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Increased Accumulation of Carbohydrates and Decreased Photosynthetic Gene Transcript Levels in Wheat Grown at an Elevated CO_2 Concentration in the Field[1]

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Repression of photosynthetic genes by increased soluble carbohydrate concentrations may explain acclimation of photosynthesis to elevated CO_2 concentration. This hypothesis was examined in a field crop of spring wheat (*Triticum aestivum* L.) grown at both ambient (approximately 360 μmol mol^{-1}) and elevated (550 μmol mol^{-1}) atmospheric CO_2 concentrations using free-air CO_2 enrichment at Maricopa, Arizona. The correspondence of steady-state levels of mRNA transcripts (coding for the 83-kD photosystem I apoprotein, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, phosphoglycerokinase, and the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase) with leaf carbohydrate concentrations (glucose-6-phosphate, glucose, fructose, sucrose, fructans, and starch) was examined at different stages of crop and leaf development and through the diurnal cycle. Overall only a weak correspondence between increased soluble carbohydrate concentrations and decreased levels for nuclear gene transcripts was found. The difference in soluble carbohydrate concentration between leaves grown at elevated and current ambient CO_2 concentrations diminished with crop development, whereas the difference in transcript levels increased. In the flag leaf, soluble carbohydrate concentrations declined markedly with the onset of grain filling; yet transcript levels also declined. The results suggest that, whereas the hypothesis may hold well in model laboratory systems, many other factors modified its significance in this field wheat crop.

Short-term exposure of terrestrial plants to $[CO_2]$ above the current ambient concentration of about 360 μmol mol^{-1} causes an immediate increase in photosynthesis and primary production in C_3 species (Kramer, 1981; Cure and Acock, 1986). However, growth for prolonged periods at elevated $[CO_2]$ may result in physiological acclimation involving a decrease in their capacity for CO_2 assimilation (Peet et al., 1986; Sage et al., 1989; Yelle et al., 1989a, 1989b) that is frequently associated with increased starch and soluble carbohydrate concentration in the source leaves (Delucia et al., 1985; Sasek et al., 1985; Stitt, 1991; Long and Drake, 1992). Several lines of indirect evidence suggest that regulation of the expression of photosynthetic genes, via increased soluble carbohydrate concentration, may underlie acclimation to growth in elevated $[CO_2]$ (Sheen, 1990, 1994; Stitt, 1991; Krapp et al., 1993; Webber et al., 1994). Decreased expression of several photosynthetic genes has occurred when sugar concentrations have been increased manipulatively by directly feeding mature spinach leaves through the transpiration stream (Krapp et al., 1991), by expression of yeast-derived invertase in transgenic tobacco plants, which directs the gene product to the cell wall to interrupt export from source leaves (Stitt, 1991), and by cooling the petiole to decrease the rate of phloem transport in intact tobacco plants (Krapp et al., 1993). Sheen (1990) demonstrated a possible mechanism for this by showing that addition of soluble carbohydrates to a maize protoplast transient expression system inhibited specifically the transcriptional activity of seven photosynthetic gene promoters, including that for *rbcS*.

Most recent research has focused on the effects of CO_2 enrichment on plant growth, leaf photosynthesis, and acclimation, but few studies have examined the effects of elevated $[CO_2]$ on gene expression in terrestrial plants. In controlled environments, a decrease of *rbcS* and *rbcL* transcript levels at elevated $[CO_2]$ was found in tomato (van

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Abbreviations: $[CO_2]$, atmospheric concentration of CO_2; $[CO_2]_{360}$, the current ambient atmospheric concentration of 360 μmol mol^{-1}; $[CO_2]_{550}$, the atmospheric concentration of CO_2 of 550 μmol mol^{-1} maintained in the FACE rings; DOY, day of year; FACE, free-air CO_2 enrichment; PGKase, phosphoglycerate kinase; PRKase, phosphoribulokinase; *psaB*, gene encoding a center apoprotein (83 kD) of PSI reaction center; *rbcL*, gene encoding the large subunit of Rubisco; *rbcS*, gene encoding the small subunit of Rubisco; SBPase, sedoheptulose-1,7-bisphosphatase; TNC, total nonstructural carbohydrate.

Oosten et al., 1994) and in N-deficient wheat (*Triticum aestivum* L.) leaves (Nie et al., 1993). However, it has not been shown that this occurs in the field, where fluctuations in temperature, light, humidity, and water supply might all modify these effects. It has also not been shown that these decreases in transcript levels are apparent at all stages of crop development.

The U.S. Department of Agriculture/Brookhaven National Laboratory FACE apparatus (Lewin et al., 1994) at the Maricopa Agricultural Center (University of Arizona, Maricopa, AZ), where wheat was grown at elevated [CO_2] in an open-field situation, provided a unique opportunity to address this question. This system avoids the direct perturbations of microclimate or artificial limitation to rooting volume that may be associated with enclosures. Furthermore, the large scale of this study allowed regular destructive sampling of leaves without significant effect on overall canopy size and structure.

The following questions were asked concerning the effects of elevated [CO_2] on photosynthesis at different crop developmental stages. (a) Are the steady-state levels of mRNAs encoding specific photosynthetic proteins decreased? (b) Is the increased accumulation of soluble carbohydrates in source leaves, which is observed in laboratory studies, also found in the field, where there is no artificial limitation of rooting volume imposed by pots? (c) Is there correspondence between increase in leaf carbohydrate concentration and decrease in mRNAs? (d) Are these differences apparent throughout different stages of crop development, at different points in the life of a single leaf, and at different points in the diurnal cycle?

MATERIALS AND METHODS

Crop Growth Conditions

Spring wheat (*Triticum aestivum* L. cv Yecora Rojo) was grown in a field on the experimental farm of the Maricopa Agricultural Center (33.075° N, 111.983° W), as described previously for cotton (Mauney et al., 1994). The seed was sown on December 15, 1992, and grown according to recommended wheat cultivation practice for the area (Dennis et al., 1976). A FACE system that was designed and built by Brookhaven National Laboratory (Upton, NY) was used to achieve controlled elevation of [CO_2] within and above the crop under field conditions (Lewin et al., 1994). Water and nutrients were applied through a subsurface drip irrigation system. Although a water-stress treatment was involved in the overall experimental design, leaves for this study were sampled only from the well-watered plots. The experimental design consisted of four replicate blocks each containing one elevated (approximately $[CO_2]_{550}$, FACE ring) and one ambient (approximately $[CO_2]_{360}$, control ring) plot (Mauney et al., 1994). The control was subsequently $[CO_2]_{360}$, and the elevated treatment was referred to as $[CO_2]_{550}$. The experimental area of the plots was 21 m in diameter, and plots were separated by at least 90 m. Elevation of [CO_2] commenced with crop emergence on January 1, 1993, and terminated at the time of grain harvest in May. Since crop emergence was the 1st DOY, DOY was equivalent to days after emergence.

RNA Study

Leaf Sampling

The uppermost, fully expanded leaves on the main stem were sampled on DOY 33 (fifth leaf), DOY 68 (eighth leaf), and DOY 75 (flag leaf). These dates corresponded to the approximate times of ligule emergence for each leaf. Leaves were collected from 20 individual wheat plants in each of the 8 rings between 9 and 10 AM on each occasion. In addition, flag leaves were sampled at four stages: (a) ligule emergence (DOY 75), (b) crop anthesis (DOY 89), (c) the soft-dough stage of grain filling (DOY 111), and (d) the hard dough stage at completion of grain filling (DOY 127). A diurnal analysis was conducted on the flag leaves from 6 AM until 7 PM, sampling at 3-h intervals on March 16, 1993 (DOY 75). It was completely dark at the first and last sampling times.

Sampling consisted of removing the 2 cm above the ligule and 2 cm below the tip of each leaf and plunging the remainder into liquid N_2. The samples were transported to the laboratory in liquid N_2 and subsequently stored at −80°C. Four or five replicates of these samples were examined for each treatment in each block on each sampling occasion.

Isolation of Nucleic Acid and Hybridization Analysis

Levels of mRNA transcripts coding for six major plastid proteins of photosynthesis were determined: large and small subunits of Rubisco (encoded by *rbcL* and *rbcS* gene, respectively), chloroplastic SBPase, PGKase, PRKase, and the reaction center apoprotein of PSI encoded by *psaB*. Total RNA was isolated from frozen leaf sections by acid-guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The purity of the RNA was determined by measuring A_{280} and A_{260}, and the quantity was determined by A_{260}. Glyoxal-denatured RNA (15 μg) was size fractionated by electrophoresis through a 1% agarose gel and transferred to Hybond-N membrane (Amersham) by capillary blotting. RNA was fixed onto the membrane by baking at 80°C for 2 h and cross-linked by exposure to UV light for 3 min. Immobilized RNA was hybridized with ^{32}P random-labeled DNA fragments specific for *rbcS* (Broglie et al., 1983), *rbcL* (Dron et al., 1982), *psaB* (Xu et al., 1993), and genes encoding for chloroplastic PGKase (Longstaff et al., 1989), PRKase (Raines et al., 1989), and SBPase (Raines et al., 1992). DNA-RNA hybridization was carried out in 6× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 50% (v/v) formamide, 5× Denhardt's solution, 0.5% (w/v) SDS, and 100 mg mL^{-1} of denatured salmon sperm DNA at 40°C (Sambrook et al., 1989). After the hybridization, the filters were washed twice for 2 min with 5× SSPE at 37°C, followed by two washes for 15 min at 37°C in 2× SSPE containing 0.1% (w/v) SDS. Autoradiography was carried out at −80°C with Kodak X-Omat film at a range of exposure times. The abundance of mRNA was quantified following autoradiography by scintillation counting. The transcript abundance of a specific mRNA was expressed as the percentage relative to the control on the same filter.

Carbohydrate Analysis

Glc, Fru, Suc, starch, fructans, and TNC were determined for parallel leaf samples. TNC includes total soluble sugar (Glc, Suc, and Fru), starch, and fructans. Preliminary HPLC analysis (Hendrix and Wei, 1994) showed that the majority of the fructans were of low molecular weight.

Leaf Sampling

Leaf blades were harvested from the field, killed by plunging into liquid N_2, and then transported to the laboratory on dry ice. The blades were lyophilized and ground to a fine powder in a miniature Wiley mill (Arthur H. Thomas Co., Philadelphia, PA). The dried leaf powder was stored at room temperature in tightly sealed glass containers until analyzed for carbohydrates.

Carbohydrate Extraction and Analysis

Thirty-milligram samples of powdered leaf tissue were extracted six times with hot (80°C) 80% ethanol (Hendrix and Peelen, 1987). The resulting ethanol extracts were treated with activated charcoal, filtered, and analyzed for ethanol-soluble carbohydrates by a microplate assay method (Hendrix, 1993). The alcohol-soluble fructans (i.e. low molecular weight) in these extracts were determined in dried aliquots as Glc and Fru released by hot (100°C) 0.1 M acetic acid. The alcohol-insoluble leaf powder residue was dried at 75°C and divided into two parts for starch and high molecular weight (i.e. alcohol insoluble) fructan determination. Starch was determined in the dried residue as Glc released by treatment with α-amylase and amyloglucosidase. High mol wt fructans were determined as Glc and Fru released from separate portions of the alcohol-insoluble residue, following treatment with hot acetic acid (Hendrix, 1993).

RESULTS

Leaves at the Completion of Blade Emergence

The steady-state mRNA levels were determined for the fifth, eighth, and flag leaf on the main stem of wheat plants grown at $[CO_2]_{360}$ and $[CO_2]_{550}$ (Figs. 1 and 2). Each of these leaves was examined on completion of its expansion and, therefore, when it was at the top of the canopy and probably the major photosynthetic organ. For the fifth leaf, five of the six mRNAs probed showed no effect of $[CO_2]_{550}$ treatment. However, the transcript for PGKase showed an increase at $[CO_2]_{550}$ compared with $[CO_2]_{360}$ (Figs. 1 and 2a). For the eighth leaf, no differences were found between $[CO_2]_{360}$ and $[CO_2]_{550}$ for any of the transcripts that were examined (Figs. 1 and 2b). However, the mRNA levels of *rbcS*, *rbcL*, and PGKase were lower in flag leaves grown at $[CO_2]_{550}$; whereas the mRNA levels of SBPase, PRKase, and *psaB* appeared unaffected (Figs. 1 and 2c).

Glc and Fru concentrations showed no detectable differences between the control and $[CO_2]_{550}$ treatment for any of these three leaves (data not shown). The mean concentrations of leaf Suc, starch, fructans, and TNC were always higher at $[CO_2]_{550}$ than at $[CO_2]_{360}$ for leaf 5, leaf 8, and the flag leaf (Fig. 3). However, the ratio of concentrations of these carbohydrates $[CO_2]_{550}/[CO_2]_{360}$ declined with crop development, from 1.48 for leaf 5 to 1.36 for leaf 8 and 1.18 for the flag leaf (Fig. 3). This pattern was apparent in TNC and its components (Fig. 3). In absolute terms, however, the carbohydrate concentration in both $[CO_2]_{360}$- and $[CO_2]_{550}$-grown flag leaves was much higher than in the fifth and eighth leaves of the respective treatments (Fig. 3). Compared with the fifth and eighth leaves, the flag leaf accumulated large amounts of fructans (Fig. 3). Starch accumulation was also enhanced by elevated $[CO_2]$ in all leaves (Fig. 3), but the absolute amount of starch was low at approximately 8% of TNC in leaves 5 and 8 and only 2% of TNC in the flag leaf (Fig. 3). Glc-6-P concentrations in the flag leaf at the time of ligule emergence were $<10\ \mu g\ g^{-1}$ and, therefore, approximately 5×10^{-5} the maximum TNC concentrations found.

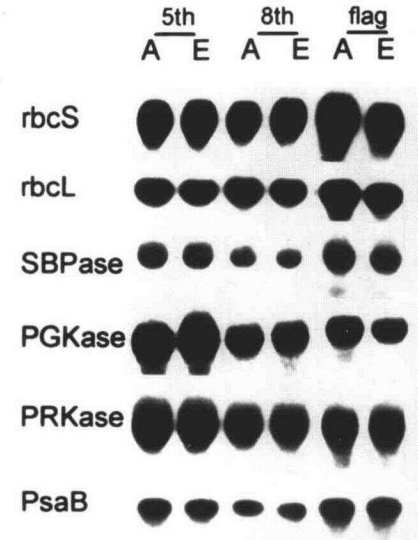

Figure 1. Northern blot analysis of total RNA that was isolated from the fifth, eighth, and flag leaf grown at $[CO_2]_{360}$ (ambient, A) and $[CO_2]_{550}$ (elevated, E). To ensure that each leaf was at the same developmental stage, samples were taken at the time of ligule emergence. Glyoxal denatured total RNA (15 μg) was size fractionated by electrophoresis and transferred to a Hybond-N membrane. Membranes were probed with ^{32}P random-labeled DNA probes specific for genes *rbcS*, *rbcL*, and *psaB*. RNA blots were also hybridized with DNA probes that were isolated from gene sequences specific for the chloroplastic SBPase, PGKase, and PRKase.

Interaction with Age for the Flag Leaf

Across the four sampling dates, a lower steady-state transcript level was indicated for all six transcripts in the flag leaf grown at $[CO_2]_{550}$ compared with $[CO_2]_{360}$ (Figs. 4 and 5), but this difference was not statistically significant for all transcripts at stages 2 and 3 (Figs. 4 and 5). Individual mRNA levels showed distinctive patterns of change with time and in relation to $[CO_2]$ treatment (Figs. 4 and 5). The decline in *rbcS* and *rbcL* transcripts was faster than for the other four transcripts (Figs. 4 and 5). Although tran-

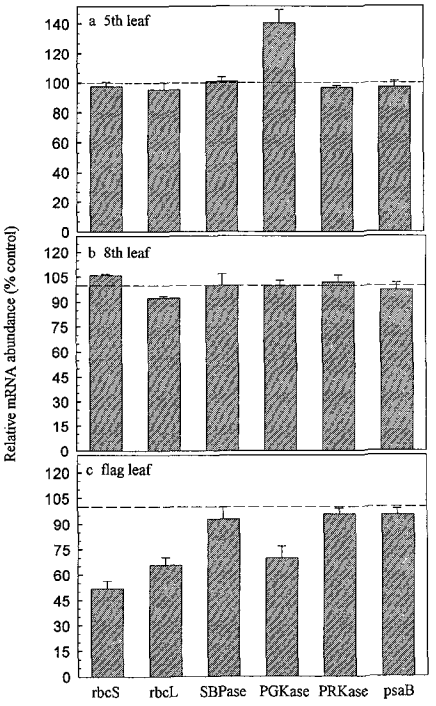

Figure 2. Quantification of northern blots as described in Figure 1, with data presented as the means (±1 SE) for isolations of RNA from the four experimental blocks. For each message, the radioactivity attached to the transcript for leaves grown at $[CO_2]_{360}$ (control) was defined as 100 (arbitrary units) and is shown by a dotted line. The relative mRNA abundance of each individual transcript in leaves grown at $[CO_2]_{550}$ was expressed as the percentage of control. a, Fifth leaf; b, eighth leaf; and c, flag leaf.

script levels of *rbcS* were much lower at $[CO_2]_{550}$ at ligule emergence (stage 1), it declined faster at $[CO_2]_{360}$ than at $[CO_2]_{550}$ after full leaf expansion, such that the differences between the treatments were almost lost by crop anthesis (stage 2) and grain filling (stage 3) (Fig. 5a). By the completion of grain filling (stage 4), the concentration of the *rbcS* transcript was just 3% of its level at ligule emergence at $[CO_2]_{360}$, whereas at $[CO_2]_{550}$ only a trace (<1%) was detectable (Figs. 4 and Fig. 5a).

Levels of *rbcL* transcripts were always lower in the flag leaves grown at $[CO_2]_{550}$ (Figs. 4 and Fig. 5b); the biggest difference was found at ligule emergence (stage 1) and the smallest was found at completion of grain filling (Figs. 4 and 5b). The level of mRNA transcripts for PGKase was markedly lower at $[CO_2]_{550}$ on leaf emergence (Figs. 4 and 5d). Unlike *rbcS* and *rbcL* transcripts, which were almost undetectable at the completion of grain filling, the levels of transcripts for PGKase at completion of grain filling (stage 4) were 50% in $[CO_2]_{360}$ and 32% in $[CO_2]_{550}$ of the amounts at ligule emergence (Fig. 5d). Transcripts for SBPase and *psaB* were lower in the leaves grown at $[CO_2]_{550}$ throughout, although the differences were only marginal at ligule emergence (Figs. 4 and 5, c and f). By the completion of grain filling (stage 4), 74% of SBPase mRNA still remained at $[CO_2]_{360}$ and 46% remained at $[CO_2]_{550}$ (Figs. 4 and 5c). By this stage, *psaB* transcript levels were still 48% at $[CO_2]_{360}$ of those at ligule emergence, but only 11% in $[CO_2]_{550}$ (Figs. 4 and Fig. 5f). The mRNA level of PRKase showed no obvious treatment effect until the completion of grain filling, when it was markedly lower at $[CO_2]_{550}$ (Figs. 4 and 5e).

At and subsequent to ligule emergence, carbohydrate concentrations were higher in the flag leaves grown at $[CO_2]_{550}$ (Fig. 6). The concentration of Suc (Fig. 6a) increased sharply after leaf emergence (stage 1, Fig. 6) and reached a maximum at anthesis (stage 2). It then declined rapidly into grain filling (stage 3) when the leaf contained only 6% of the concentration found at anthesis. The concentration showed a final increase after grain filling (Fig. 6a). Leaves grown at $[CO_2]_{550}$ contained 30% more Suc at crop anthesis than control leaves, but at the three other sampling times there were no obvious differences between the two $[CO_2]$ treatments (Fig. 6a). Under both growth conditions, fructan concentration was highest at leaf emergence and declined sharply until grain filling was complete (Fig. 6b). The leaf always appeared to contain higher concentrations of fructans at $[CO_2]_{550}$; however, this difference proved to be statistically significant only at anthesis and during grain filling (Fig. 6b). For starch (Fig. 6c), the absolute concentration in the leaf was quite low compared with other types of carbohydrate, although it was markedly higher at $[CO_2]_{550}$ on ligule emergence (Fig. 6c). The dynamic pattern of TNC accumulation (Fig. 6d) was similar to that of Suc (Fig. 6a). TNC content increased after leaf emergence and reached a peak at crop anthesis; it then decreased rapidly during grain filling but increased again at the end of grain filling (Fig. 6d). TNC was considerably higher at $[CO_2]_{550}$ than at $[CO_2]_{360}$ at leaf emergence and at anthesis, but there was little difference during grain filling (Fig. 6d).

Diurnal Analysis

On March 16, 1993, a day with clear sky from dawn to dusk, the PRKase transcript levels remained approximately

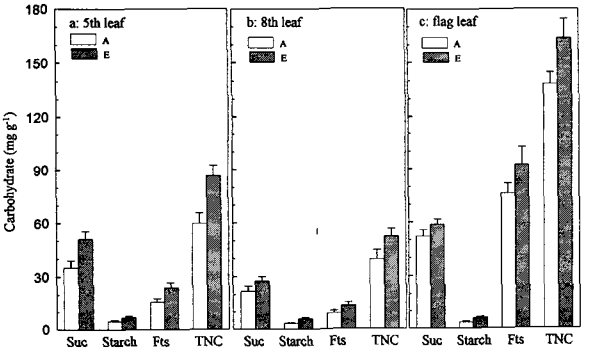

Figure 3. Carbohydrate contents of the leaves described in Figure 1. The leaves were grown at $[CO_2]_{360}$ (ambient, A) and $[CO_2]_{550}$ (elevated, E). The concentrations (expressed as mg carbohydrate per g dry weight of leaf) of Suc, starch, fructans (Fts), and TNC are illustrated. Data points are the means (±1 SE) for extractions from leaves from each of the four experimental blocks. a, Fifth leaf; b, eighth leaf; and c, flag leaf.

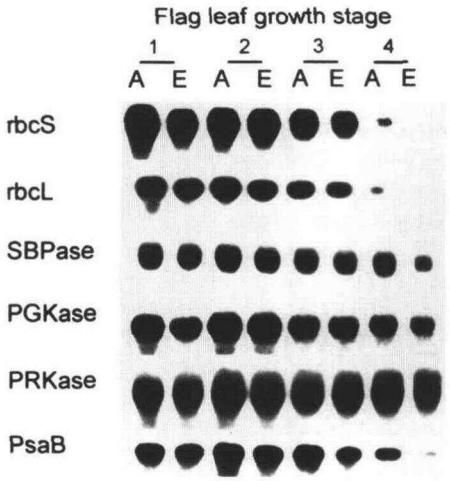

Figure 4. Northern blot analysis of total RNA isolated from the flag leaf grown at $[CO_2]_{360}$ (ambient, A) and $[CO_2]_{550}$ (elevated, E). The leaves were sampled at the following growth stages: 1, Ligule emergence (DOY 75); 2, crop anthesis (DOY 89); 3, soft-dough stage of grain filling (DOY 111); and 4, hard-dough stage at completion of grain filling (DOY 127). Visualization of mRNA levels was as described in Figure 1.

constant throughout the day (Fig. 7e), whereas all other transcripts showed some degree of diurnal variations (Fig. 7). In general, transcript levels oscillated from a maximum at approximately dawn to a minimum in the late afternoon (Fig. 7) and were essentially antiparallel to the pattern of leaf carbohydrate concentration (Fig. 8). The amplitude of this fluctuation in transcript levels during the day was small and less than 2-fold (Fig. 7).

Transcript concentrations for *rbcS* (Fig. 7a), *rbcL* (Fig. 7b), and PGKase (Fig. 7d) were lower in the leaves grown at $[CO_2]_{550}$ compared to $[CO_2]_{360}$ throughout the day, although treatment differences were most profound in the morning, especially before dawn (Fig. 7, a, b, and d). Relatively smaller differences between the treatments were observed in the late afternoon (approximately 5 PM), especially for *rbcL* and *rbcS* (Fig. 7, a and b). After sunset the differences between $[CO_2]_{550}$ and $[CO_2]_{360}$ became larger again for these three transcripts (Fig. 7, a, b, and d), although the mRNA level of SBPase (Fig. 7c) and *psaB* (Fig. 7f) also fluctuated diurnally, with the highest level in the early morning, declining to a minimum at approximately noon, and then recovering in the evening.

Parallel samples were taken on the same day to examine the diurnal fluctuation in nonstructural carbohydrate contents. All TNCs were at a minimum at 6 AM, predawn, with leaves in $[CO_2]_{550}$ showing slightly more (approximately 15 mg g^{-1}) than controls. Except for starch, all of the carbohydrates showed a rapid increase in concentration after sunrise, leading to an approximately 5-fold increase in TNC, Suc, and fructan by the end of the day (Fig. 8). By dusk, Glc-6-P concentrations were slightly higher in $[CO_2]_{550}$, at 7.4 ± 0.2 $\mu g\ g^{-1}$, compared to 6.3 ± 0.5 $\mu g\ g^{-1}$ in $[CO_2]_{360}$. The concentration of starch stayed at a minimum for the first 1 to 2 h of the day and then it increased monotonically during daylight hours (Fig. 8c). After sunrise, the content of all TNCs increased more rapidly in the leaves grown at $[CO_2]_{550}$ relative to $[CO_2]_{360}$ (Fig. 8). This was particularly marked for starch content, which increased 4.5 times at $[CO_2]_{360}$ compared to 6.5 times at $[CO_2]_{550}$ throughout the day (Fig. 8c). Thus, absolute differences between the treatments were greatest at dusk, with leaves growing at $[CO_2]_{550}$ containing approximately 60 mg g^{-1} more than controls (Fig. 8). However, differences in some TNCs had become pronounced by mid-day, with leaves growing in $[CO_2]_{550}$ containing approximately 20 mg g^{-1} more Suc than controls (Fig. 8a).

DISCUSSION

The hypothesis that increased soluble carbohydrate concentration causes repression of the genes coding for Rubisco and other photosynthetic proteins in leaves in elevated $[CO_2]$ provides an attractive explanation of acclimation of photosynthetic capacity (reviewed by Webber et al., 1994). However, Arp (1991) and Thomas and Strain

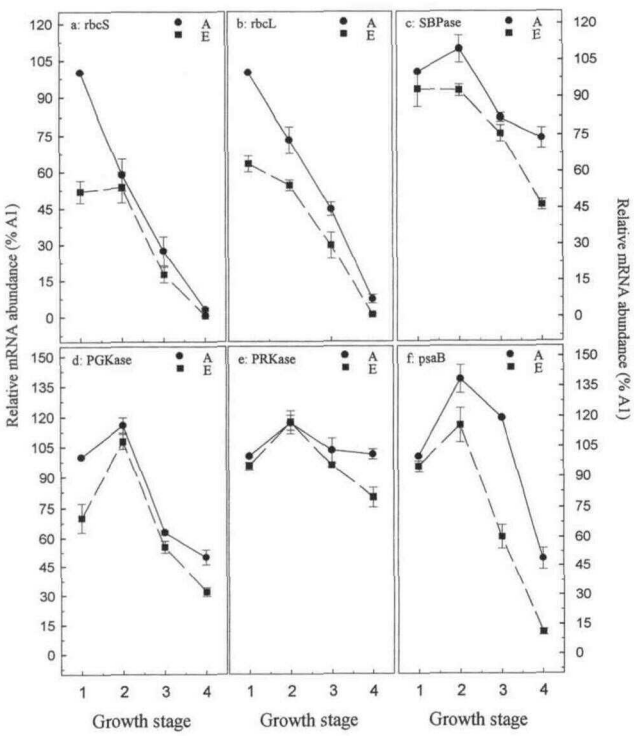

Figure 5. The effect of elevated $[CO_2]$ on the steady-state mRNA transcripts in the flag leaf at the four growth stages as described in Figure 4. The radioactivity attached to each transcript from leaves grown at $[CO_2]_{360}$ (A, ●) and at developmental stage 1 (A1) was defined as 100%. Abundance of transcripts at other developmental stages and for leaves grown at $[CO_2]_{550}$ (E, ■) are expressed relative to this value. Data points are the means (±1 SE) for isolations of RNA from the four experimental blocks. Northern blots were hybridized with probes specific for gene *rbcS* (a) and *rbcL* (b) and with probes for the genes coding for the chloroplastic enzymes SBPase (c), PGKase (d), and PRKase (e) and for *psaB* (f).

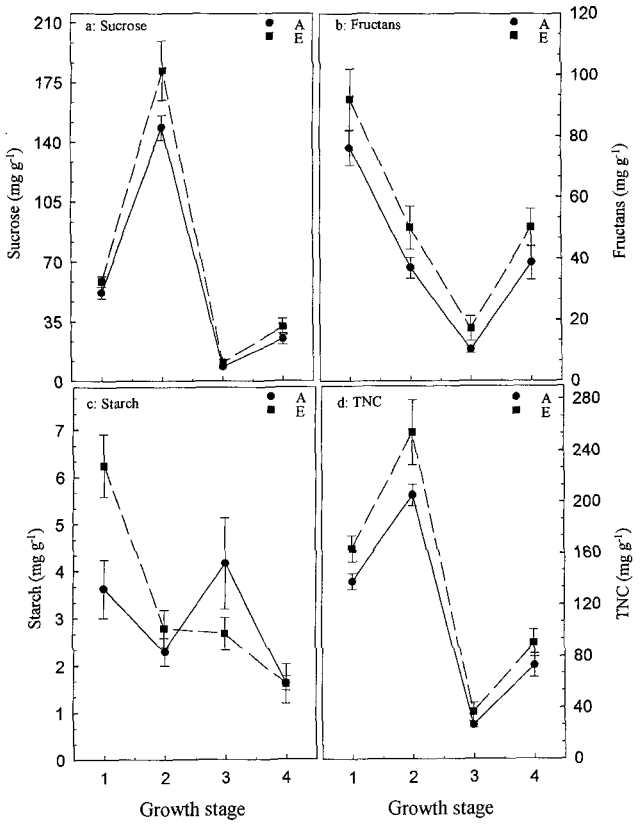

Figure 6. Carbohydrate content of the flag leaf grown at $[CO_2]_{360}$ (A, ●) and $[CO_2]_{550}$ (E, ■) at the four growth stages as described in Figure 4. Data points are the means (±1 SE) for extractions from leaves from each of the four experimental blocks. The concentrations of Suc (a), fructans (b), starch (c), and TNC (d) are illustrated.

(1991) argued that acclimatory loss of photosynthetic capacity in elevated $[CO_2]$ may be an experimental artifact produced by a restricted rooting volume. Our results show that in a field crop of spring wheat, elevated $[CO_2]$ induces an increase in leaf nonstructural carbohydrates and a decrease in the concentration of gene transcripts coding for specific proteins of the photosynthetic apparatus, despite the absence of any artificial restriction of rooting volume and despite a good supply of water and nutrients. However, the effect on levels of gene transcripts varied markedly with the stages of both crop and leaf development and with time of day.

Differences in the carbohydrate content of flag leaves between the two $[CO_2]$ treatments was only apparent after dawn. At dawn there was no significant difference, suggesting that despite the increased photosynthesis of these leaves sufficient additional sink capacity had developed to remove any accumulation of carbohydrates overnight. Both Suc and Glc have been shown to repress genes in higher-plant transient expression systems. No significant differences in Glc concentration between $[CO_2]$ treatments were detected. However, midday Suc concentration in the $[CO_2]_{550}$-grown flag leaves had reached 82 mg g^{-1} compared to 60 mg g^{-1} for the $[CO_2]_{360}$ leaves (Fig. 6). Although approximately 30 times more Suc is required to induce the same change in gene expression (Sheen, 1994), an increase in Suc could have an effect via formation of hexose phosphates following hydrolysis by acid invertase or Suc synthase (Goldschmidt and Huber, 1992).

The wheat crop was exposed to a season-long CO_2 concentration of 550 μmol mol^{-1} using FACE. To ensure that the different leaves were sampled at the same point in their individual development, each was examined at the time of completion of blade emergence, i.e. ligule appearance. Elevated $[CO_2]$ affected the steady-state transcript levels to a different extent in each of the leaves. The relative increase in soluble carbohydrate concentration was greatest in leaf 5 and least in the flag leaf. The relative decrease in *rbcS*, one of the genes known to be suppressed by soluble carbohydrates (Sheen, 1990), was greatest in the flag leaf and least in leaf 5 (Fig. 2). Why is the apparent suppression of gene expression greatest in the leaf that shows the smallest relative increase in carbohydrate concentration? There are three possible explanations.

1. Development was slightly faster in the wheat crop grown in elevated $[CO_2]$. At the point of flag leaf ligule

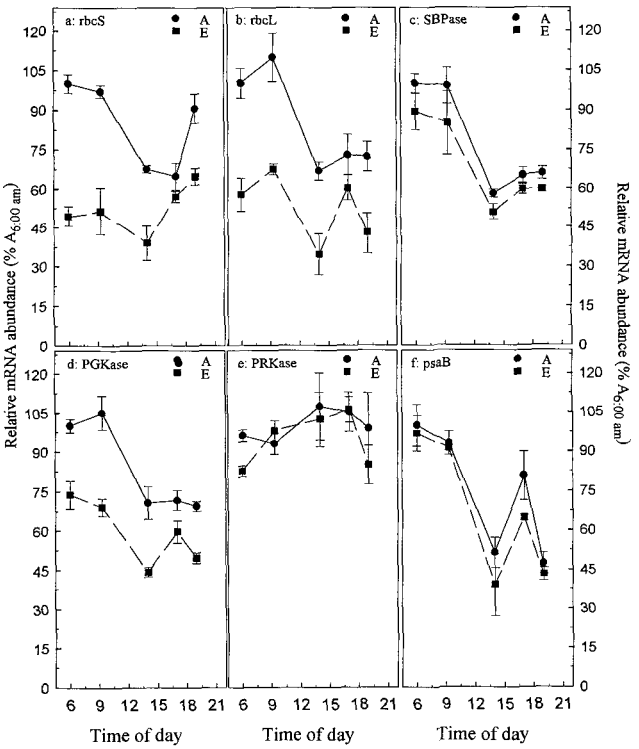

Figure 7. The steady-state abundance of mRNA in flag leaves sampled from 6 AM to 7 PM at approximately 3-h intervals on March 16, 1993 (DOY 75). The leaves were grown at $[CO_2]_{360}$ (A, ●) and $[CO_2]_{550}$ (E, ■). For each transcript, the signal of the flag leaf grown at $[CO_2]_{360}$ at 6 AM (Mountain Standard Time) ($A_{6:00am}$) was defined as 100%, and abundance at other times of day and in the $[CO_2]_{550}$-grown leaves is expressed relative to this initial value ($A_{6:00am}$). Data points are the means (±1 SE) for isolations of RNA from the four experimental blocks. Gene probes used with the northern blots are as described for Figure 5.

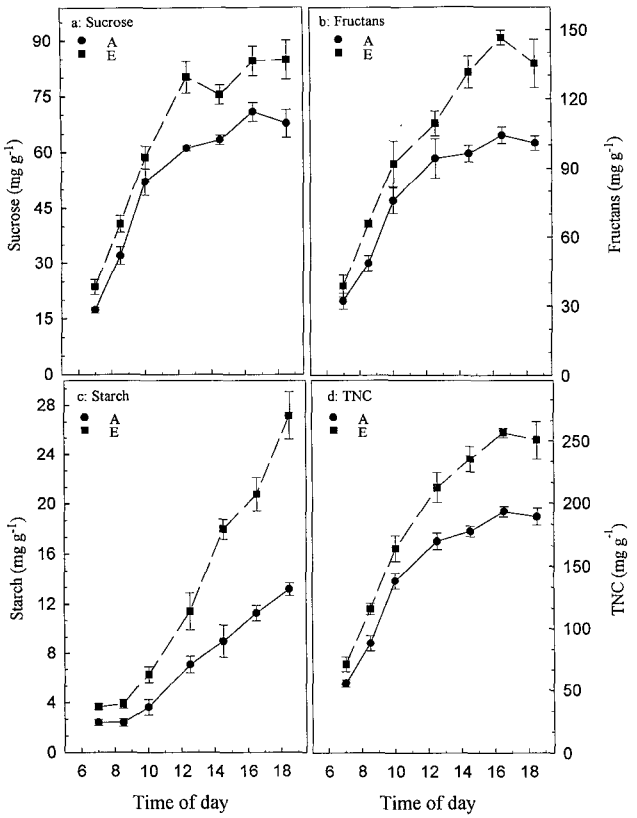

Figure 8. Diurnal variations in carbohydrate content in the flag as described in Figure 7. Leaves were grown at $[CO_2]_{360}$ (A, ●) and $[CO_2]_{550}$ (E, ■). Data points are the means (±1 SE) for extractions from leaves from each of the four experimental blocks. The concentrations of Suc (a), fructans (b), starch (c), and TNC (d) are illustrated.

appearance, the crop in $[CO_2]_{550}$ was estimated to be about 1 d ahead of the controls in terms of wheat developmental stage (Pinter et al., 1993). Figure 4 shows that transcript levels decline rapidly between ligule emergence (DOY 75) and anthesis (DOY 89). Thus, a crop that is developmentally 1 d more advanced could be expected to show lower transcript levels for that reason alone. However, on examining *rbcS* levels in Figure 4 and assuming a linear decline during the 14-d interval, <10% of the difference could be accounted for by accelerated crop development.

2. In both control and $[CO_2]_{550}$-grown leaves, the carbohydrate concentration in the flag leaves was considerably higher than it had been in its predecessors when their blades completed emergence. If gene repression results when carbohydrate concentrations exceed a threshold, then it is possible that, although the relative increase in the flag leaf was less than in leaves 5 and 8, the greater absolute increase was sufficient to exceed the threshold. However, most of the difference in TNC between the flag leaf and leaves 5 and 8 results from increased accumulation of fructans (Fig. 3). Since fructans are stored in the vacuole (Pollock, 1986; Chatterton et al., 1987), they are unable to produce a direct effect on gene expression; however, they may reflect a capacity to produce higher levels of hexoses in the cytoplasm.

3. Suc and Glc are likely to affect gene expression via their metabolic products, possibly via hexose phosphate synthesis rather than by direct action (Sheen, 1994). If, for example, phosphorylation is required, then the effective concentration of Glc will change according to capacity for the subsequent step. Only very small differences in Glc-6-P contents were found at the completion of flag leaf emergence. This, however, may reflect the difficulty in resolving differences for a substrate that occurs at such low concentrations in wheat leaves. If Suc acts through hexose phosphate synthesis, then levels of invertase or Suc synthase may alter the concentration of Suc required to affect a given reduction in gene expression.

Although cereal leaves retain the ability to synthesize Rubisco after expansion, synthesis and turnover remain very low (Peterson et al., 1973). After full expansion, there is a coordinated decline in synthesis of both subunits up to senescence (Gutteridge and Keys, 1985). At the completion of blade emergence, an increased soluble carbohydrate concentration in the flag leaves developed at $[CO_2]_{550}$ and corresponded to a marked decrease in *rbsS* transcript levels (Figs. 2–5). A marked decrease in the abundance of *rbcS* and *rbcL* on completion of expansion has been widely reported (Speirs and Brady, 1981; Kasemir et al., 1988; Bate et al., 1991). However, after anthesis and several days after completion of blade expansion, a dramatic (approximately 90%) decline in TNC, and especially in Suc content, of the flag leaf occurred (Fig. 5, a and d). If soluble carbohydrates dominate expression of the Rubisco genes throughout the life of the flag leaf, then a recovery of transcript levels could be expected during this period when it is assumed that the strong demands of the developing grain are causing rapid mobilization of TNC in the flag leaf. However, decline in *rbcS* and *rbcL* continued unabated through this period (Fig. 5). The lack of increase in any of the transcripts during this marked decline in TNC contradicts the suggestion that carbohydrate accumulation, through gene repression, may drive leaf senescence (Hensel et al., 1993). This suggests that factors other than carbohydrate level dominate the decline in transcript levels in the flag leaf of wheat. The rapid decline in *rbcS* and *rbcL* mRNA observed here is consistent with the finding that the Rubisco content of the flag leaf declined rapidly relative to other proteins following blade emergence and that the decline in both of the polypeptide subunits was accelerated by the $[CO_2]_{550}$ treatment (Nie et al., 1995). A larger depression of levels of *rbcS* and *rbcL* by $[CO_2]_{550}$ on completion of leaf emergence and during the course of the diurnal cycle is consistent with the theoretically lower requirement for Rubisco, relative to other photosynthetic proteins, in elevated $[CO_2]$ (Long and Drake, 1992; Webber et al., 1994).

Levels of the six transcripts revealed partially independent changes, both in relation to time and $[CO_2]$ treatment. Whereas transcripts of *rbcS*, *rbcL*, and PGKase were lower at ligule emergence in $[CO_2]_{550}$, by comparison with the control, transcripts of PRKase showed no effect of $[CO_2]$ treatment at any stage of either crop or leaf development

(Figs. 2 and 4). At the time of anthesis, when differences in TNC between the two $[CO_2]$ treatments were greatest, a significant decrease in *psaB* transcript levels became apparent (Fig. 4f). Like *rbcL*, *psaB* is chloroplast encoded and thus regulated by a different pathway from that operating in the nucleus. Nevertheless, their repression has also been linked to hexose accumulation (Criqui et al., 1992).

At completion of grain filling, all of the mRNAs were lower at $[CO_2]_{550}$ than at $[CO_2]_{360}$ (Fig. 5). By this stage, however, the development of the $[CO_2]_{550}$-grown crop was more than 2 d ahead of the controls (Pinter et al., 1993; Nie et al., 1995) and this suggests that a significant part of this final difference resulted from earlier flag leaf senescence at $[CO_2]_{550}$. Earlier senescence in elevated $[CO_2]$ may reflect the strongly determinate developmental pattern of this annual crop. In other species, elevated $[CO_2]$ can result in a delay of senescence (Long and Hutchin, 1991).

The steady-state level of mRNAs, except PRKase mRNA, was found to oscillate during the day (Fig. 8). The diurnal magnitude of oscillation of these mRNAs was less profound compared with the widely reported oscillation of *cab* transcript levels (Kloppstech, 1985; Spiller et al., 1987; Paulsen and Bogorad, 1988). The relatively small-scale oscillation of these mRNAs observed in this study was consistent with previous reports (Piechulla and Gruissem, 1987; Adamska et al., 1991; Pilgrim and McClung, 1993). The mRNA level was highest at 6 AM and declined through the morning to reach a minimum after midday (Fig. 7). This pattern corresponded to that of carbohydrate accumulation, in particular Suc, which reached and maintained a maximum at approximately midday. The levels of mRNA transcripts of all genes except PRKase were reduced throughout the day at $[CO_2]_{550}$ by comparison with levels at $[CO_2]_{360}$. Thus, whereas transcript levels changed diurnally, lower expression in elevated $[CO_2]$ was apparent throughout (Fig. 8).

In conclusion, this study has shown that growth at an elevated $[CO_2]$ of approximately 50% above current levels does result in both increased leaf soluble carbohydrate concentrations and decreased mRNA transcripts for some genes coding for the photosynthetic apparatus, in particular *rbcS* and *rbcL*. This occurs despite the growth of this crop without restriction of root growth. However, when different stages of both crop and leaf development are considered, a complex interaction with $[CO_2]$ treatment is apparent. Any effect of $[CO_2]$ treatment appeared to be highly dependent on the time at which samples were taken. This suggests that when studies have been limited to just one developmental stage very different conclusions could be drawn according to the stage of development selected. Furthermore, correspondence between increased soluble carbohydrate content and decreased gene transcript levels was often lacking, suggesting that other internal and external signals may be more dominant in the field. Although repression of photosynthetic genes by accumulation of soluble carbohydrates in laboratory-grown plants remains an attractive mechanistic explanation of acclimation of photosynthetic capacity in elevated $[CO_2]$, the variable correspondence of transcript level with soluble carbohydrate accumulation at different stages of development in the field show that other factors significantly modify this effect in nature.

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