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Doubling the CO_2 Concentration Enhanced the Activity of Carbohydrate-Metabolism Enzymes, Source Carbohydrate Production, Photoassimilate Transport, and Sink Strength for *Opuntia ficus-indica*[1]

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After exposure to a doubled CO_2 concentration of 750 μmol mol^{-1} air for about 3 months, glucose and starch in the chlorenchyma of basal cladodes of *Opuntia ficus-indica* increased 175 and 57%, respectively, compared with the current CO_2 concentration of 370 μmol mol^{-1}, but sucrose content was virtually unaffected. Doubling the CO_2 concentration increased the nocturnal malate production in basal cladodes by 75%, inorganic phosphate (Pi) by 32%, soluble starch synthase activity by 30%, and sucrose-Pi synthase activity by 146%, but did not affect the activity of hexokinase. Doubling CO_2 accelerated phloem transport of sucrose out of the basal cladodes, resulting in a 73% higher dry weight for the daughter cladodes. Doubling CO_2 increased the glucose content in 14-d-old daughter cladodes by 167%, increased nocturnal malate production by 22%, decreased total amino acid content by 61%, and increased soluble starch synthase activity by 30% and sucrose synthase activity by 62%. No downward acclimation of photosynthesis during long-term exposure to elevated CO_2 concentrations occurs for *O. ficus-indica* (M. Cui, P.M. Miller, P.S. Nobel [1993] Plant Physiol 103: 519–524; P.S. Nobel, A.A. Israel [1994] J Exp Bot 45: 295–303), consistent with its higher source capacity and sink strength than under current CO_2. These changes apparently do not result in Pi limitation of photosynthesis or suppression of genes governing photosynthesis for this perennial Crassulacean acid metabolism species, as occur for some annual crops.

Increases in the atmospheric CO_2 concentration generally enhance the rates of photosynthesis and growth for various C_3 plants, although the enhancement can diminish during long-term exposure to elevated CO_2 concentrations for annual plants with limited sinks (Sasek et al., 1985; Peet et al., 1986; Stitt, 1991). Such plants commonly respond to elevated CO_2 concentrations with a decrease of carboxylating enzyme activity and an increase of carbohydrate accumulation (Yelle et al., 1989a, 1989b; Köner et al., 1995). Because Rubisco activity remains in excess of that needed to support the photosynthetic rates observed under elevated CO_2 concentrations (Sage et al., 1989), the acclimation of photosynthesis may be a result of excess starch and sugar accumulation, which can cause feedback inhibition of photosynthesis (Sharkey and Vanderveer, 1989; Yelle et al., 1989a, 1989b; Stitt, 1991). Based mainly on studies with annual crops and Arabidopsis, two hypotheses have been proposed to explain the feedback inhibition: (a) accumulation of starch and Suc can reduce the rates of their own synthesis, causing sugar phosphates to build up and deplete Pi pools in the chloroplasts and cytosol, thereby inhibiting photophosphorylation (Sharkey and Vanderveer, 1989; Stitt, 1991); and (b) buildup of sugars, especially Glc and Suc, can suppress (down-regulate) the expression of genes governing photosynthesis (Oosten et al., 1994; Sheen, 1994; Nie et al., 1995), resulting in a lowered photosynthetic rate.

On the other hand, some C_3 tree species and perennial CAM plants do not show acclimation of photosynthesis during long-term exposure to elevated CO_2 concentrations (Idso and Kimball, 1994; Nobel and Israel, 1994; Ceulemans et al., 1995; Liu and Teskey, 1995). Doubling the atmospheric CO_2 concentration to 750 μmol mol^{-1} air increases CO_2 uptake and productivity of the perennial CAM species *Opuntia ficus-indica* by 25 to 70% (Cui et al., 1993; Nobel and Israel, 1994) and also decreases the activities of the carboxylating enzymes PEP carboxylase and Rubisco (Israel and Nobel, 1994). One reason may be that such species can generate numerous new sink organs, in contrast to annual crops such as wheat, rice, and tomato, which usually produce limited sink organs during a single growing season. Limitations in sink capacity can accelerate the acclimation of photosynthesis to high CO_2 concentrations due to a lack of efficient transport of photosynthates out of source leaves (Behboudian, 1994; Oosten et al., 1994). So far, the impacts of high atmospheric CO_2 concentrations on source-sink relations, metabolite levels, and carbohydrate-metabolism enzymes have not been collectively studied in an integrated manner.

Source-sink transport, carbohydrate partitioning, and sink growth characteristics can profoundly influence photosynthesis in source organs and hence whole plant growth. Therefore, a detailed study on how these processes are affected by elevated CO_2 concentrations can improve the understanding of plant responses to elevated CO_2 con-

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centrations in perennial CAM plants and how such plants differ from annual crops or trees. The present study examined the effects of a doubled CO_2 concentration on source-sink transport and carbohydrate metabolism of *O. ficus-indica*. Suc-Pi synthase and starch synthase, whose activity levels are sensitive to environmental conditions (Vassey et al., 1991; Hawker and Jenner, 1993), can regulate the synthesis of Suc and starch and also affect the rate of photosynthesis; hexokinase and invertase play pivotal roles in regulating photosynthetic gene expression in source organs under elevated CO_2 concentrations (Sheen, 1994). Invertase, Suc synthase, and starch synthase in sink organs are involved in acquiring Suc from the phloem and are closely related to sink strength (Sung et al., 1989; Vassey, 1989; Black, 1993; Hawker and Jenner, 1993). Thus, these enzymes were assayed in source and sink organs of *O. ficus-indica* to determine whether their activities were affected by doubling the CO_2 concentration and to what extent changes in their activities accounted for responses to elevated CO_2 concentrations at the whole-plant level.

MATERIALS AND METHODS

Plant Material

Mature 1-year-old cladodes (flattened stem segments) of *Opuntia ficus-indica* (L.) Miller (Cactaceae) from the Agricultural Research Station (University of California, Riverside) were planted in 0.005-m^3 pots containing soil from the Agricultural Research Station mixed with an equal part of quartz sand and were maintained in Conviron E-15 environmental chambers (Controlled Environments, Pembia, ND). Day/night air temperatures were 25/15°C, and the photoperiod was 12 h with a total daily photosynthetic photon flux (400–700 nm) of 22 mol m^{-2} d^{-1} in the planes of the cladodes examined (instantaneous value of 500 μmol m^{-2} s^{-1}). Ten plants were maintained under the current ambient CO_2 concentration of 370 μmol mol^{-1} air and an additional 10 plants were maintained under an approximately doubled CO_2 concentration of 750 μmol mol^{-1}. The doubled CO_2 concentration was achieved by releasing 100% CO_2 via a massflow meter connected to a needle valve into an environmental chamber whose CO_2 concentration was monitored with a LI-6200 portable photosynthesis system (Li-Cor, Lincoln, NE). Plants were watered twice weekly with one-tenth-strength Hoagland solution.

To assess the dependency of sink organ growth on the supply of photoassimilate from a source tissue, first-order daughter cladodes growing on the planted basal cladodes were removed periodically so that only two of equal ages remained. After 2 to 3 months with the plants in either the current or the doubled CO_2 concentration, a 1-d-old daughter cladode on each basal cladode was covered with a neutral-density nylon net to attenuate the photosynthetic photon flux by 88%, leading to 2.6 mol m^{-2} d^{-1} in the planes of the shaded daughter cladodes, approximately the light compensation point for *O. ficus-indica* (Nobel and Hartsock, 1983); the other 1-d-old daughter cladode was unshaded. The shaded and unshaded cladodes were harvested 13 d later and dried at 80°C in a forced-draft oven to determine dry weight. For ^{14}C-labeling experiments, the daughter cladodes were harvested at 22 d.

$^{14}CO_2$ Labeling

At 14 d after the appearance of daughter cladodes, $^{14}CO_2$, generated by adding 1.0 cm^3 of 4 × 10^3 mol m^{-3} HCl to 9.2 × 10^6 Bq $NaH^{14}CO_3$, was introduced into sealed chambers 10 cm in diameter placed on both sides at the center of basal cladodes for 20 min at midnight. Immediately after labeling and at 10:00 AM on the subsequent 7 d, three tissue samples were removed from the labeling region with a cork borer 0.5 cm in diameter, six were removed from surrounding areas on the basal cladodes, and three were removed from the daughter cladodes. For basal cladodes, the chlorenchyma was separated from the water-storage parenchyma using a razor blade and then weighed. For daughter cladodes, the water-storage parenchyma was not readily distinguishable from the chlorenchyma at the sampling times, so the two tissues were not separated. After combining the three to six samples of a particular type, the samples were boiled for 3 min in distilled water, blended with a high-speed tissue homogenizer, and then centrifuged. The supernatant fluid was used for ^{14}C counting to determine the radioactivity of photoassimilate per gram of fresh weight. The insoluble portion of the tissue extract was resuspended and then centrifuged three times with methanol:chloroform:water (12:5:3, v/v/v) and twice with distilled water to remove pigments, lipids, and remaining solutes. The remaining precipitate was mixed with 2 cm^3 of distilled water and then heated at 100°C for 2 h to suspend the starch, which was hydrolyzed to Glc with amyloglucosidase (EC 3.2.1.3) at 55°C for 20 h prior to ^{14}C counting (Haissig and Dickson, 1979).

Phloem sap was collected using stylets of cochineal insects (*Dactylopius opuntiae* Cockrell) that infested the basal cladodes (Wang and Nobel, 1995). Colonies of cochineal insects on the region between the ^{14}C-labeled area and the daughter cladodes (the pathway for source-sink photoassimilate transport) were gently wiped away with tissue paper soaked in 80% ethanol, leaving the severed stylets protruding from the cladode surface. Droplets of phloem sap were collected 2 h after ^{14}C labeling at midnight and at 10:30 AM on the subsequent 7 d. The exuded phloem sap evaporated to semi-dried viscous droplets within 1 h after the stylets were severed, so contamination by microorganisms was minimal. After dissolving in distilled water, phloem sap was used for ^{14}C counting and to determine the specific activity of solutes.

Aliquots of the soluble tissue extract and the phloem sap were also separated into acidic, basic, and neutral fractions by passing through 0.1 cm^3 of Sephadex ion-exchange columns (Fisher and Wang, 1993), and each fraction was counted for ^{14}C activity. ^{14}C-labeled metabolites in the neutral fraction of phloem sap were separated on high-performance thin-layer chromatographic plates at 75°C using acetonitrile:water (3:1, v/v) as the mobile phase and then autoradiographed with x-ray film to identify labeled solutes.

Chemical Analysis of Metabolites

At various times during the day, 0.2 to 1.0 g of chlorenchyma from basal cladodes and of stem cores from 14-d-old daughter cladodes was harvested; one-half was immediately frozen using dry ice and the other half was dried at 80°C to determine water content. To obtain sugars, amino acids, and starch, the frozen samples were pulverized together with dry ice and then ground in 0.5 to 1.0 cm^3 of methanol:chloroform:water to precipitate proteins, followed by extraction at 25°C with 6.0 cm^3 of distilled water. After heating to 95°C for 3 min to inactivate enzymes and centrifugation, the decanted supernatant was mixed with 7 cm^3 of chloroform and centrifuged to separate out pigments and lipids. The upper, aqueous phase (containing sugars and amino acids) was collected and freeze-dried; distilled water equal in amount to that in the tissue (calculated from its water content) was added. To obtain starch and other polysaccharides, the insoluble portion of the tissue was treated as for ^{14}C-labeled starch. After pulverizing the frozen sample, grinding was done in 6.0 cm^3 of distilled water at 0°C for malate extraction and in 6.0 cm^3 of 3% perchloric acid at 25°C for Pi extraction (Sharkey and Vanderveer, 1989). For extraction of soluble protein, the frozen samples were pulverized with dry ice and extracted at 0°C in 1.0 cm^3 of 50 mol m^{-3} Mops-KOH (pH 7.5), 2 mol m^{-3} DTT, 2 mol m^{-3} EDTA, 20 kg m^{-3} polyvinylpolypyrrolidone (insoluble), 0.5% (v/v) Triton X-100, and 0.5 mol m^{-3} PMSF. After centrifugation, the supernatant was immediately used to determine soluble protein content with a Pierce Coomassie blue plus protein assay kit using BSA as the standard.

Suc and Glc were determined enzymatically (Jones et al., 1977; Sturgeon, 1990). Fru was determined using H_2SO_4-anthrone-Trp (Somani et al., 1987). Total amino acids were determined by a ninhydrin-binding method (Moore and Stein, 1954). Malate was determined with malate dehydrogenase (EC 1.1.1.37; Osmond et al., 1989). Pi was measured spectrophotometrically using ammonium molybdate-ascorbic acid (Cooper, 1977). Fructan and other oligosaccharides were separated on high-performance thin-layer chromatographic plates at 75°C using acetonitrile:water (3:1, v/v) as the mobile phase, and the plates were sprayed with anthrone for fructan (Cairns and Pollock, 1988) and with phenol-sulfuric acid for other carbohydrates (Touchstone, 1992). Osmolality of the tissue extract was measured with a Wescor (Logan, UT) model 5500 vapor pressure osmometer. Starch in the insoluble portion was analyzed using amyloglucosidase. All chemicals (except for determining soluble protein) were from Sigma.

Measurement of Enzyme Activities

At various times during the day, 0.1 to 0.2 g of chlorenchyma from basal cladodes and of stem cores from 14-d-old daughter cladodes were harvested and ground in ice-chilled mortars containing 1.0 cm^3 of 50 mol m^{-3} Mops-KOH (pH 7.2), 5 mol m^{-3} DTT, 2 mol m^{-3} EDTA, and 5 kg m^{-3} BSA. The homogenate was centrifuged for 1 min at 16,000g at 0°C in a microcentrifuge, and the supernatant was used for assays of Suc synthase (EC 3.4.1.13), which was determined spectrophotometrically (Macleod and Duffus, 1988), and soluble starch synthase (EC 2.4.1.21), which was determined using a ^{14}C-isotope method (Hawker and Jenner, 1993). To extract Suc-Pi synthase (EC 2.4.1.14), hexokinase (EC 2.7.1.1), and soluble invertase (EC 3.2.1.26), the tissue samples were ground in 2 cm^3 of 50 mol m^{-3} Hepes-NaOH (pH 7.2), 5 mol m^{-3} $MgCl_2$, 1 mol m^{-3} EDTA, 15 mol m^{-3} KCl, 20 kg m^{-3} polyvinylpolypyrrolidone, 5 mol m^{-3} DTT, and 5 kg m^{-3} BSA. After desalting with Bio-Gel P-2 columns (Bio-Rad), activities of Suc-Pi synthase (Vassey, 1989; Vassey et al., 1991), hexokinase, and acid invertase (Rao et al., 1990) were determined spectrophotometrically.

Statistical significance of differences between the current and the doubled CO_2 concentrations was analyzed using Student's t test. Data are presented as means ± SE (n = 4 plants, unless specified otherwise).

RESULTS

Photoassimilate Transport and Sink Organ Growth

Doubling the CO_2 concentration accelerated ^{14}C-photoassimilate movement from basal cladodes (source) to initially 14-d-old daughter cladodes; the time to decrease to 50% of maximum specific activity was 3.3 d under 370 μmol CO_2 mol^{-1} and 2.1 d under 750 μmol CO_2 mol^{-1} (Fig. 1A). Suc, which accounted for about 90% of total radioactivity in the phloem sap, reached maximum specific activity in the phloem sap of basal cladodes at 3.5 d under the current CO_2 concentration and 2.5 d under the doubled CO_2 concentration (Fig. 1B). For the daughter cladodes (both shaded and unshaded), ^{14}C-photoassimilate reached maximum specific activity at 4.5 d under the current CO_2 concentration and 3.4 d under the doubled CO_2 concentration (Fig. 1C). Under the doubled CO_2 concentration, unshaded and shaded daughter cladodes averaged 10% longer ($P > 0.05$), 27% wider ($P < 0.05$), and 34% thicker ($P < 0.05$) compared with the current CO_2 concentration, resulting in a 73% greater dry weight ($P < 0.05$) for 14-d-old daughter cladodes under the doubled CO_2 concentration (Table I). Shading did not significantly affect ^{14}C kinetics (Fig. 1C), size, or dry weight of daughter cladodes (Table I) for either CO_2 concentration.

Daily Changes in Metabolite Contents

Fru, Glc, and Suc contents in the chlorenchyma of basal cladodes generally followed similar daily patterns under the current and the doubled CO_2 concentrations, the highest values usually occurring in late afternoon (Fig. 2, A–C). Fru averaged 0.45 ± 0.02 μmol g^{-1} fresh weight over a 24-h period under both CO_2 concentrations (Fig. 2A). Glc increased from 0.40 ± 0.03 μmol g^{-1} fresh weight over 24 h under the current CO_2 concentration to 1.11 ± 0.14 μmol g^{-1} under the doubled CO_2 concentration ($P < 0.05$; Fig. 2B). Suc averaged 5.00 ± 0.20 μmol g^{-1} fresh weight over 24 h for both CO_2 concentrations ($P > 0.05$; Fig. 2C). The Pi content averaged 19.6 ± 3.1 μmol g^{-1} fresh weight for the

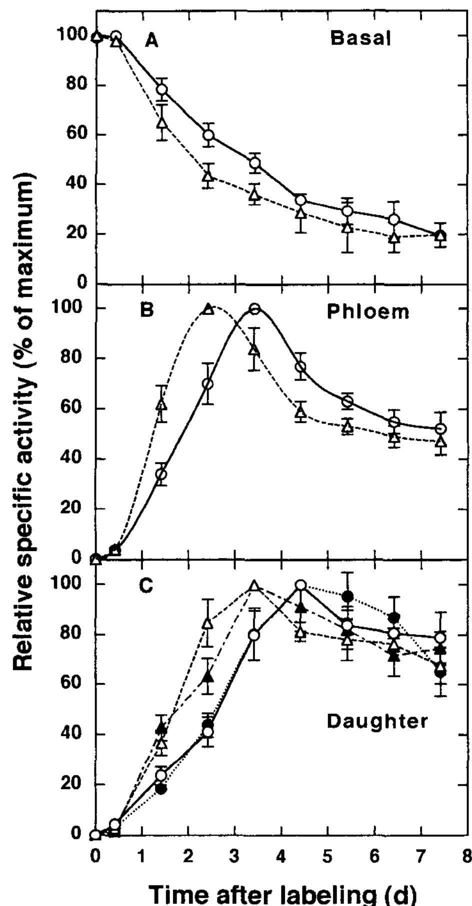

Figure 1. Kinetics of ^{14}C in the chlorenchyma of labeled basal cladodes (A), the phloem between the labeled region and daughter cladodes (B), and daughter cladodes (C) of *O. ficus-indica.* Basal cladodes on plants growing under 370 (○) or 750 (△) μmol CO_2 mol^{-1} were labeled with $^{14}CO_2$ at 14 d after the appearance of daughter cladodes. The photosynthetic photon flux was 22 mol m^{-2} d^{-1} (○, △) or reduced 88% at cladode appearance (●, ▲) for one of the two daughter cladodes per basal cladode. Data are means ± SE (n = 4 plants).

chlorenchyma of basal cladodes under the current CO_2 concentration and 25.9 ± 0.7 μmol g^{-1} under the doubled CO_2 concentration (mean ± SE for n = 4 plants; $P > 0.05$); for the water-storage parenchyma, Pi averaged 8.9 ± 1.0 μmol g^{-1} fresh weight under the current CO_2 concentration, increasing to 14.1 ± 0.8 μmol g^{-1} under the doubled CO_2 concentration ($P < 0.05$).

For the stem cores of 14-d-old daughter cladodes, the patterns of daily changes in sugar contents varied, and sugar contents were relatively high compared with basal cladodes (Fig. 2, D–F). Over a 24-h period for unshaded cladodes, Fru averaged 3.5 ± 0.5 μmol g^{-1} fresh weight for both CO_2 concentrations (Fig. 2D); Glc averaged 14.4 ± 1.4 μmol g^{-1} under the current CO_2 concentration, increasing 167% ($P < 0.05$) under the doubled CO_2 concentration (Fig. 2E); and Suc averaged 1.5 ± 0.2 μmol g^{-1} for both CO_2 concentrations (Fig. 2F). Shading daughter cladodes increased the average Fru content by 94% ($P < 0.05$) under the current CO_2 concentration and by 294% ($P < 0.05$) under the doubled CO_2 concentration (Fig. 2D); Glc was not affected by shading under the current CO_2 concentration and decreased 24% ($P < 0.05$) under the doubled CO_2 concentration (Fig. 2E); and Suc content was not affected by shading at either CO_2 concentration (Fig. 2F).

The content of total amino acids in the chlorenchyma of basal cladodes and in the stem cores of unshaded and shaded daughter cladodes was essentially constant over a 24-h period but was reduced by doubling the CO_2 concentration. In basal cladodes, total amino acids averaged 3.80 ± 0.04 μmol g^{-1} fresh weight over 24 h under the current CO_2 concentration and 3.50 ± 0.08 μmol g^{-1} under the doubled CO_2 concentration ($P < 0.05$). For unshaded daughter cladodes, total amino acids averaged 14.7 ± 0.4 μmol g^{-1} fresh weight under the current CO_2 concentration, decreasing to 5.7 ± 0.5 μmol g^{-1} under the doubled CO_2 concentration ($P < 0.05$). For shaded daughter cladodes, the total amino acid averaged 23.2 ± 0.8 μmol g^{-1} fresh weight for both CO_2 concentrations.

The starch content in the chlorenchyma of basal cladodes was maximal at the end of the daytime, becoming 15.4 μmol Glc equivalents g^{-1} fresh weight under the current CO_2 concentration and 57% higher ($P < 0.05$) under the doubled CO_2 concentration; the starch content rapidly decreased during the night under both CO_2 concentrations (Fig. 3A). In contrast, the malate content was minimal at the end of the daytime and rapidly increased during the night, becoming 121 μmol g^{-1} fresh weight under the current CO_2 concentration and 27% higher ($P < 0.05$) under the doubled CO_2 concentration (Fig. 4A). The nocturnal decrease of starch was 78% greater ($P < 0.05$) and the nocturnal increase of malate was 75% greater ($P < 0.05$) under the doubled CO_2 concentration compared with the current CO_2 concentration (Figs. 3A and 4A). For unshaded 14-d-old daughter cladodes, the starch content was relatively low; it varied irregularly during the day under the current CO_2 concentration and became maximal in the afternoon (1.2 ± 0.1 μmol Glc equivalents g^{-1} fresh weight under the current CO_2 concentration and 2.3 ± 0.1 μmol Glc equivalents g^{-1} under the doubled CO_2 concentration; Fig. 3B). The nocturnal increase of malate for unshaded daughter cladodes (Fig. 4B) was 47% of that in basal cladodes (Fig. 4A) under the current CO_2 concentration and increased 22% ($P < 0.05$; Fig. 4B) under the doubled CO_2 concentration. For shaded daughter cladodes, starch and malate contents were lower ($P < 0.05$) than for unshaded cladodes and were virtually constant over 24-h periods (Figs. 3B and 4B). For unshaded daughter cladodes, the osmolality averaged 0.277 ± 0.008 mol kg^{-1} under the current CO_2 concentration, increasing to 0.333 ± 0.008 mol kg^{-1} under the doubled CO_2 concentration ($P < 0.05$).

Daily Changes in Enzymatic Activities

The activity of soluble starch synthase in the chlorenchyma of basal cladodes was correlated with the daytime changes of starch content, becoming maximal at the end of the daytime and minimal at midnight; its mean activity over 24 h under the current CO_2 concentration was 61.9

Table I. *Properties of daughter cladodes of O. ficus-indica*

Data are for 14-d-old daughter cladodes growing under the current or the doubled CO_2 concentration and are means ± SE for $n = 5$ plants in environmental chambers at a photosynthetic photon flux of 22 mol m^{-2} d^{-1} or with one of the two daughter cladodes per basal cladode shaded 88%.

Cladode Properties	CO_2 Concentration			
	370 μmol mol^{-1}		750 μmol mol^{-1}	
	Unshaded	Shaded	Unshaded	Shaded
Length (cm)	16.6 ± 0.4	17.3 ± 0.9	18.4 ± 0.5	18.8 ± 1.5
Width (cm)	6.3 ± 0.7	5.7 ± 0.3	8.3 ± 0.3	6.9 ± 0.4
Thickness (cm)	0.54 ± 0.03	0.43 ± 0.02	0.71 ± 0.04	0.59 ± 0.03
Dry wt (g)	2.49 ± 0.10	2.20 ± 0.10	4.22 ± 0.20	3.90 ± 0.48

nmol Glc equivalents g^{-1} fresh weight min^{-1}, increasing 30% ($P < 0.05$) under the doubled CO_2 concentration (Fig. 5A). The activity of Suc-Pi synthase also showed daily fluctuations, being highest near noon and lowest at midnight; its mean activity over 24 h under the current CO_2 concentration was 43.7 nmol Suc g^{-1} fresh weight min^{-1}, increasing 146% ($P < 0.05$) under the doubled CO_2 concentration (Fig. 5B). The activity of hexokinase was similar throughout the day under the current CO_2 concentration but was highest at midnight under the doubled CO_2 concentration ($P < 0.05$); its mean activity over 24 h under the current CO_2 concentration was 174 nmol hexose-Pi g^{-1} fresh weight min^{-1}, decreasing 4% ($P > 0.05$) under the doubled CO_2 concentration (Fig. 5C). The activity of acid invertase was barely detectable (less than 0.5 nmol g^{-1} fresh weight min^{-1}) in the chlorenchyma of basal cladodes under both CO_2 concentrations.

Contrary to the case for basal cladodes, the activity of soluble starch synthase in unshaded daughter cladodes was virtually constant over 24 h, averaging 36.3 ± 0.9 nmol g^{-1} fresh weight min^{-1} under the current CO_2 concentration and increasing 30% ($P < 0.05$) under the doubled CO_2 concentration (Fig. 6A). For shaded daughter cladodes, the activity of soluble starch synthase was also virtually constant over 24 h, averaging 25.3 ± 0.8 nmol g^{-1} fresh weight min^{-1} for both CO_2 concentrations (Fig. 6A). For unshaded daughter cladodes, the activity of Suc synthase was constant under the current CO_2 concentration at 101 ± 6 nmol

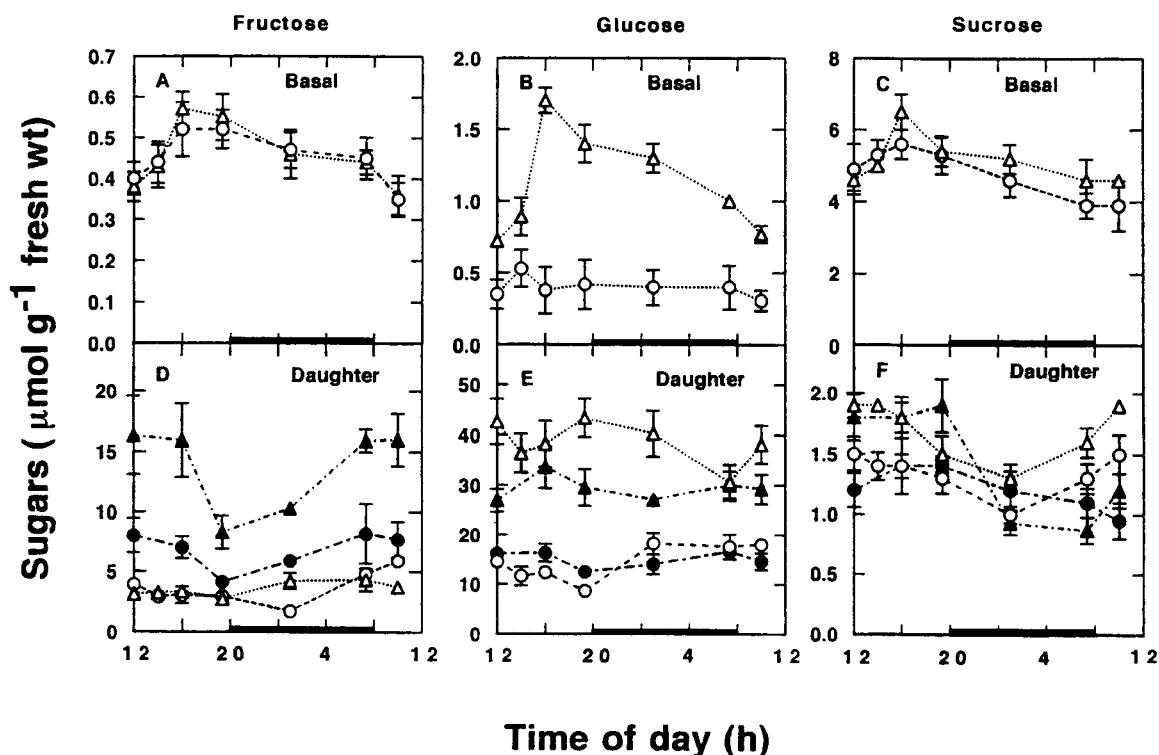

Figure 2. Daily time course for the content of Fru (A and D), Glc (B and E), and Suc (C and F) in the chlorenchyma of basal cladodes (A–C) and cores of 14-d-old daughter cladodes (D–F) growing under 370 (○, ●) or 750 (△, ▲) μmol CO_2 mol^{-1}. One of the two daughter cladodes per basal cladode was shaded 88% (●, ▲). Data are means ± SE ($n = 4$ plants). Dark bars indicate night.

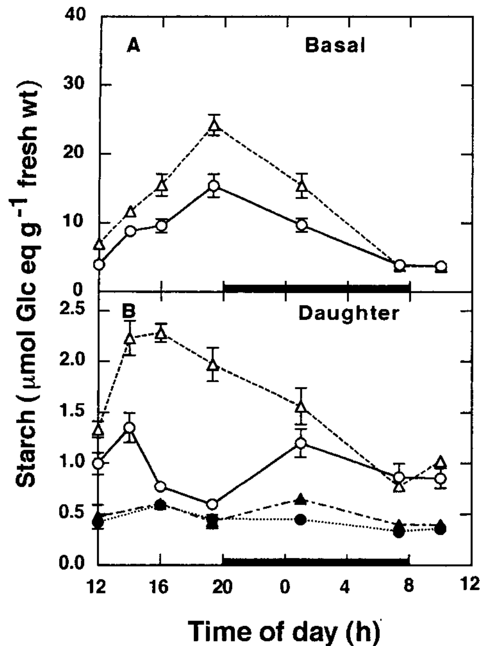

Figure 3. Daily time course for starch content in the chlorenchyma of basal cladodes (A) and in cores of 14-d-old daughter cladodes (B) growing under 370 (○, ●) or 750 (△, ▲) μmol CO_2 mol^{-1}. One of the two daughter cladodes per basal cladode was shaded 88% (●, ▲). Data are means ± SE (n = 4 plants). Dark bars indicate night.

g^{-1} fresh weight min^{-1} and increased 62% ($P < 0.05$) under the doubled CO_2 concentration, with maximal activity at mid-afternoon and the end of the night (Fig. 6B). For shaded daughter cladodes, the activity of Suc synthase was also virtually constant over 24 h, averaging 95 ± 6 nmol g^{-1} fresh weight min^{-1} for both CO_2 concentrations (Fig. 6B). The activity of acid invertase in unshaded daughter cladodes was 8.1 ± 1.1 nmol g^{-1} fresh weight min^{-1} under the current CO_2 concentration and 7.7 ± 0.7 nmol g^{-1} min^{-1} under the doubled CO_2 concentration.

The total soluble protein content in basal cladodes was virtually constant over 24 h at 3.29 ± 0.15 mg g^{-1} fresh weight under the current CO_2 concentration and 3.36 ± 0.18 mg g^{-1} under the doubled CO_2 concentration (n = 4–5 plants). For unshaded and shaded daughter cladodes, the total soluble protein content was also virtually constant over 24 h but was lower under the doubled CO_2 concentration ($P < 0.05$). Specifically, the total soluble protein content was 3.94 ± 0.17 mg g^{-1} fresh weight for unshaded daughter cladodes under the current CO_2 concentration, decreasing to 3.17 ± 0.19 mg g^{-1} ($P < 0.05$) under the doubled CO_2; for shaded daughter cladodes it was 3.49 ± 0.27 mg g^{-1} under the current CO_2 concentration, decreasing to 2.66 ± 0.09 mg g^{-1} ($P < 0.05$) under the doubled CO_2 concentration (n = 4–6 plants).

DISCUSSION

Under long-term exposure of the perennial CAM species *O. ficus-indica* to a doubled CO_2 concentration, the source capacity, including CAM activity, carbohydrate production, and source-sink photoassimilate transport, as well as sink strength, were greatly enhanced. This is consistent with the lack of acclimation of photosynthesis during long-term exposure to elevated CO_2 concentrations for this species (Cui et al., 1993; Nobel and Israel, 1994). In particular, these coordinated responses may be responsible for its continued higher rate of photosynthesis and productivity under the doubled CO_2 concentration.

Doubling the CO_2 concentration increased the levels of starch and Glc as well as the rates of starch and Suc synthesis in basal cladodes of *O. ficus-indica.* Contrary to some C_3 species whose starch level in source leaves often decreases in late afternoon accompanying a reduced photosynthetic rate (Servaites et al., 1989), the starch content increased linearly during the daytime in basal cladodes of *O. ficus-indica* and was higher under the doubled CO_2 concentration, indicating that starch synthesis was not limiting photosynthesis due to feedback inhibition. Similarly, accumulation of starch following girdling of the petioles of mature leaves of some starch-storage species also does not directly cause feedback inhibition of photosynthesis (Goldschmidt and Huber, 1992). The Suc content and its daily changes in basal cladodes were similar under both CO_2 concentrations, suggesting that doubling the CO_2 concentration did not cause accumulation of Suc in source organs of *O. ficus-indica.* Moreover, the higher activities of Suc-Pi synthase and soluble starch synthase in basal cladodes and the faster rates of phloem Suc transport and sink growth under the doubled CO_2 concentration indicate that the rates of Suc and starch synthesis were higher (to meet the

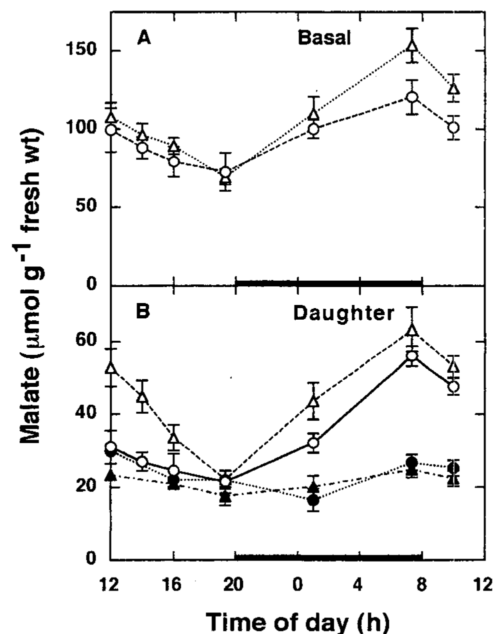

Figure 4. Daily time course for malate content in the chlorenchyma of basal cladodes (A) and in cores of 14-d-old daughter cladodes (B) growing under 370 (○, ●) or 750 (△, ▲) μmol CO_2 mol^{-1}. One of the two daughter cladodes per basal cladode was shaded 88% (●, ▲). Data are means ± SE (n = 4 plants). Dark bars indicate night.

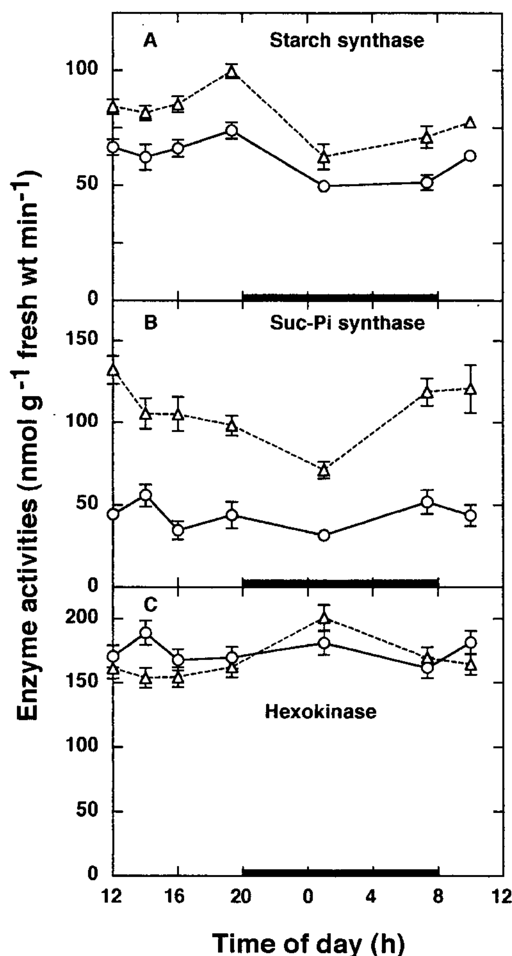

Figure 5. Daily time course for the activities of soluble starch synthase (A), Suc-Pi synthase (B), and hexokinase (C) in the chlorenchyma of basal cladodes growing under 370 (○) or 750 (△) μmol CO_2 mol^{-1}. Data are means ± SE (n = 4 plants). Dark bars indicate night.

increased demand from sink organs) compared with those under the current CO_2 concentration. About 80% of the starch synthesized during the daytime was utilized for malate synthesis during the night in basal cladodes. Nocturnal malate content, which reflects CAM activity, increased linearly throughout the night in both basal and daughter cladodes of *O. ficus-indica* and was higher under the doubled CO_2 concentration, leading to more acid storage for decarboxylation and greater Calvin cycle activity during the subsequent daytime.

Doubling the CO_2 concentration, which reduces the activities of enzymes for carboxylation in *O. ficus-indica* (Israel and Nobel, 1994), increased the activities of soluble starch synthase and Suc-Pi synthase, enzymes involved in carbohydrate production. A high carbohydrate content also leads to the retention of enzymes required for glycolysis and loss of enzymes needed for photosynthesis (Krapp and Stitt, 1994). The substantial increase of Suc-Pi synthase activity in basal cladodes of *O. ficus-indica* caused by doubling the CO_2 concentration could increase the Suc level if the rate of phloem transport into sink organs, such as young daughter cladodes, were not increased, resulting in feedback inhibition of photosynthesis. However, Suc transport into sink organs was increased by doubling the CO_2 concentration, so no Suc accumulated and the rate of Suc synthesis in basal cladodes of *O. ficus-indica* was not reduced compared with plants under the current CO_2 concentration. The increase in soluble starch synthase activity caused by doubling the CO_2 concentration may be partly responsible for the higher starch levels in basal cladodes.

When the rate of CO_2 uptake in basal cladodes of *O. ficus-indica* is increased by doubling the CO_2 concentration (Cui et al., 1993; Nobel and Israel, 1994), part of the excess carbohydrate is transported to the daughter cladodes, leading to their large increase in dry weight compared with daughter cladodes under the current CO_2 concentration. The dry weight of shaded, 14-d-old daughter cladodes was about 90% of that for unshaded daughter cladodes under both the current and the doubled CO_2 concentrations, indicating that the growth of these young sink organs depended almost exclusively on the supply of photoassimilate from basal cladodes. Although shading the young daughter cladodes affected their dry weight accumulation only slightly compared with the unshaded daughter cladodes, it greatly increased the contents of Fru and total amino acids under both CO_2 concentrations, possibly due to a lowered respiration rate that generally occurs with shading (Taiz and Zeiger, 1991). Shading the young daughter cladodes also drastically reduced their CAM activity,

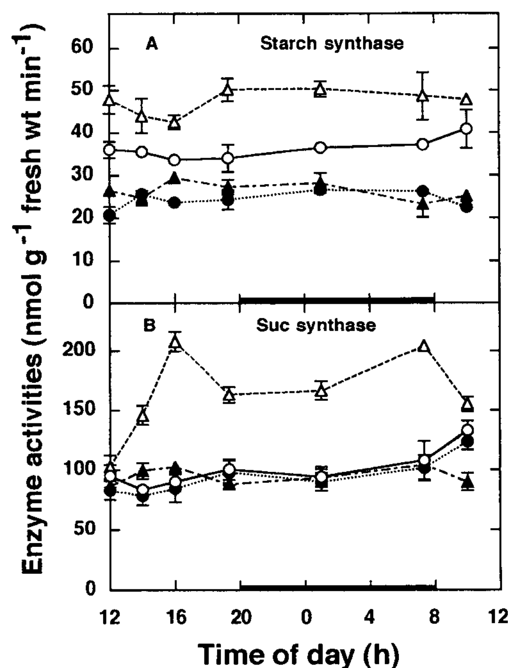

Figure 6. Daily time course for the activities of soluble starch synthase (A) and Suc synthase (B) in cores of 14-d-old daughter cladodes growing under 370 (○, ●) or 750 (△, ▲) μmol CO_2 mol^{-1}. One of the two daughter cladodes per basal cladode was shaded 88% (●, ▲). Data are means ± SE (n = 4 plants). Dark bars indicate night.

since malate content increased only slightly throughout the night under both CO_2 concentrations.

In addition to an increase in source capacity for carbohydrate production and photoassimilate transport, doubling the CO_2 concentration also increased the sink strength of daughter cladodes of *O. ficus-indica.* Doubling the CO_2 concentration increased the levels of Glc and malate as well as the osmolality in sink cells of 14-d-old daughter cladodes of *O. ficus-indica*. The higher osmolality in the surrounding tissues can in turn lower the turgor pressure in the phloem of these sink organs (Ho, 1988), resulting in a more rapid movement of photoassimilate from basal to daughter cladodes and higher dry weight of daughter cladodes than under the current CO_2 concentration. Although the 14-d-old daughter cladodes depend mainly on the supply of photoassimilate from basal cladodes for their growth, their CAM activity was substantial and became 22% higher when the CO_2 concentration was doubled. Under the current CO_2 concentration, the small pools of Glc and starch did not decrease during the night, but under the doubled CO_2 concentration about 70% of the malate synthesized in the daughter cladodes was derived from Glc and starch, whose levels decreased substantially during the night. Doubling the CO_2 concentration also lowered the level of total amino acid in the daughter cladodes, allowing a switch of energy from nitrate reduction and amino acid synthesis to carbohydrate metabolism (Huber and Huber, 1992).

Doubling the CO_2 concentration enhanced the activities of Suc synthase and soluble starch synthase for the 14-d-old daughter cladodes of *O. ficus-indica*, which also indicates that the sink strength for this perennial CAM species was increased (Black, 1993; Jenner and Hawker, 1993). In addition, the correlation of such enhanced activities with the increased rate of Suc transport to the sink organs of *O. ficus-indica* under the doubled CO_2 concentration suggests that these enzymes were involved in regulating photoassimilate import. Suc synthase was responsible for Suc breakdown in the daughter cladodes, whereas invertase, whose activity was less than 10% of that of Suc synthase, played only a secondary role, which also occurs for other actively filling sink organs (Sung et al., 1989). Consequently, when more Suc moved from basal to daughter cladodes under the doubled CO_2 concentration, it was hydrolyzed to Glc and Fru by Suc synthase, resulting in a higher Glc content than that under the current CO_2 concentration. Because the content of Glc in the 14-d-old daughter cladodes was higher than that of Fru under both CO_2 concentrations, Fru may be the preferred substrate for respiration by *O. ficus-indica*. Although the starch level in daughter cladodes was only about 9% of that for basal cladodes, their activity of soluble starch synthase was about 60% of that for starch-accumulating basal cladodes and was increased by doubling the CO_2 concentration, suggesting that starch synthesis in these sink organs was regulated by other mechanisms in addition to direct responses to starch synthase.

The present study suggests that Pi-limited feedback inhibition of photosynthesis under a doubled CO_2 concentration may not occur for source organs of *O. ficus-indica*. First, the rates of Suc and starch synthesis in basal cladodes were not reduced by doubling the CO_2 concentration, although more starch and Suc were produced. Second, even though photosynthesis becomes limited by low Pi, such limitation is probably only transitory (Stitt, 1991), since accumulation of starch in the chloroplasts during long-term exposure to elevated CO_2 concentrations can regenerate Pi for ATP synthesis (Makino, 1994). Third, the plants used were grown under a luxuriant level of Pi, leading to high concentrations of Pi in the chlorenchyma and the water-storage parenchyma, and the Pi content in both tissues was greatly increased by doubling the CO_2 concentration. Fourth, a higher rate of Suc synthesis in basal cladodes of *O. ficus-indica* under the doubled CO_2 concentration will allow more Pi to be moved into the chloroplasts in exchange for triose phosphate moving into the cytosol (Stitt and Quick, 1989). Fifth, chloroplasts isolated from CAM plants require less Pi for optimum oxygen evolution than do chloroplasts from C_3 plants (Monson et al., 1983).

The hypothesis that suppression of photosynthetic gene expression in source organs by increased Suc and Glc concentration under elevated CO_2 concentrations may not apply to *O. ficus-indica*. Although this hypothesis is supported for annual crops (Sheen, 1994; Nie et al., 1995), little support occurs for trees or perennial CAM species. The Suc concentration was about 5 mol m^{-3} in basal cladodes of *O. ficus-indica* under both CO_2 concentrations, 60 times less than that needed to suppress gene expression in annual crops, and the Glc concentration was about 1.1 mol m^{-3}, 10 times less (Sheen, 1994). Furthermore, the action of Suc and Glc in suppressing genes responsible for photosynthesis is via hexose-Pi after Suc is hydrolyzed by invertase to Glc, which is then phosphorylated by hexokinase (Sheen, 1994). The activity of invertase was extremely low in basal cladodes of *O. ficus-indica*, however, and the activity of hexokinase was slightly decreased by doubling the CO_2 concentration. The near absence of invertase activity in basal cladodes may indicate that Suc breakown in these source organs is via Suc synthase or via acid hydrolysis, as occurs for lime fruit (Echeverria and Burns, 1989), because the pH in the vacuoles of the chlorenchyma for basal cladodes of *O. ficus-indica* is about 4.0 during the daytime and even lower at night (N. Wang and P.S. Nobel, unpublished observations). The higher hexokinase activity at midnight compared with the daytime under the doubled CO_2 concentration may reflect a higher demand for hexose-Pi needed for nocturnal malate synthesis.

In conclusion, no downward acclimation of photosynthesis during long-term exposure to elevated CO_2 concentrations occurs for *O. ficus-indica* (Cui et al., 1993; Nobel and Israel, 1994), which is consistent with its continued higher source capacity, higher rate of phloem Suc transport, and stronger sink strength than under the current CO_2 concentration. These changes in source and sink relations and starch and Glc accumulation by doubling the CO_2 concen-

tration apparently do not result in Pi limitation of photosynthesis or suppression of genes for photosynthesis for this perennial CAM species, as occur for some annual crops. Although further research is necessary to understand various genetic aspects, it is clear that *O. ficus-indica* can benefit from long-term exposure to elevated CO_2 concentrations with respect to its carbohydrate metabolism, photoassimilate transport, and hence productivity.

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