Sucrose cycling, Rubisco expression, and prediction of photosynthetic acclimation to elevated atmospheric CO₂

B. d. MOORE, S.-H. CHENG, J. RICE & J. R. SEEMANN

Department of Biochemistry, University of Nevada, Reno, Reno, NV 89557–0014, USA

ABSTRACT
Photosynthetic acclimation to elevated CO₂ cannot presently be predicted due to our limited understanding of the molecular mechanisms and metabolic signals that regulate photosynthetic gene expression. We have examined acclimation by comparing changes in the leaf content of RuBP carboxylase/oxygenase (Rubisco) with changes in the transcripts of Rubisco subunit genes and with leaf carbohydrate metabolism. When grown at 1000 mm³ dm⁻³ CO₂, 12 of 16 crop species at peak vegetative growth had a 15–44% decrease in leaf Rubisco protein, but with no specific association with changes in transcript levels measured at midday. Species with only modest reductions in Rubisco content (10–20%) often had a large reduction in Rubisco small subunit gene mRNAs (> 30%), with no reduction in large subunit gene mRNAs. However, species with a very large reduction in Rubisco content generally had only small reductions in transcript mRNAs. Photosynthetic acclimation also was not specifically associated with a change in the level of any particular carbohydrate measured at midday. However, a threshold relationship was found between the reduction in Rubisco content at high CO₂ and absolute levels of soluble acid invertase activity measured in plants grown at ambient or high CO₂. This relationship was valid for 15 of the 16 species examined. There also occurred a similar, albeit less robust, threshold relationship between the leaf hexose/sucrose ratio at high CO₂ and a reduced photosynthetic capacity ≥ 20%. These data indicate that carbohydrate repression of photosynthetic gene expression at elevated CO₂ may involve leaf sucrose cycling through acid invertase and hexokinase.

Key-words: acid invertase; carbohydrate signalling; elevated CO₂; gene expression; Rubisco; sucrose cycling

INTRODUCTION
Growth of many herbaceous and woody plant species at elevated [CO₂] often results in a decrease in leaf photosynthetic capacity due to a decrease in leaf content of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Sage, Sharkey & Seemann 1989; Webber, Nie & Long 1994). However, the magnitude of this acclimation response can vary not only between different species, but also among different varieties or cultivars of the same species grown under controlled conditions (e.g. Campbell 1997; Kalina & Ceulemans 1997). Furthermore, the extent by which photosynthetic capacity may be reduced at elevated CO₂ in a given species can be modulated by a number of factors including light (Ghannoum et al. 1997; Sims, Seemann & Luo 1998), nutrient availability (Tissue et al. 1993; Seneeweera et al. 1994; Nakano et al. 1997), water availability (Roden & Ball 1996; Scarascia-Mugnozza et al. 1996), and tissue developmental stage (Van Oosten & Besford 1995; Xu, Gifford & Chow 1996). However, there presently is no basis to predict photosynthetic acclimation to elevated CO₂ in large part due to a lack of understanding of the regulatory mechanisms and metabolic signals involved.

Leaf carbohydrates commonly accumulate during long-term growth at high CO₂ (Long & Drake 1992) as result of a limited capacity for their utilization in growing sink tissues (Stitt 1991). An increase in leaf carbohydrates has long been associated with an inhibition of photosynthesis (e.g. Neals & Incoll 1968) and may account for the down-regulation of photosynthetic capacity that can occur in plants exposed to elevated CO₂ (Van Oosten, Wilkins & Besford 1994). However, end-product inhibition of photosynthesis is not due to increased carbohydrate content per se, but instead is associated with the metabolism of hexoses probably derived from sucrose hydrolysis (Goldschmidt & Huber 1992; Krapp et al. 1993). Hexokinase has been proposed to function as a sugar sensor in the cytosol of plant mesophyll cells (Jang & Sheen 1994; Jang et al. 1997). During phosphorylation of hexoses, hexokinase is hypothesized to initiate a signal cascade that results in the repressed expression of a number of photosynthetic genes (Sheen 1994; Smeekens & Rook 1997), but there are many aspects of this process that are not known.

In order to understand and therefore possibly predict photosynthetic acclimation at elevated CO₂, we are interested in determining the process by which carbohydrates generate a mesophyll signal and the molecular targets and processes that are modulated at high CO₂. If hexokinase can function as a flux sensor (Graham, Denby & Leaver 1994; Koch 1996), an internal signal could be generated.
simply by increased provision of substrate in the mesophyll cytoplasm. However, there was no evidence for the presence of a significant level of daytime cytosolic hexoses in leaves of three species grown at elevated CO₂ (Moore, Palmquist & Seemann 1997). Alternatively, sucrose hydrolysis by soluble acid invertase (Goldschmidt & Huber 1992) or hydrolytic starch degradation (Kruger 1990) could generate increased substrates for the hexokinase reaction. Thus, hexoses might not accumulate in the cytosol depending on their rate of transport from the vacuole or chloroplast and the catalytic activity of hexokinase.

Plant growth at elevated CO₂ can result in repression of a number of photosynthetic genes, but the effects are known to vary between species as well as within species. Nuclear-encoded genes such as \textit{rbcS} (Rubisco small subunit genes) and \textit{cab} (chlorophyll a/b binding protein genes) are more sensitive to repression than are chloroplast-encoded genes such as \textit{rbcL} (Rubisco large subunit genes) or \textit{psaA-B} (photogene; Van Oosten & Besford 1995). Furthermore, growth at high CO₂ can result in a differential repression of \textit{rbcS} gene-specific mRNAs within even a given species (Arabidopsis; Cheng, Moore & Seemann 1998).

In this study, we have two main objectives in examining photosynthetic acclimation in a number of species grown at high CO₂. First, we have evaluated acclimation (as a reduction in Rubisco content) in relation to leaf carbohydrate metabolism and in relation to \textit{rbcS} and \textit{rbcL} transcript levels to determine which, if any, parameters may be useful for predicting acclimation. Carbohydrate metabolism was examined by measuring the accumulation of specific leaf sugars and the activities of sucrose-phosphate synthase (SPS) and soluble acid invertase. Activities of these two enzymes are important to consider since they largely control the level of sucrose accumulation and hexose production within leaves (Huber 1989; Goldschmidt & Huber 1992). Secondly, we have used the correlative information to help elucidate the mechanism for carbohydrate signalling at elevated CO₂ and to identify affected molecular mechanisms that control Rubisco content.

**MATERIALS AND METHODS**

**Plant material**

Bean (\textit{Phaseolus vulgaris} L. cv. Little Linden), bugle (\textit{Ajuga reptans} L. cv. Bronze Beauty), corn (\textit{Zea mays} L. cv. Early Sunglow hybrid, cotton (\textit{Gossypium hirsutum} [L.]), tobacco (\textit{Nicotiana tabacum} \textit{L}.) and tomato (\textit{Lycopersicon esculentum} Mill. cv. Early Girl) were grown to a peak vegetative growth stage (5–8 weeks depending on species) in 5 dm³ pots in greenhouses with natural irradiance, a 28°/15 °C day/night thermoperiod, and either 400 or 1000 cm³ m⁻³ CO₂. Pea (\textit{Pisum sativum} L. cv. Little Marvel), radish (\textit{Raphanus sativus} L. cv. Cherry Belle), and wheat (\textit{Triticum aestivum} L. cv. Twin Spring) were grown for 3–4 weeks in flats in the greenhouses. Plants were watered daily with Peter’s nutrient solution (15, 16, 17% N, P, K; 5·7 mm N, 1·5 mm P, 2·4 mm K). Arabidopsis (\textit{Arabidopsis thaliana} [L.] Hennh. ecotype Columbia) were grown in growth chambers for 4–5 weeks as described previously (Cheng, Moore & Seemann 1998). Youngest, full-sized leaves or rosettes (Arabidopsis) were collected into liquid N₂ at midday (12:00) and visible leaf veins were removed prior to biochemical analyses.

**Carbohydrate measurements**

Leaf material (0·25 g FW) was extracted in boiling 80% ethanol as previously described (Moore, Palmquist & Seemann 1997). Residual material from whole-leaf extracts was autoclaved and the starch from replicate aliquots was hydrolysed as described by Schulze et al. (1991). Soluble carbohydrates were measured by HPLC using a Dionex DX 300 system with a pulsed amperometric detector and a CarboPac PA1 or occasionally an MA1 column (Dionex Corp., Sunnyvale, CA, USA) as previously described for parsley (Moore, Palmquist & Seemann 1997).

**Rubisco and Chl content assays**

Rubisco content was measured as described elsewhere (Evans & Seemann 1984). Chl content of whole-leaf material was determined after extraction in ethanol (Wintermans & De Mots 1965).

**Leaf enzyme extraction and activity assays**

Leaf samples (0·25 g FW) were extracted with a mortar and pestle in 2 cm³ of 50 mm Mops-KOH (pH 7·5), 5 mm MgCl₂, 1 mm EDTA, 2·5 mm dithiothreitol, 0·05% Triton X-100, and 10% (w/w) polyvinylpolypyrrolidone. Extracts were filtered through Miracloth and centrifuged at 10 000 g for 1 min. The supernatants were immediately desalted by centrifugal filtration using 5 cm³ Sephadex G-25 columns equilibrated in extraction buffer minus the Triton X-100.

Soluble acid invertase (β-fructosidase, EC 3.2.1.16) was assayed at 25 °C using components described by Huber (1989). Reactions were initiated by addition of extract and aliquots were removed for boiling at 0 and 30 or 60 min. Amounts of glucose plus fructose were determined by endpoint enzyme assays coupled through glucose 6-phosphate dehydrogenase (from \textit{Leuconostoc}) as described by Doehlert (1989), but with all coupling enzymes used at 2 units cm⁻³.

Sucrose-phosphate synthase (EC 2.4.1.14) was assayed at 30 °C by measuring under \( v_{\text{max}} \) conditions the fructose-6-P-dependent formation of sucrose-6-phosphate + sucrose from UDP-glucose (Huber et al. 1989). Sucrose products were measured using an anthrone reagent (Van Handel 1968).
Leaf RNA extraction and northern analysis

Total leaf RNA was isolated as in Cheng & Seemann (1997). The purity and quantity of the RNA samples was monitored by measuring UV absorbance at 230, 260 and 280 nm. The ratios of A260/A230 and A260/A280 were each about 2, indicating little or no contamination by carbohydrates or proteins. The average RNA yield was about 500 μg (g FW)^{-1} of leaf tissue.

Northern analyses were carried out using 1 μg (for tobacco and Arabidopsis) or 5 μg (for all other species) of total RNA as described by Cheng, Moore, & Seemann (1998). cDNAs used as probes were a 520 bp HindIII fragment of tobacco rbcS (pTSSU3) kindly provided by Dr Graham Hudson, a 1.2 kb EcoRI/XbaI fragment of corn rbcS (SS1, a generous gift from Dr Jen Sheen), a 750 bp Sall/NotI fragment of Arabidopsis rbcS (ID no. 11C1T7P) obtained from the Arabidopsis Biological Research Center at Ohio State University, and a 1.2 kb plastid BamH1/EcoRI fragment of tobacco rbcL (Shinozaki & Sugiyama 1982). These DNA fragments were labelled with [α-32P]dCTP using the Prime-a-Gen Labeling system (Promega). For hybridization of rbcS mRNA, 32P-labelled Arabidopsis rbcS cDNA was used for Arabidopsis, bean, bugle, cotton, parsley, pea, plantain, radish, spinach, sunflower and wheat; labelled tobacco rbcS cDNA was used for cucumber, soybean, tobacco, and tomato, and labelled corn rbcS cDNA was used for corn. 32P-labelled tobacco rbcL cDNA was used for all species. In each blot, a dilution series of an RNA sample was included to ensure that 32P-labelled probes were in excess. The hybridization conditions were as described by Cheng et al. (1998). Following hybridization, the blots were washed twice for 5 min at room temperature in 2X SSC/0.1% SDS, and then twice for 10 min either in 0.2X SSC/0.1% SDS at 60 °C (for rbcS mRNA of Arabidopsis, corn and tobacco), in 0.5X SSC/0.1% SDS at 56 °C (for rbcS mRNA of all other species), or in 0.5X SSC/0.1% SDS at 60 °C (for rbcL mRNA). Hybridization signals were quantified with a Phosphor Imager (Bio-Rad Laboratories, Inc.) to determine the relative amount of RNA present in each lane.

RESULTS

In this study, we have examined possible correlations between photosynthetic acclimation and specific biochemical and molecular characteristics of mature leaves of 16 species grown at ambient or elevated CO2. Rubisco protein content was measured as a biochemical indicator of leaf photosynthetic capacity. The species were grouped according to the relative reduction in leaf Rubisco protein content that occurred in plants grown at high CO2 (Table 1). Some of the species in the different groups include corn and spinach with no reduction (group 1), cotton and wheat with a moderate reduction (10–20%, group 2), cucumber and soybean with a substantial reduction (20–30%, group 3), and Arabidopsis and tobacco with a very large reduction (30–45%, group 4).

The leaf amounts of rbcS and rbcL mRNAs were measured from the same samples used to measure Rubisco contents (Fig. 1, Table 1). Relative leaf Rubisco protein content at high CO2 generally did not correlate with the expression level of Rubisco transcripts (both measured at midday). In many species, rbcS and rbcL mRNAs were the same or higher at elevated CO2 (e.g. corn and spinach), including some species which had a reduced Rubisco content (e.g. plantain and tobacco; Table 1). In other species, though, rbcS mRNA amounts were substantially reduced, but rbcL mRNA levels were not much affected, if any (e.g. parsley, cotton and wheat). Only in Arabidopsis were transcript amounts of both gene products reduced at high CO2 to about the same extent as Rubisco content. Species of group 2 had highly reduced rbcS mRNA levels (> 30%), with only a moderate reduction in Rubisco protein content (e.g. cotton and sunflower). Species in group 4 had the most reduction in Rubisco content, yet both rbcS and rbcL transcript levels were frequently not much affected (e.g. radish and tobacco). These responses indicate that photosynthetic acclimation cannot be predicted by changes in message level (Fig. 2).

A decline in relative Rubisco content of species at high CO2 was generally associated with some decrease in leaf Chl content, but the latter to a lesser extent (Fig. 3, e.g. radish, 23% decrease in Chl, 34% decrease in Rubisco). However, there was substantial variation in the relative influence of high CO2 on Rubisco protein and Chl contents. In plantain and sunflower, leaf Chl contents did not decline although Rubisco contents did. Such variation indicates that one cannot accurately predict photosynthetic acclimation based on the response of leaf Chl content. Whether photosynthetic components other than leaf Chl and/or Rubisco content were affected in this study by growth at elevated CO2 is not known.

Next, we examined to what extent particular aspects of carbohydrate metabolism were correlated with photosynthetic acclimation. The accumulation of total non-structural carbohydrates (TNC) in leaves varied substantially between species grown at either ambient or elevated CO2 (Fig. 4). Species with the greatest amounts of TNC (cotton, cucumber, soybean, tomato and parsley) had relatively high levels of starch. The present carbohydrate data notably are closer to actual mesophyll levels than those reported in previous studies due to our removal of as much vein material as reasonably possible prior to analyses. None the less, the absolute amount of TNC at high CO2 was not closely correlated with photosynthetic acclimation. For example, at high CO2 Arabidopsis had relatively low carbohydrate levels and greatly reduced Rubisco protein content, while parsley had relatively high TNC levels but no reduction in Rubisco content. Furthermore, cotton, cucumber and soybean accumulated the greatest amounts of TNC, yet none of these species had a very large reduction in Rubisco protein content (all < 25%). Photosynthetic acclimation also was not associated with the accumulation of any particular sugar. For example, a number of species such as cotton, soybean and tomato had high levels of...
starch at high CO₂ and had a reduced Rubisco content (Fig. 5). However, this relationship was not consistent. Parsley had a substantial amount of starch and no reduction in Rubisco content, while several species had a reduced Rubisco level but a relatively low amount of starch (e.g. Arabidopsis, plantain and radish).

Although total TNC levels were not associated with photosynthetic acclimation, we examined whether changes in the relative leaf Rubisco content in plants grown at high CO₂ may be a function of the relative change in TNC (Fig. 6) or in total hexoses (Fig. 7). A decline in leaf Rubisco content was not necessarily associated with increased TNC. TNC changed very little in several species which had reduced Rubisco levels (e.g. sunflower, wheat and radish). Also, TNC increased 3-fold in spinach, but Rubisco content did not decline. Similarly, changes in leaf hexose content were not specifically associated with

Table 1. Influence of growth CO₂ concentration on Rubisco protein and transcript levels in mature leaves collected at midday. Rubisco protein content is expressed as nmol of CABP binding sites. Values are means ± SD (n = 3). Percentages are expressed relative to corresponding values for plants grown at ambient CO₂.

| Group/species (cm⁻³ m⁻³) | Rubisco protein | rbcS (%) | rbcL (%) |
|---------------------------|-----------------|----------|----------|
| I. Species with no significant reduction in Rubisco protein content |                  |          |          |
| Corn 400                  | 38.5 ± 2.2      | 95       | 152 ± 1  |
| 1000                      | 36.5 ± 2.4      | 103      | 60 ± 1   |
| Parsley 400               | 77.5 ± 3.5      | 104      | 110 ± 1  |
| 1000                      | 84.2 ± 3.4      | 104      | 112 ± 6  |
| Pea 400                   | 87.3 ± 1.0      | 104      | 110 ± 1  |
| 1000                      | 56.1 ± 3.9      | 101      | 135 ± 9  |
| Spinach 400               | 56.6 ± 4.3      | 101      | 99 ± 11  |
| II. Species with a moderate reduction in Rubisco protein content |                  |          |          |
| Bugle 400                 | 59.6 ± 5.8      | 83       | 60 ± 2   |
| 1000                      | 49.2 ± 3.5      | 85       | 54 ± 3   |
| Cotton 400                | 162.7 ± 1.7     | 80       | 69 ± 2   |
| 1000                      | 138.5 ± 5.3     | 80       | 133 ± 1  |
| Sunflower 400             | 162.9 ± 19.0    | 80       | 61 ± 1   |
| 1000                      | 130.3 ± 10.7    | 80       | 106 ± 3  |
| Wheat 400                 | 130.0 ± 6.8     | 80       | 61 ± 1   |
| 1000                      | 112.1 ± 3.6     | 80       | 106 ± 3  |
| III. Species with a substantial reduction in Rubisco protein content |                  |          |          |
| Cucumber 400              | 138.3 ± 4.0     | 77       | 98 ± 8   |
| 1000                      | 105.9 ± 2.7     | 77       | 109 ± 4  |
| Plantain 400              | 50.2 ± 0.4      | 75       | 125 ± 9  |
| 1000                      | 37.5 ± 1.3      | 75       | 148 ± 25 |
| Soybean 400               | 206.4 ± 3.5     | 77       | 73 ± 4   |
| 1000                      | 159.6 ± 3.8     | 77       | 90 ± 7   |
| IV. Species with a very large reduction in rubisco protein content |                  |          |          |
| Arabidopsis 400           | 33.7 ± 0.5      | 66       | 40 ± 3   |
| 1000                      | 22.3 ± 2.5      | 64       | 85 ± 4   |
| Bean 400                  | 161.7 ± 11.3    | 64       | 85 ± 4   |
| 1000                      | 103.8 ± 6.4     | 64       | 111 ± 8  |
| Radish 400                | 117.6 ± 8.3     | 66       | 83 ± 5   |
| 1000                      | 78.1 ± 6.7      | 66       | 91 ± 5   |
| Tobacco 400               | 94.5 ± 2.3      | 62       | 92 ± 2   |
| 1000                      | 58.5 ± 0.6      | 62       | 92 ± 5   |
| Tomato 400                | 71.9 ± 7.6      | 56       | 81 ± 3   |
| 1000                      | 40.5 ± 1.4      | 56       | 90 ± 6   |

Figure 1. Northern analysis of rbcS and rbcL expression in leaves of representative species grown at ambient (400 cm⁻³ m⁻³, Amb) or elevated (1000 cm⁻³ m⁻³, HC) CO₂. Leaves were collected at midday. 25S RNA is shown stained with methylene blue. Tob = tobacco; Arab = Arabidopsis.
adjustments in Rubisco content at high CO₂. For example, leaf hexoses increased 2.5-fold in spinach, but Rubisco content did not change. Also, there were minimal changes in leaf hexoses at high CO₂ in a number of species which had substantial declines in Rubisco content (e.g. bean, radish and sunflower).

Next, we measured activities of SPS (under V_max conditions) and soluble acid invertase (Table 2) in the same leaf material used for the previously described measurements. SPS activities declined by $\approx 35\%$ in a few species grown at high CO₂ (e.g. bean, cotton, cucumber and plantago), with smaller differences in other species. Acid invertase activities also declined substantially in only a few species at high CO₂ (e.g. radish, soybean and wheat; about a 25% decline). For all species grown at high CO₂, the relative mean SPS activity was 87% and the relative mean soluble acid invertase activity was 82%. The SPS activities of species did not correlate with measured leaf sucrose levels, nor were acid invertase activities closely associated with total hexose or total sucrose levels (analyses not shown). The general decline in leaf SPS activities at high CO₂ was not correlated with specific decreases in Rubisco content (Fig. 8). Since a number of species had similar relative leaf SPS activities, but very different relative Rubisco contents, one cannot predict photosynthetic acclimation based on measured SPS activities.

Although growth at high CO₂ did not substantially affect leaf acid invertase activity in most species (Table 2),
Figure 6. Relative leaf Rubisco protein content in plants grown at high CO₂ as a function of relative leaf content of total non-structural carbohydrates. The fitted line is a first-order, linear regression ($r^2 < 0.01$).

Figure 7. Relative leaf Rubisco protein content in plants grown at high CO₂ as a function of relative leaf hexose content. The fitted line is a first-order, linear regression ($r^2 = 0.02$).

Table 2. Influence of growth CO₂ concentration on sucrose phosphate synthase and acid invertase activities in mature leaves at midday. Values are means ± SD of three or more extractions. Percentages are expressed relative to corresponding values for plants grown at ambient CO₂. Sucrose phosphate synthase activity was measured under V_max conditions.

| Species   | [CO₂] | Sucrose-P synthase | Acid invertase |
|-----------|-------|--------------------|----------------|
|           | [µmol (h g FW)^{-1}] | %               | [µmol (h g FW)^{-1}] | %               |
| Arabidopsis | 400 | 3.5 ± 0.1 | 23.9 ± 2.9 | 1000 | 2.8 ± 1.4 | 18.6 ± 5.5 |
| Bean      | 400 | 19.8 ± 0.6 | 3.9 ± 0.7 | 1000 | 12.6 ± 3.0 | 4.8 ± 3.2 |
| Bugle     | 400 | 11.8 ± 1.2 | 16.0 ± 4.4 | 1000 | 10.6 ± 1.9 | 11.5 ± 2.6 |
| Corn      | 400 | 83.3 ± 6.7 | 3.2 ± 0.7 | 1000 | 86.2 ± 1.3 | 1.8 ± 0.1 |
| Cotton    | 400 | 10.9 ± 2.2 | 20.4 ± 3.5 | 1000 | 5.1 ± 1.1 | 16.1 ± 2.5 |
| Cucumber  | 400 | 17.8 ± 1.4 | 22.1 ± 1.0 | 1000 | 9.0 ± 0.8 | 18.8 ± 1.4 |
| Parsley   | 400 | 9.5 ± 1.5 | 6.0 ± 1.5 | 1000 | 9.2 ± 1.8 | 4.5 ± 2.1 |
| Pea       | 400 | 14.2 ± 0.3 | 3.0 ± 0.4 | 1000 | 15.6 ± 3.0 | 2.6 ± 1.4 |
| Plantain  | 400 | 5.5 ± 1.8 | 34.4 ± 2.1 | 1000 | 2.1 ± 0.3 | 41.3 ± 0.8 |
| Radish    | 400 | 6.3 ± 0.4 | 129.7 ± 3.6 | 1000 | 6.3 ± 0.3 | 94.9 ± 4.2 |
| Soybean   | 400 | 25.2 ± 1.2 | 141.0 ± 4.8 | 1000 | 27.3 ± 2.5 | 108.9 ± 8.7 |
| Spinach   | 400 | 26.3 ± 3.3 | 5.3 ± 1.1 | 1000 | 29.7 ± 4.1 | 4.4 ± 2.1 |
| Sunflower | 400 | 6.2 ± 1.5 | 20.3 ± 1.6 | 1000 | 6.7 ± 0.9 | 27.1 ± 7.2 |
| Tobacco   | 400 | 17.7 ± 0.9 | 60.9 ± 4.1 | 1000 | 16.8 ± 3.3 | 51.6 ± 5.1 |
| Tomato    | 400 | 13.4 ± 2.8 | 37.1 ± 5.0 | 1000 | 15.8 ± 3.4 | 35.6 ± 5.5 |
| Wheat     | 400 | 7.7 ± 2.7 | 54.7 ± 1.3 | 1000 | 6.7 ± 1.0 | 40.1 ± 2.4 |
photosynthetic acclimation was related to the absolute level of acid invertase activity measured from plants grown at either ambient or high CO₂ (Fig. 9). High acid invertase activities were always associated with a decrease in photosynthetic capacity at high CO₂. Also, the absence of photosynthetic acclimation at high CO₂ (group 1 species in Table 1) occurred only in species with low activities of acid invertase. The data indicate that a threshold relationship exists between the level of acid invertase activity and photosynthetic acclimation to high CO₂. Notably, in plants grown at ambient CO₂, there was a 3-fold increase in invertase activity from the highest of the non-acclimating species to the lowest of the acclimating species. The threshold relationship between enzyme activity and acclimation was valid for 15 of the 16 species examined. The sole exception was bean which had only a low activity of acid invertase, but did have a substantial reduction in leaf Rubisco content when grown at high CO₂.

Since acid invertase catalyses the hydrolysis of sucrose to glucose plus fructose, we also examined to what extent one can predict photosynthetic acclimation at high CO₂ based on the amounts of leaf hexose-carbon that are present relative to sucrose-carbon (Fig. 10). High hexose/sucrose ratios (> 2·0) were present only in Arabidopsis, radish, sunflower and tomato. These species all showed substantial photosynthetic acclimation (≥ 20% decline). A number of species that also showed photosynthetic acclimation had more moderate values of the leaf hexose/sucrose ratio, but these values increased substantially at high CO₂. These species included cucumber, plantain, soybean and tobacco, with respective ratios at high CO₂ of 0·29, 1·4, 0·39 and 0·97. Thus, a hexose/sucrose ratio > 0·25 for leaves collected at high CO₂ was associated with a ≥ 20% decline in photosynthetic capacity in eight of
nine species. The sole exception again was bean. However, a moderate decline in Rubisco content (10–20%) at high CO₂ was not associated with either a high leaf hexose/sucrose ratio or an increased value in that ratio.

**DISCUSSION**

In this study, we found that species with relatively high leaf activities of soluble acid invertase showed photosynthetic acclimation (defined as a reduction in leaf Rubisco content) when grown at an elevated [CO₂] (Fig. 9). The use of leaf Rubisco content as a biochemical indicator of the photosynthetic capacity of a species is generally reasonable, since within a given species adjustments in Rubisco content commonly result in corresponding adjustments in the photosynthetic rate (Evans & Seemann 1989; Stitt & Schulze 1994). However, the differential adjustments amongst species in leaf Chl and Rubisco contents that occurred at elevated CO₂ (Fig. 3) indicate that the responses of different photosynthetic components are not necessarily linked. None the less, the relationship between leaf acid invertase activity and Rubisco content is important for identifying the mechanism of photosynthetic acclimation, for use as a tool to predict acclimation, and also possibly as a target for plant transformation.

With regard to the mechanism, the data suggest that so-called 'futile' cycling of sucrose may in fact be a regulatory component involved in leaf sugar sensing. Goldschmidt & Huber (1992) provided evidence that sucrose cycling may occur in mesophyll cells of mature leaves under sink-limited conditions through sucrose hydrolysis by vacuolar acid invertase coupled with hexose phosphorylation by cytosolic hexokinase. If hexokinase can function as a flux sensor (Graham, Denby, & Leaver 1994; Koch 1996), then the increased provision of hexoses from sucrose cycling could account for the generation of a primary carbohydrate signal. There is apparently no direct evidence from any metabolite labelling studies that sucrose cycling does occur in leaves. None the less, there are several lines of indirect evidence that such can occur. First, overexpression of a yeast invertase gene in the vacuole of mature tobacco leaves resulted in an accumulation of leaf hexoses and an inhibition of photosynthesis (Sonnenthal et al. 1991). Secondly, an analogous pathway of sucrose cycling that involves sucrose synthase rather than acid invertase has been shown to occur in potato tubers and certain other non-photosynthetic tissues (Geigenberger & Stitt 1993). Thirdly, sucrose cycling at high CO₂ through acid invertase might be expected to increase the leaf hexose/sucrose ratio as observed in many species in this study (Fig. 10), in part due to the relatively low activity of leaf hexokinase (e.g. Huber 1989).

Photosynthetic acclimation to elevated CO₂ was related to a threshold level of soluble acid invertase activity, resulting in an ability to predict in 15 of 16 species whether photosynthetic capacity would decrease (Fig. 9). After petiole girdling, a comparable threshold relationship was previously observed between photosynthetic capacity or sucrose accumulation, and soluble acid invertase activity (Goldschmidt & Huber 1992). Photosynthetic acclimation at high CO₂ similarly could be predicted from leaf hexose/sucrose ratios at high CO₂, but only in cases of a ≥ 20% decrease in photosynthetic capacity (Fig. 10). In cold-stored tubers from a number of potato cultivars, the hexose/sucrose ratio was linearly dependent on the extractable activities of soluble acid invertase (Zrenner, Schuller & Sonnewald 1996). In this study, species activities of soluble invertase were generally, but not rigorously, related to the leaf hexose/sucrose ratio (data not shown). Photosynthetic acclimation could not be predicted though from either the absolute activities or relative change in SPS activities (Vmax; Tables 1 and 2; Fig. 8) or from the absolute amounts or relative changes in particular leaf carbohydrates (Figs 4, 5, 6 & 7).

Leaf soluble acid invertase has two other characteristics that further strengthens its use in predicting photosynthetic acclimation. Leaf soluble invertases are not thought to be metabolically regulated in vivo other than by substrate availability (Kruger 1990). Also, leaf soluble acid invertase activity is not much affected under sink-limited conditions that induce carbohydrate repression of gene expression (Table 2; see also Goldschmidt & Huber 1992). This response is in contrast to strong glucose-repression of yeast acid invertase expression (Gancedo 1992) and carbohydrate-dependent regulation of maize invertase genes in certain sink tissues (Koch 1996).

Bean (Phaseolus vulgaris) had an anomalous response at elevated CO₂, since photosynthetic acclimation occurred although soluble acid invertase activity was quite low (Fig. 9). There is some evidence that plant carbohydrate signalling can also occur by transport of external hexoses or even sucrose across the plasmalemma (Koch 1996; Smeekens & Rook 1997). One such mechanism may involve leaf sucrose hydrolysis by cell wall invertase, thus establishing a futile sucrose cycle between the apoplast and mesophyll cell (Foyer 1987). The transport of hexoses into the mesophyll cell and/or their subsequent phosphorylation may result in carbohydrate signalling, as apparently occurs in tobacco plants transformed to express yeast invertase in the leaf apoplast (Stitt, von Schawen & Willmitzer 1990). Whether any such alternate mechanisms may account for bean photosynthetic acclimation remains to be determined (see also below). However, the low hexose/sucrose ratio in bean at elevated CO₂ is indirect evidence against any invertase-related signalling mechanism.

Rubisco protein expression is normally co-ordinated with rbcS and rbcL gene expression during leaf development (Jiang & Rodermerl 1995). Growth at elevated CO₂ altered this coordination, and in different ways, in many of the species examined in this study (Table 1). The adjustments in plant development that commonly occur at elevated CO₂ (Bowes 1993) can complicate comparisons between species. In this study though, similar relative adjustments were repeatedly observed in leaf Rubisco protein and message levels in different, mature plants during vegetative growth.
growth and, when examined, in leaves just older than the youngest, full-sized (data not shown). The relative lack of coordination between Rubisco content and message levels at elevated CO$_2$ (Fig. 2) more probably reflects the complexity of molecular control of Rubisco content. The control of Rubisco content involves a number of processes, including transcription, post-transcription (e.g. message stability), translation and/or post-translation (e.g. protein turnover) events (Berry et al. 1986; Shirley & Meagher 1990; Wanner & Gruissem 1991). In some species, there may have even occurred an altered diurnal rhythm in mRNA accumulation. Nonetheless, during growth of species at elevated CO$_2$, there apparently did occur differential adjustments in the control of these processes, which are described as follows.

In a few species, transcript levels of both $rbcS$ and $rbcL$ genes were increased at high CO$_2$ even though Rubisco content was unchanged (e.g. corn) or even decreased (e.g. plantain). The reason for such stimulation of transcript levels is not clear, but the overall response suggests that translation of messages of both $rbcS$ and $rbcL$ genes can be directly or indirectly affected at high CO$_2$. In a second response pattern to high CO$_2$, there occurred a large decrease (> 30%) in $rbcS$ mRNA, no decline in $rbcL$ mRNA, and generally only a modest decrease in Rubisco content (e.g. parsley plus group 2 species). The simplest explanation for this response is that $rbcS$ gene transcription may be reduced and either $rbcL$ mRNA is not efficiently translated (as in tobacco $rbcS$ antisense plants; Rodermel et al. 1996) or large subunit protein may accumulate in excess (as has been observed in tomato at high CO$_2$; Fig. 4, Van Oosten & Besford 1995). In these species, Rubisco protein content may be controlled in large part by the abundance of small subunit protein. In a third response pattern, there occurred a very large reduction in Rubisco content in the absence of appreciable adjustments in the levels of either transcript (e.g. bean, radish and tobacco). The control of Rubisco content in these species is probably rather complex. Possibly, either one or both of the subunit mRNAs are not efficiently translated, or Rubisco protein turnover may increase at high CO$_2$. In these species, the primary target(s) for gene repression may involve specific translation factors for mRNA of either small or large subunit genes, specific proteases, and/or some dysfunction in the processing of small subunit protein or assembly of holoenzyme. Finally, in a fourth response pattern that occurred only in Arabidopsis, levels of $rbcS$ and $rbcL$ mRNAs and Rubisco content all substantially decreased at high CO$_2$ in an apparently co-ordinated manner. This response suggests that control of Rubisco content can be determined largely by message level, as discussed in more detail previously (Cheng, Moore & Seemann 1998).

Carbohydrate modulation of photosynthetic gene expression probably also occurs in ambient CO$_2$ conditions since a decrease in [CO$_2$] also alters $rbcS$ mRNA levels (Krapp et al. 1993; Majeau & Coleman 1996). However, whether leaf sucrose cycling may occur under ambient CO$_2$ growth conditions is not known. Since a reduction of vacuolar invertase activity by co-suppression in tomato leaves did not affect leaf photosynthetic rates (Scholes et al. 1996), sucrose cycling may be evident only under pronounced sink-limited conditions. This possibility is strengthened by the demonstration that in tobacco transformed to overexpress yeast invertase in the apoplast or vacuole, source but not sink leaves have bleached and/or necrotic sectors that are associated with reduced photosynthesis (Sonnewald et al. 1991). Based on diurnal leaf responses, night-time starch metabolism was previously suggested as one component of carbohydrate signalling (Cheng, Moore & Seemann 1998), and hydrolytic starch degradation also could generate substrate for the hexokinase reaction. Starch-accumulating species are often noted for their susceptibility to down-regulation of photosynthetic capacity under sink-limited conditions (e.g. Goldschmidt & Huber 1992; Farrar & Williams 1991). In this study, though, we did not observe a close coupling between starch accumulation and photosynthetic acclimation (Fig. 5). One extension of the idea that starch metabolism is in some aspects a buffer to sucrose metabolism (Stitt 1984), is the possibility that starch metabolism in part may function to minimize leaf sucrose cycling. From this viewpoint, species that are more active in starch metabolism may be more likely to experience sink-limited growth conditions, as often occurs at high CO$_2$.

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