerol | 22.2 ± 4.7 | 24.0 ± 5.4 |
| | (11.7 ± 2.5) | (15.1 ± 3.4) |
| Total lipid | 185.6 ± 12 | 157.6 ± 4.9 |

tochondrial biogenesis. The results (Table II) show clearly that labeling was very much increased for young tissue from wheat grown under elevated $CO_2$. On a total labeling/g fresh weight basis there was a 5.7-fold increase and as a percent of total radiolabeled lipids there was a 6.3-fold increase. These data indicate a very significant elevation in diphosphatidylglycerol formation, commensurate with an increase in mitochondrial biogenesis.

We have identified a specific stage in the cellular development of the young wheat leaf, i.e. in cells 12 h postmitosis when dramatic changes in mitochondria are evident in response to an increased level of $CO_2$. Significant increases were found in mitochondrial number, in the accumulation of the mitochondrial proteins chaperonin, the PDC, and the OGDC, and in the formation of diphosphatidylglycerol. This dramatic response to elevated $CO_2$ very early in wheat leaf development suggests that the many documented positive effects of elevated $CO_2$ are initiated as early as 12 h postmitosis. We have previously recorded significant changes in cell size and chloroplast size in mature leaf cells grown under elevated $CO_2$. The effects of these changes on the future development of the leaf grown in elevated $CO_2$ remain to be determined.

## ACKNOWLEDGMENT

We acknowledge the skilled assistance of A. Liddell to C.J.L.

Received December 1, 1994; accepted February 22, 1995.


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