Interactions of Nitrate and CO_2 Enrichment on Growth, Carbohydrates, and Rubisco in Arabidopsis Starch Mutants. Significance of Starch and Hexose[1]

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Wild-type (wt) Arabidopsis plants, the starch-deficient mutant TL46, and the near-starchless mutant TL25 were grown in hydroponics under two levels of nitrate, 0.2 versus 6 mM, and two levels of CO_2, 35 versus 100 Pa. Growth (fresh weight and leaf area basis) was highest in wt plants, lower in TL46, and much lower in TL25 plants under a given treatment. It is surprising that the inability to synthesize starch restricted leaf area development under both low N (N_L) and high N (N_H). For each genotype, the order of greatest growth among the four treatments was high CO_2/N_H > low CO_2/N_H, > high CO_2/N_L, which was similar to low CO_2/N_L. Under high CO_2/N_L, wt and TL46 plants retained considerable starch in leaves at the end of the night period, and TL25 accumulated large amounts of soluble sugars, indicative of N-limited restraints on utilization of photosynthates. The lowest ribulose-1,5-bisphosphate carboxylase/oxygenase per leaf area was in plants grown under high CO_2/N_L. When N supply is limited, the increase in soluble sugars, particularly in the starch mutants, apparently accentuates the feedback and down-regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase, resulting in greater reduction of growth. With an adequate supply of N, growth is limited in the starch mutants due to insufficient carbohydrate reserves during the dark period. A combination of limited N and a limited capacity to synthesize starch, which restrict the capacity to use photosynthate, and high CO_2, which increases the potential to produce photosynthate, provides conditions for strong down-regulation of photosynthesis.

In plants grown under elevated CO_2 there is often acclimation, reducing the capacity of photosynthesis. A variety of factors have been proposed to contribute to the down-regulation of photosynthesis under prolonged elevated CO_2, including limited sink capacity, N limitation, end-product limitation, excess accumulation of starch, a decrease in photosynthetic enzymes such as Rubisco, and accelerated senescence.

Rubisco small subunit (*rbcS*) transcripts often decrease in elevated CO_2, for example, as reported in wheat (*Triticum aestivum*; Nie et al., 1995), Arabidopsis (Cheng et al., 1998), pea (*Pisum sativum*; Majeau and Coleman, 1996), and tomato (*Lycopersicon esculentum*; Van Oosten and Besford, 1995). This correlates with a decrease in Rubisco activity (Van Oosten and Besford, 1995; Majeau and Coleman, 1996) and content (Cheng et al., 1998; Moore et al., 1998). The decrease in Rubisco has been associated with an increase in the levels of soluble sugars in some studies (Van Oosten and Besford, 1995; Majeau and Coleman, 1996; Cheng et al., 1998), implicating sugar-mediated repression of photosynthetic gene expression of which hexokinase is one of the likely sensors in the signaling pathway (Jang and Sheen, 1994; 1997; Pego et al., 2000; Smeekens, 2000). The decrease in gene transcripts is not always correlated with absolute levels of soluble sugars (Nie et al., 1995; Moore et al., 1998), and sugar repression of photosynthesis has been observed to correlate with acid invertase activity, and therefore increased hexoses from Suc cycling (Goldschmidt and Huber, 1992; Moore et al., 1998).

The accumulation of carbohydrates in elevated CO_2 may be due to limited sink capacity, and therefore a limitation on the use of photosynthate (Stitt, 1991). Arp (1991) found that photosynthetic capacity under elevated CO_2 was decreased, in line with a decline in sink capacity resulting from factors such as low N and restricted root growth. In tomato, removal of sink by the detachment of young leaves resulted in elevated hexose levels and a more substantial decrease in photosynthetic gene transcripts in elevated CO_2 (Van Oosten et al., 1994). In a converse manner, when sink capacity is increased relative to the source, down-regulation of photosynthesis was not observed in ryegrass (*Lolium perenne*; Rogers et al., 1998). Some studies suggest that the observed accumulation of carbohydrates under elevated CO_2 may not be due to sink limitation. The decline in photosynthesis has been ascribed to a limitation on triose-P utilization for synthesis of products like starch and Suc (Cure et al., 1991; Ludewig et al., 1998). Photosynthesis may exceed the rate of end-product synthesis, therefore, the recycling of P_i is decreased and can feedback to

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limit the rate of photosynthesis. A decline in P_i and an increase in phosphoglyceric acid will also induce starch synthesis (Preiss, 1982). In clover (*Trifolium subterraneum*), when photosynthesis was stimulated by growth in high irradiance, there was no accumulation of starch, in contrast to plants grown in elevated CO_2, which had similar photosynthetic rates (Morin et al., 1992). Therefore, in high irradiance, carbon is exported from leaves, indicating there was not a limitation on sink capacity. It is postulated that the decrease in photorespiration in elevated CO_2 is causing a decrease in P_i, and ATP synthesis, thereby altering carbon partitioning (Morin et al., 1992).

The accumulation of starch grains has been suggested to disrupt chloroplast structure (Cave et al., 1981) and increase diffusive resistance to CO_2 (Nafziger and Koller, 1976; Grub and Mächler, 1990). There is often a more pronounced down-regulation of photosynthesis in starch-accumulating species when sink capacity is limiting (Goldschmidt and Huber, 1992), and in elevated CO_2-grown plants, a decline in CO_2 assimilation has been seen to correlate with increased leaf starch (Ehret and Jolliffe, 1985). This might occur because starch is metabolized to Glc in the dark period, which may then function in sugar signaling to further decrease photosynthetic genes (Cheng et al., 1998). However, others have found no relationship between starch accumulation and a decrease in photosynthesis (Van Oosten et al., 1994; Moore et al., 1998). In potato (*Solanum tuberosum*) with antisense inhibition of leaf AGPase, resulting in a reduction in starch content, CO_2 assimilation was lower in antisense plants than wild type (wt) when grown in elevated CO_2; therefore, in this case, acclimation is not caused by an accumulation of starch (Ludewig et al., 1998).

Another explanation for the decline in photosynthetic capacity in elevated CO_2 is accelerated senescence. Earlier leaf senescence in elevated CO_2 was responsible for the decreased photosynthetic rates, Rubisco activity, and chlorophyll in tobacco (*Nicotiana tabacum*; Miller et al., 1997). In agreement, *rbcS* and other photosynthetic genes declined in elevated CO_2, which was also attributed to accelerated senescence (Ludewig and Sonnewald, 2000). There is evidence for interaction between N supply and plant response to growth in elevated CO_2 (Stitt and Krapp, 1999). N limitation leads to decreased growth (Paul and Stitt, 1993; Scheible et al., 1997a, 1997b) and an accumulation of starch (Rufty et al., 1988; Paul and Stitt, 1993; Paul and Driscoll, 1997; Scheible et al., 1997a). Under low N, sink strength is decreased and acclimation of photosynthesis to elevated CO_2 is usually more marked (Pettersson and McDonald, 1994; Sage, 1994; Bowler and Press, 1996). For example, in tobacco grown under elevated CO_2, *rbcS* was decreased in low N supply, but not when grown with sufficient N (Geiger et al., 1999). The decrease in Rubisco and other photosynthetic proteins in elevated CO_2 has also been ascribed to a general decrease in leaf protein due to N limitation, which is accentuated in elevated CO_2 (Nakano et al., 1997).

In a previous paper (Sun et al., 1999), we investigated the performance of starch mutants of Arabidopsis (TL46 and TL25) grown in soil under low and high light regimes. Under these conditions, photosynthesis and growth were correlated with the capacity for starch production. In the following study, we examined the effects of elevated CO_2 and N availability on wt and starch mutants of Arabidopsis during growth in hydroponics. We aimed at providing insight into the physiological significance of starch and hexose in relation to photosynthate production and utilization under different CO_2 and N levels. Our results indicate that there is a complex interplay between N and C that affects the extent of starch accumulation and starch turnover and, in turn, plant growth.

RESULTS

C and N Composition and Growth

Figure 1 shows the results of growth of Arabidopsis wt and leaf starch mutants (TL46, a starch-deficient mutant, and TL25, a near-starchless mutant), under low N (N_L) and high N (N_H) nutrition and low (C_L) and high (C_H) CO_2, on the C and N composition of shoots and roots. It is apparent that the total C content of the shoots and roots was similar across all treatments (approximately 40% [w/w]), whereas the total N content decreased substantially under N_L nutrition. Under N_H nutrition, the N content across all treatments, low versus high CO_2, and across the three genotypes was 5% to 6.5% (w/w) of the dry weight, with shoots from wt plants having a slightly lower N content than those from the starch mutants. Thus, on average, the C/N ratio of shoots under the N_H treatment was about 6 for the starch mutants and approximately 7 for the wt. With growth under N_L nutrition, the N content of the tissue decreased, resulting in an increase in the C/N ratio of the tissue. In leaf tissue, the largest increase in C/N ratio under N_L nutrition occurred in wt plants compared with the starch mutants. In roots, the increase in the C/N ratio in N_L-grown plants was very similar across the three genotypes; the increase was slightly higher in the low CO_2-grown plants.

Measurements of fresh weight as an indicator of growth showed a decrease in the ratio of top (aerial)/root fresh weight under N_L across all three genotypes and under low and high CO_2 (Fig. 2). This ratio decreased under N_L due to a large decrease in the top growth, whereas the growth of roots was less affected. In wt and TL46 plants, the root growth was very similar across treatments; however, in TL25, the root growth was less, and it was lowest in the N_L

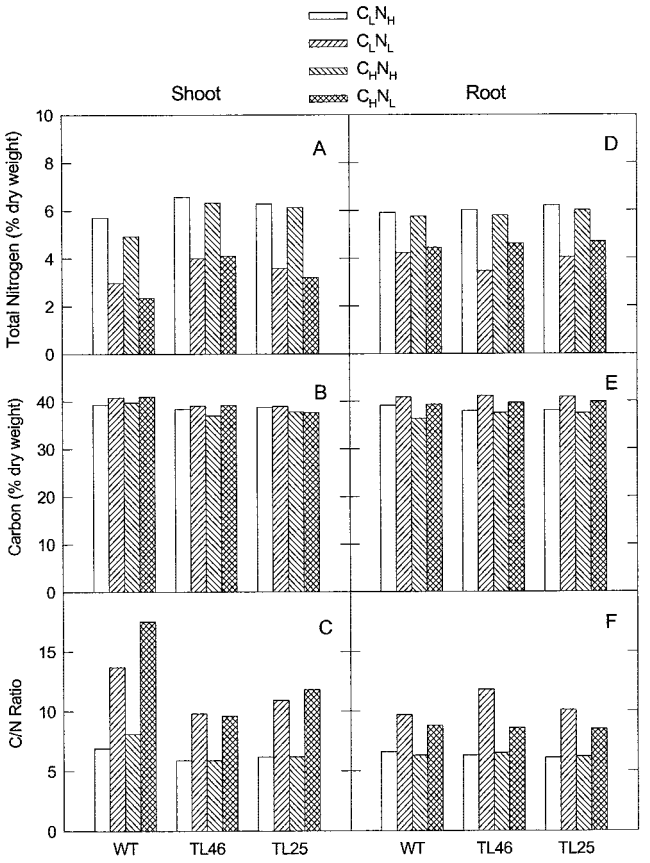

Figure 1. C and N content of roots and tops of wt and starch mutants of Arabidopsis grown under different levels of nitrate and CO_2. Material from three individual plants was pooled and used for analysis. Plants were grown under different levels of CO_2 and N nutrition during the last 16 d of growth as follows: C_LN_H, growth under 35 Pa CO_2, 6 mM nitrate; C_LN_L, growth under 35 Pa CO_2, 0.2 mM nitrate; C_HN_H, growth under 100 Pa CO_2, 6 mM nitrate; and C_HN_L, growth under 100 Pa CO_2, 0.2 mM nitrate.

plants. In wt plants, the top growth under N_H was significantly enhanced under high CO_2. The average growth of tops in wt plants under higher N was higher than in TL46, although with the degree of variation, it was not significantly different. However, the growth of tops and roots of TL25 was much lower than wt and TL46 plants under all treatments. The differences in cumulative leaf area per plant (Fig. 3) were very similar to that of top fresh weight (Fig. 2A) across all treatments.

Leaf Starch and Soluble Carbohydrates

Leaf starch was analyzed at the end of the light (Fig. 4A) and dark periods (Fig. 4B). In wt plants under C_LN_H, a moderate amount of starch accumulated during the day and it was largely used during the dark period. Under C_HN_H, there was a substantial increase in starch levels during the day, and although starch turnover rate was twice that observed under C_LN_H, substantial levels remained at the end of the dark period. In the N_L treatments, 2.5- to 3-fold increases in starch levels were observed at the end of the light period compared to C_LN_H. As there was little difference in starch turnover in N_L treatments versus C_LN_H, the bulk of the starch remained in N_L plants at the end of the dark period. TL46 plants showed only moderate increases in starch levels across all treatments, with the apparent

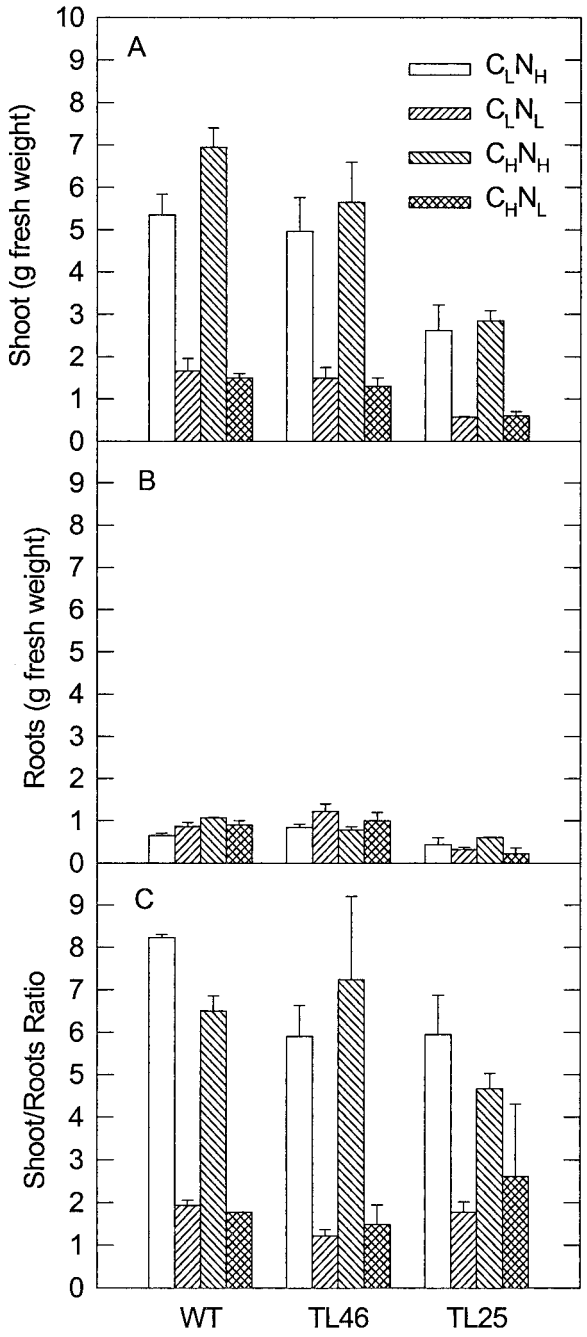

Figure 2. Growth of shoots and roots of wt and starch mutants of Arabidopsis under different CO_2 and N treatments on a fresh weight basis. Analyses were made after 35 d (±2 d) of growth. See Figure 1 for growth conditions.

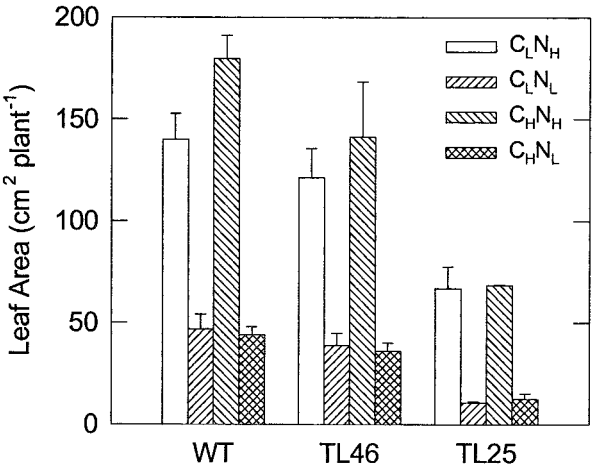

Figure 3. Cumulative leaf area of wt and starch mutants of Arabidopsis under different CO_2 and N treatments. Analyses were made after 35 d (±2 d) of growth. See Figure 1 for growth conditions.

exception of C_HN_L. In C_HN_L-treated TL46 plants, much of the starch remained at the end of the night period due to substantially reduced turnover. In TL25, starch content was very low under all conditions. Under N_H, the cumulative leaf area per plant increased with increasing synthesis and turnover of starch across genotypes and CO_2 levels. Under N_L, cumulative leaf area was smaller when synthesis and turnover of starch was negligible (in TL25 mutant), whereas there was little or no difference with increasing starch turnover (Fig. 5).

The level of Suc in leaves at the end of the day was very similar in all treatments in wt and TL46 plants (Fig. 6). At the end of the dark period, the level of Suc in wt and TL46 plants was higher in C_LN_L plants than in C_LN_H plants, whereas the highest levels of Suc in these genotypes at the end of the dark period was in the C_HN_L plants. In TL25 plants, the level of Suc in C_LN_H plants at the end of the day was similar to that in wt and TL46 plants. However, in the other treatments of TL25 plants, there were large increases in Suc levels during the day. Under C_HN_L and C_LN_L treatments, there was substantial Suc remaining in the leaves at the end of the dark period, whereas under C_HN_H treatment, Suc levels decrease to levels similar to that evident in wt and TL46.

In wt and TL46 plants, the highest levels of Glc and Fru occurred in C_HN_L plants, with levels being similar at the end of the light and dark periods. This coincides with the highest level of starch accumulation during the day and retention during the night. At the end of the light period, the levels of Glc and Fru in wt and TL46 were also higher under the C_LN_L treatment than in the N_H treatments. Thus, N_L results in an increase in these soluble sugars.

In TL25 plants, the levels of Glc and Fru at the end of the light period were considerably higher than in wt and TL46 plants across all C-N treatments. At the end of the dark period, high levels of Glc and Fru remained in TL25 in the N_L treatments, with the highest in C_HN_L, whereas in the N_H treatments, the levels of these sugars were low and similar to that in wt and TL46 plants.

Rubisco

The effects of growth under N_H, N_L, C_H, and C_L on Rubisco activity and content and total soluble protein per unit leaf area were determined (Figs. 7 and 8). For each genotype, the initial extractable activity of Rubisco (which is dependent on the amount of

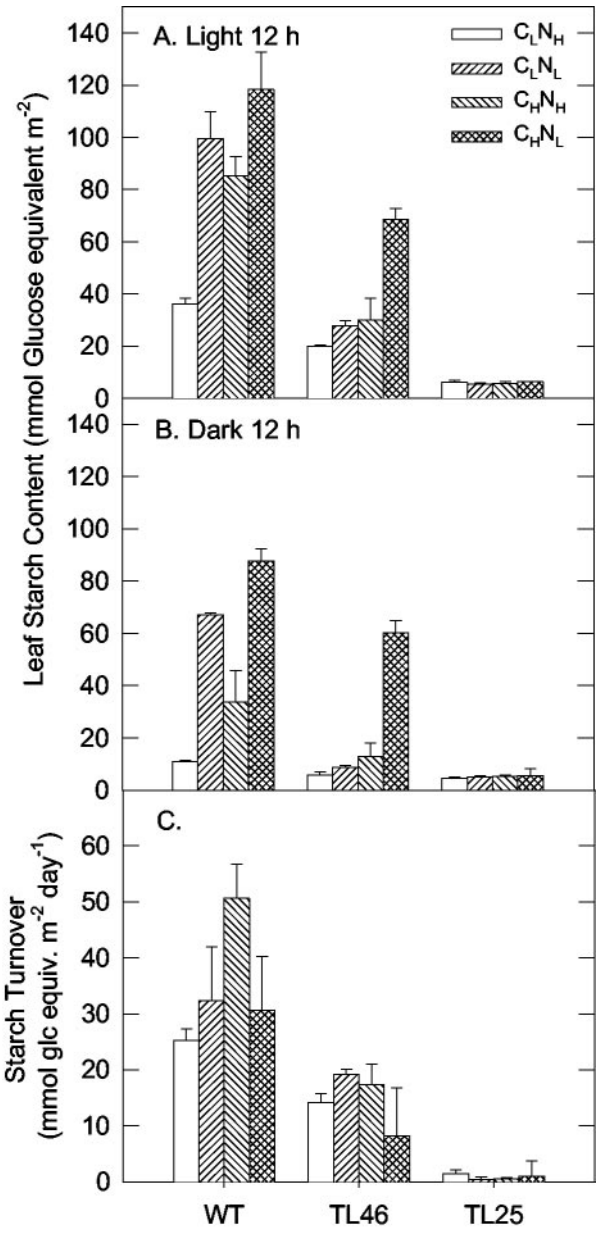

Figure 4. Leaf starch content at the end of the light period (A, 12 h of light) and the end of the dark period (B, 12 h of dark) and diurnal starch turnover (C) in wt and starch mutants of Arabidopsis under different CO_2 and nitrogen treatments. See Figure 1 for growth conditions.

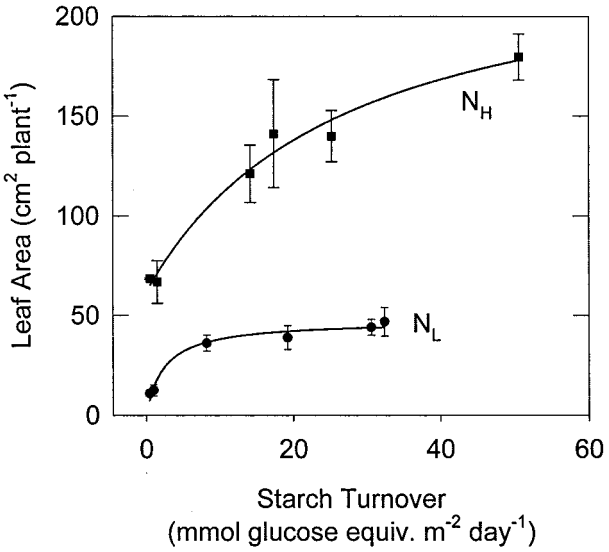

Figure 5. Correlation of cumulative leaf area with starch turnover in plants grown with N_L and N_H in wt and starch mutants of Arabidopsis. See Figure 1 for growth conditions.

Rubisco and its state of activation) showed a general pattern, with decreasing activity in the following order: C_LN_H and C_LN_L being the highest, followed by C_HN_H, and C_HN_L. For each C-N treatment, the mutant TL46 and TL25 plants had lower initial extractable activities than the wt.

Also, for each genotype there was a similar pattern in change in total Rubisco activity (the maximum activity after activation in vitro) and Rubisco protein per unit leaf area (the correlation coefficient across all treatments was R = 0.88**; results not shown). For each genotype, the highest total activity and Rubisco protein was in C_LN_H, plants; the C_LN_L and C_HN_H plants had a similar reduction in total activity and Rubisco protein, whereas the C_HN_L plants had the largest reduction. Although the initial Rubisco activity was lower in mutants than in wt plants (Fig. 7A), the three genotypes were very similar in total activity (Fig. 7B) and content of Rubisco (Fig. 8A) in response to the CO_2 and N treatments, except for C_HN_L. In the C_HN_L treatment, it was apparent that the total activity and Rubisco content progressively decreased from wt to TL46 to TL25. The calculated in vivo state of activation of Rubisco (initial/total × 100) indicates the initial state of activation for each genotype was lowest in the high CO_2-grown plants. Also, the results showed a pattern of declining state of activation from wt to TL46 to TL25 for a given C-N treatment.

The changes in Rubisco protein content for the C-N treatments and the total soluble protein showed a very similar relationship (Fig. 8, A and B). For each genotype, the C_LN_H plants had the highest Rubisco and highest soluble protein, and the C_HN_L plants had the lowest Rubisco and soluble protein. The amount of leaf soluble protein excluding that in Rubisco was calculated (Fig. 8C) to show how the remaining pool of soluble proteins change per leaf area under the various treatments. In wt and TL46, there was little effect of C-N treatments on the remaining total soluble proteins. With the exception of the C_HN_L treatment, the remaining soluble proteins tended to be higher in TL25 than in wt or TL46.

Rubisco total activities as well as Rubisco protein were correlated with leaf Glc concentration (Fig. 9). Rubisco content and activity decreased with increas-

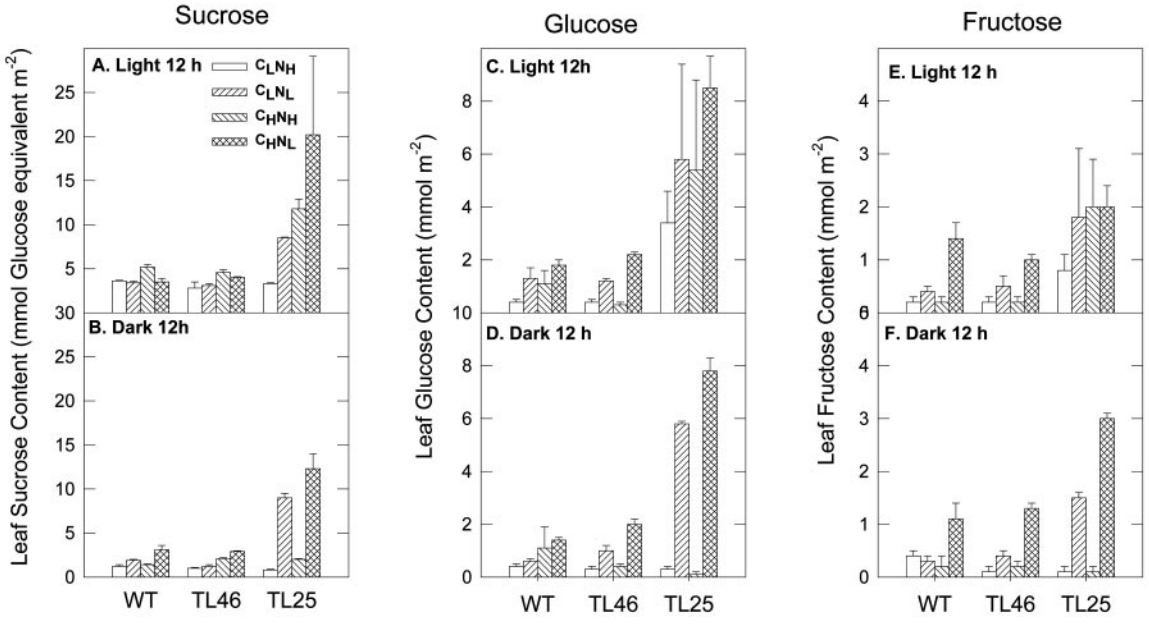

Figure 6. Leaf soluble carbohydrates, Suc, Glc, and Fru at the end of the light period (12 h of light) and the end of the dark period (12 h of dark) in wt and starch mutants of Arabidopsis under different CO_2 and N treatments. See Figure 1 for growth conditions.

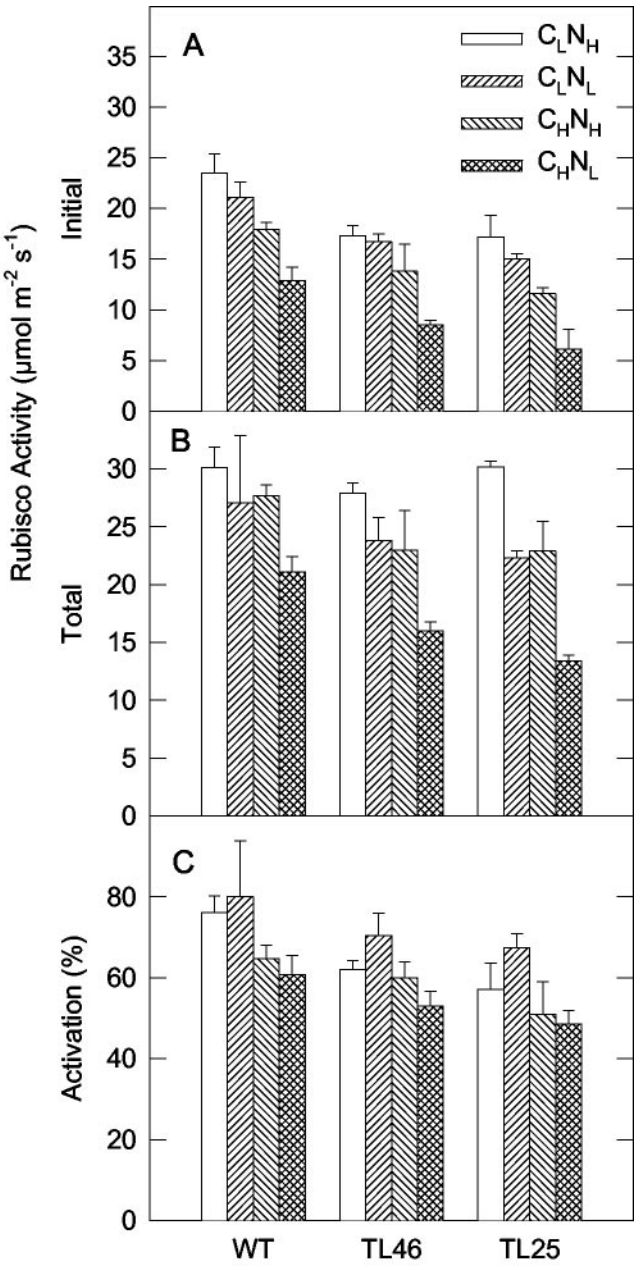

Figure 7. Initial and total extractable activity of Rubisco, and percentage of activation, of wt and starch mutants of Arabidopsis under different CO_2 and N treatments. Analyses were made after 35 d (±2 d) of growth. See Figure 1 for growth conditions.

ing levels of Glc in all three genotypes. There was a large shift in the response in TL25, with decreasing Rubisco occurring at higher Glc levels (Fig. 9). Plots of Fru and Suc versus Rubisco showed a less significant correlation (see legend to Fig. 9).

DISCUSSION

Growth and Source versus Sink Limitation

With growth of wt plants under N_L nutrition, the N content of the tissue decreased, resulting in an increase in the C/N ratio. There was a large reduction in shoot growth (decreased shoot/root ratio) and leaf area under N_L nutrition, indicating N supply was limiting for growth (Paul and Stitt, 1993). With CO_2 enrichment under N_H in wt plants, there was an enhancement of growth (top fresh weight and leaf area per plant), whereas with CO_2 enrichment under N_L, there was no enhancement of growth. Similar results have been found in several other species (Bowler and Press, 1996; Rogers et al., 1996a, 1996b; Ziska et al., 1996; Geiger et al., 1999). Hence, when N

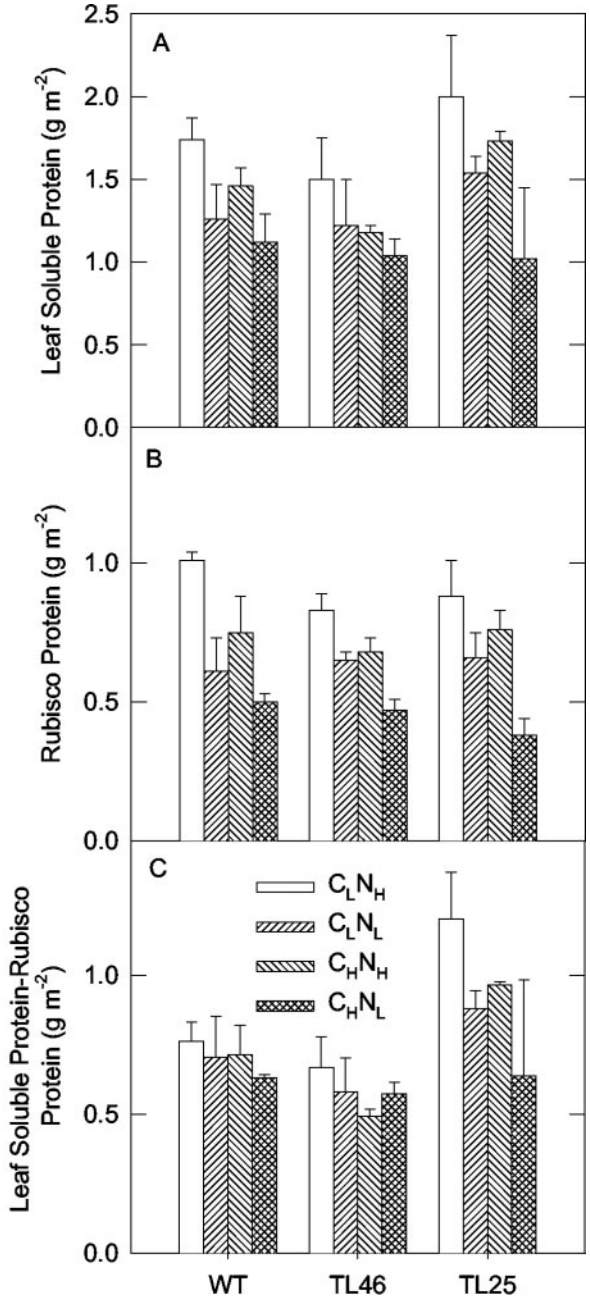

Figure 8. Rubisco content and total soluble protein on leaf area basis of wt and starch mutants of Arabidopsis under different CO_2 and N treatments. See Figure 1 for growth conditions.

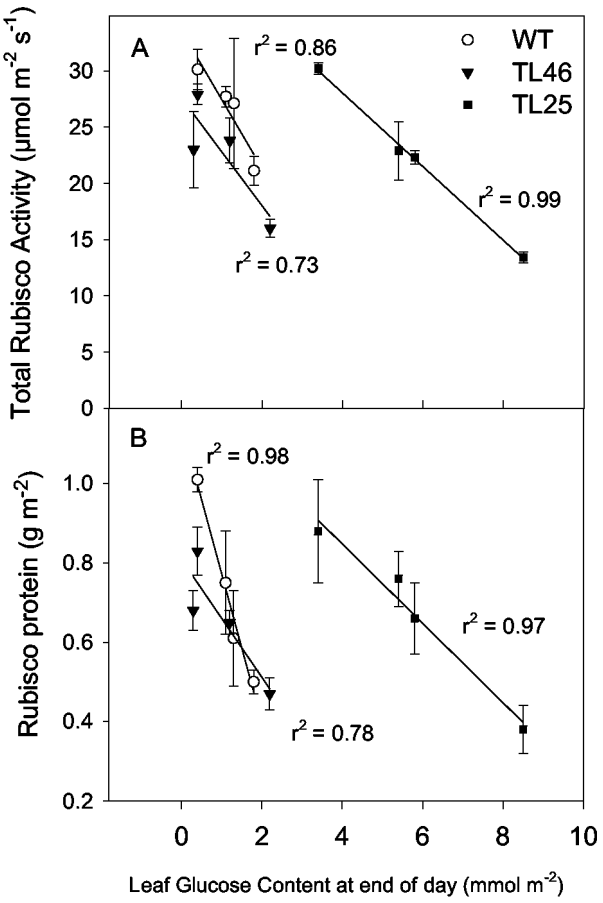

Figure 9. The relationship between Rubisco total activity (A) and Rubisco protein (B) and leaf Glc concentration. For each genotype, the four data points are values under the two different CO_2 and nitrogen regimes. r^2 is the correlation of the different Rubisco factors with Glc concentration. r^2 values for Rubisco versus Suc and Fru; total activity versus Suc: wt $r^2 = 0.06$, TL46 $r^2 = 0.33$, TL25 $r^2 = 0.95$. Rubisco protein versus Suc: wt $r^2 = 0.03$, TL46 $r^2 = 0.26$, TL25 $r^2 = 0.85$. Total activity versus Fru: wt $r^2 = 0.93$, TL46 $r^2 = 0.8$, Tl25 $r^2 = 0.66$. Rubisco protein versus Fru: wt $r^2 = 0.55$, TL46 $r^2 = 0.83$, TL25 $r^2 = 0.46$.

is limiting, it has a more dominant role than photosynthetic capacity in affecting plant growth and development.

With the growth of starch mutants under N_H nutrition, the C/N ratio of plant tissue was very similar to that of wt plants. Also, as in wt plants, with growth under N_L, the N content of the tissue decreased, resulting in an increase in the C/N ratio. It was evident for both mutants, as in wt, that N was limiting for growth in the N_L treatment from measurements of fresh weights of shoots and roots, and total leaf area per plant. Leaf expansion is decreased when nitrate becomes limiting, possibly through interactions with a nitrate-cytokinin signaling pathway (Forde, 2002).

In comparisons of growth of the three genotypes based on fresh weight and leaf area measurements, it is clear that in each C-N treatment there is a strong reduction in growth of TL25 plants compared with that of wt and TL46. Wt and TL46 plants had similar growth, but in general, growth of TL46 plants was lower. The differences in growth between genotypes may be a function of the photoperiod; with a 12-h dark period, starch reserves are important for growth because when starch mutants were grown under continuous light, differences were not evident (Caspar et al., 1985).

The highest growth occurred in wt plants in the C_HN_H treatment. TL46 plants also had high growth in the C_HN_H treatment, although not significantly higher than in C_LN_H. Although wt plants produced more starch during the day and used more at night (Fig. 4), TL46 plants might partly compensate by increasing partitioning into Suc during the day (Sun et al., 1999). The results suggest in the C_HN_H treatment, growth is not limiting in wt by supply of photosynthate as there is substantial starch remaining at the end of the dark period. In TL25 plants, growth in the C_HN_H treatment was only about 40% to 50% of that in wt and TL46 plants, which indicates that the inability of TL25 to make starch as a carbon reserve, for use in the dark is limiting growth. Although TL25 plants accumulate substantial Suc, Glc, and some Fru in the light period, this is not sufficient to compensate for its inability to synthesize starch as a dark period carbon reserve. In a previous study, it was shown in TL25 plants grown under normal CO_2 that there is only a partial compensation during photosynthesis with exposure to high levels of $^{14}CO_2$ for loss of capacity for starch synthesis by increased Suc synthesis in TL25 plants (Sun et al., 1999). Also, some of the sugars produced in the light during photosynthesis in TL25 plants may be lost by respiration in the dark period and thus be unavailable to support growth because an increase in respiration correlates with increased carbohydrate, and therefore respiratory substrates (Wullschleger et al., 1994). An increase in respiration was also found to contribute to decreased growth in other starchless Arabidopsis mutants (Caspar et al., 1985; Schulze and Schulze, 1994).

Wt plants in the C_LN_H treatment had lower growth than in the C_HN_H treatment. This may occur by higher Suc production during the day, and increased starch use at night in the C_HN_H- compared with C_LN_H-grown plants (55 versus 25 mmol Glc equivalents m^{-2} used in the dark, respectively; Fig. 4). This suggests the capacity for production of photosynthate is limiting growth in C_LN_H plants. On the other hand, C_HN_H plants are producing more starch than they can use at night, indicating some limitation on sink capacity. In TL46 plants, there was no significant difference in growth under C_LN_H and C_HN_H conditions; that could be due to limited capacity for production of starch, and a similar degree of starch use in the dark period (Fig. 4). Under C_LN_H growth, the level of Suc at the end of the light period in TL25

plants was similar to that in wt and TL46 plants, whereas the level of Glc and Fru was higher (Fig. 6). This suggests there is some excess production of Suc in the light, which is not exported, but is converted to Fru and Glc by hydrolysis in leaves.

In wt plants under the C_LN_L treatment there is a large reduction in growth due to limited N compared with the C_LN_H treatment. This results in a large accumulation of starch during the light period, although the amount of starch used at night (33 mmol Glc equivalents m^{-2}) is slightly higher than in C_LN_H-grown plants. The large accumulation of starch during the day in C_LN_L plants indicates there is limited capacity to use Suc due to N limitation for synthesis of amino acids and development of sinks to use carbohydrate. Therefore, the capacity for photosynthesis and carbohydrate synthesis is greater than sink capacity. In the C_LN_L treatment, TL46 was slightly more restricted in growth than the wt. In contrast to the wt, TL46 showed only a moderate increase in starch levels under C_LN_L. However, this level was sufficient to sustain growth nearly to the level of the wt under the limiting N treatment. In the C_LN_L treatment, the TL46 and TL25 plants had high levels of Glc and Fru at the end of the light and dark periods, whereas in wt, there was less accumulation. This suggests that under limiting N, some of the Suc may be hydrolyzed to Glc and Fru, or starch may be converted to Glc and not exported from the leaf. In the TL25 plants, the growth under C_LN_L was much lower than in wt and TL46 plants. This indicates that even when N is limiting, the inability to synthesize starch can limit growth.

In the C_HN_L treatments, the growth of wt and TL46 plants was strongly suppressed, similar to that in C_LN_L-grown plants, again due to the N limitation. This resulted in a large accumulation of starch during the light, and a substantial retention of starch at the end of the dark period. In wt, there is greater accumulation of starch under conditions of N_L in both CO_2 treatments. Leaves have an increased capacity for starch synthesis when N is low, possibly through increased *agpS* transcript expression, which encodes the regulatory subunit of ADP-Glc pyrophosphorylase (AGPase; Scheible et al., 1997a; Geiger et al., 1999) and allosteric activation of AGPase catalytic activity. In all genotypes, and especially TL25, the levels of Glc and Fru were high at the end of the light and dark period. Therefore, the inability of TL25 plants to produce starch when N is low results in elevated levels of soluble sugars and limited growth, which indicates that N is limiting for synthesis of amino acids and sink development. However, in wt, increased starch production through increased AGPase activity may reduce the accumulation of soluble sugars, thereby limiting feedback effects on photosynthesis. Lack of sink demand contributed to the reduced growth in a starch-deficient Arabidopsis mutant at the rosette stage because relative growth rate increased during flowering when sink strength was increased (Schulze et al., 1994). Therefore, the effect of a lack of sinks in TL25 plants may be exacerbated under limiting N supply. In plants grown under limiting N manipulated to have a decreased source:sink ratio through partial removal of the source leaves in ryegrass (Rogers et al., 1998) or shading in tobacco (Paul and Driscoll, 1997), there was no accumulation of carbohydrates, and down-regulation of photosynthesis was absent. Therefore, sink capacity plays a major role in the acclimation to N_L (Paul and Driscoll, 1997) as well as elevated CO_2 (Rogers et al., 1998).

When plants have sufficient N available, the capacity of sinks to use carbohydrates is increased. In N_H-grown Arabidopsis, the correlation between cumulative leaf area and production and use of starch across genotypes and CO_2 levels indicates the importance of starch reserves in the dark period to plant growth. Unexpectedly, under N limited growth, cumulative leaf area also increased with increased rate of use of starch, even though starch mutants had increased soluble sugars that were not fully used in the dark period. Excess sugars may lead to excess respiration and loss of CO_2, storage of hexoses in compartments where they are not available for metabolism (e.g. in vacuoles or the apoplastic space), and sugar signaling that down-regulates the capacity for photosynthesis. An increase in sugars in the apoplastic space could also cause a loss of turgor and limits on cell/leaf expansion.

Feedback Regulation of Rubisco

In each genotype, there was a progressive decrease in the initial extractable Rubisco activity on a leaf area basis under growth conditions from C_LN_H to C_LN_L to C_HN_H to C_HN_L. Also, for each CO_2/N treatment, the initial extractable activity of Rubisco was higher in the wt than in the starch mutants. This suggests feedback regulation of Rubisco due to limitations on sink capacity. The N_L treatments will limit development of sinks due to limiting supply of amino acids. The starch mutants are limited in capacity for synthesis of carbohydrates. Under N_L supply, elevated CO_2 often leads to a decrease in Rubisco activity as seen in pea (Riviere-Rolland et al., 1996) and tobacco (Geiger et al., 1999), although this may depend on the level of N supplied (Riviere-Rolland et al., 1996).

What is particularly interesting is that even under N_L, which limits growth, the starch mutants have lower initial extractable activity of Rubisco and lower growth than wt. This suggests that synthesis of starch is important for growth even when N is limiting. One reason for this may be that the starch mutants have increased synthesis of soluble sugars that are not as effectively used for growth (e.g. stored in vacuoles, apoplastic space, or respired) as starch as noted ear-

lier. In an alternate manner, down-regulation of Rubisco in the starch mutants may be mediated by increased soluble sugars, resulting in limited capacity for photosynthesis compared with the wt. There are two ways to account for lower initial extractable activity of Rubisco: control of Rubisco synthesis and control of Rubisco state of activation. The results indicate both contribute to the lower initial extractable activity.

Rubisco total activity and content decreased with increasing Glc levels, and this occurred to a greater degree in TL25 where Glc levels were higher due to limited ability to synthesize starch. Because the decrease in Rubisco protein was much greater than the decrease in other soluble protein, it indicates some selectivity in the down-regulation of Rubisco. A selective decrease in Rubisco relative to other proteins under N_L in elevated CO_2 has been reported in spinach (*Spinacia oleracea*; Evans and Terashima, 1988) and bean (*Phaseolus vulgaris*; Nakano et al., 1998) where other photosynthetic proteins remained constant. This is also supported by Cheng et al. (1998) who found a decrease in Rubisco transcripts in Arabidopsis with elevated CO_2. The results are consistent with the proposed sugar-mediated repression of photosynthetic genes due to increased hexose metabolism (Graham et al., 1994; Jang and Sheen, 1994; Cheng et al., 1998). The down-regulation of Rubisco is more pronounced in N_L, when Glc levels were at their highest. Also, sugar-mediated down-regulation of photosynthesis is particularly effective at N_L supply (Nielsen et al., 1998). Rubisco may be used as an N store and mobilized, as a result of sugar repression, when N becomes limiting (Paul and Stitt, 1993). Because there is an interaction between N and sugar signaling, the increased C:N ratio in N_L (Fig. 1C) may contribute to triggering the sugar-mediated gene repression (Lam et al., 1994; Paul and Driscoll, 1997). The relationship between leaf sugars and Rubisco activity will be influenced not only by compartmentation of sugars in the leaf, but by interaction with other metabolites involved in gene regulation. For example, in antisense potato plants with decreased capacity for starch production, there was an increase in hexoses in elevated CO_2. However, this did not result in an inhibition of Rubisco activity or *rbcS* transcripts, although photosynthesis was decreased. The decreased photosynthetic rate was not due to sugar repression, rather it was limited by end-product synthesis and triose-P use (Ludewig et al., 1998).

When photosynthesis is limited by sink capacity, and if down-regulation of Rubisco synthesis is insufficient to balance the capacity of the source with sink, then further regulation may occur through feedback and decreased state of activation of Rubisco. The state of activation of Rubisco was lower in the starch mutants than wt plants, and the lowest states of activation occurred under CO_2 enrichment. Limited capacity for synthesis of starch (starch mutants) or limited capacity to use Suc (under N deficiency) can result in accumulation of organic phosphates, reduction in P_i and synthesis of ATP in the chloroplast, and decreased state of activation of Rubisco, which is dependent on ATP (Sharkey, 1990).

In summary, this study on Arabidopsis indicates that when synthesis of starch is limiting with an adequate supply of N, growth is limited due to insufficient carbohydrate reserves during the dark period (also supported by Sun et al., 1999). When synthesis of starch is restricted under conditions where N supply is limited, the large increase in soluble sugars apparently accentuates the feedback and down-regulation of Rubisco, resulting in greater reduction of growth.

MATERIALS AND METHODS

Plant Growth

Arabidopsis cv Columbia wt, starch-deficient TL46 (10%–40% starch of the wt compared on a w/v basis), and near starchless TL25 (Lin et al., 1988b, 1988a) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). TL46 contains a missense mutation of *adg2* gene that codes the large subunit structural gene of AGPase (Wang et al., 1997). TL25 contains a mutation of the *adg1* gene that codes the small subunit structural gene of AGPase (Lin et al., 1988b).

Plants were grown in controlled environmental growth chambers with a 12-h photoperiod and photosynthetic photon flux density of 300 μmol m^{-2} s^{-1} provided by metal halide lamps. Day and night temperatures were 24°C ± 1°C and 18°C ± 1°C, respectively. Relative humidity in the growth chambers was 70%. Plants were cultured hydroponically in modified Hoagland solution (Hoagland and Arnon, 1950). Hydroponic culture was adapted from the previous reports (Sun et al., 1996; Gibeaut et al., 1997). Polyethylene boxes (32L; Rubbermaid, Wooster, OH) were used, and a sheet of Plexiglas was placed on top of the cover. Holes (2.8 cm) were cut through the Plexiglas and the cover to hold 35 to 59 No. 6 rubber stoppers (depending on the plant size). A 1.3-cm hole was drilled in the center of each plug to accept rockwool cylinders (1.5 × 4 cm), which was used for supporting the seedlings with one plant per hole. Seeds were placed on rockwool for germination.

The hydroponic solution consisted of one-quarter-strength Hoagland macronutrients, full-strength Hoagland micronutrients, and various nitrate levels (0.625 mM K_2SO_4, 0.5 mM $MgSO_4$, 0.25 mM KH_2PO_4, 3 mM Ca, 20 μM Fe-EDTA, 35 μM 330 Fe [Sequestrene 330; Ciba-Geigy, Greensboro, NC], 46 μM H_3BO_3, 9 μM $MnCl_2$, 0.76 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 0.12 μM $NaMoO_4$, and various nitrates).

Plants were first established by growth under normal atmospheric levels of CO_2 and medium levels of nitrate (3 mM) for 3 weeks (i.e. before the rapid phase of leaf expansion). The plants were then grown under two different levels of nitrate, at 6 mM, designated as N_H, and at 0.2 mM, designated as N_L (plus another 0.1 mM every week), and two different levels of CO_2, 100 Pa (C_H) versus 35 Pa (C_L) for 16 ± 2 d.

Leaf Area and Fresh Weight

Leaf areas were determined with a leaf area meter (Li-3000; LI-COR, Lincoln, NE). Leaf area and fresh weight measurements were taken at 35 ± 2 d after germination.

Nitrogen Analysis

Three plants from each treatment were dried, pooled, and ground to a powder. The samples were combusted, and C was measured by infrared absorption and N was determined by thermal conductivity (LECO CNS 2000; LECO, St. Joseph, MI).

Starch, Suc, and Hexoses (Glc and Fru) Determination

Leaf starch, Suc, and hexoses were extracted and determined as previously described (Angelov et al., 1993). Leaf discs (2 × 0.33 cm^2), acquired using a paper punch, were extracted with 80% (v/v) ethanol several times until the extract was colorless. The ethanol soluble fractions from each sample were pooled, dried at 55°C under vacuum (speed-vac, Savant, Farmingdale, NY), resolubilized in 0.5 mL of distilled water, and frozen (−20°C) until analyzed for sugars. The leaf residue was briefly air dried and was then homogenized in 0.2 mL of 0.5 M KOH. The homogenate was then boiled for 30 min, and the pH was adjusted to approximately 5.5 by the addition of 0.2 mL of 1 M acetic acid. Amyloglucosidase (Sigma, St. Louis), which was used to digest starch, was dissolved in 50 mM MOPS, pH 7.5, centrifuged to remove starch, and desalted to remove sugar. To convert starch to Glc, samples were incubated with amyloglucosidase (10 units in a sample volume of 0.4 mL) at 55°C for 2 h (preliminary tests showed no additional sugars were released beyond 2 h). Free sugars were determined spectrophotometrically in each extract by the coupled enzyme methods as previously described (Angelov et al., 1993; Winder et al., 1998).

Rubisco Enzyme Extraction and Assay

Arabidopsis leaves were collected about 2 h into the light period and were stored in liquid nitrogen until analysis. The leaves were extracted and analyzed the same day as sampled. Protein content was determined using the Bradford procedure with bovine serum albumin as the standard (Bradford, 1976).

Rubisco Activity

Two leaf discs (0.3 cm^2) were acquired using a paper punch that was precooled in liquid nitrogen and they were homogenized in 200 μL of solution containing 100 mM Bicine, pH 8.0, 15 mM $MgCl_2$, 0.5 mM EDTA-Na_2, 0.01% (v/v) Triton, and 5 mM dithiothreitol. The homogenate was centrifuged in a microcentrifuge (model 235; Fisher Scientific, Pittsburgh) at maximum speed (approximately 12,000*g*) for 1 min at 4°C. Ten microliters of the supernatant was assayed in a reaction mixture (final volume of 100 μL) containing 70 mM Bicine, pH 8.0, 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 20 mM $NaH^{14}CO_3$ (1 Ci mol^{-1}), and 1 mM RuBP at 25°C. To determine initial activity, the enzyme was added to the above mixture. To determine total activatable activity, the enzyme was incubated in the above mixture for 5 min in the absence of RuBP, and then the reaction was initiated by addition of RuBP. After incubating at 25°C for 1 min, the reaction was stopped by addition of 30% (v/v) acetic acid. The mixture was dried at 55°C and then 100 μL of distilled water was added to dissolve the sample. Ten milliliters of scintillation cocktail was then added and the radioactivity was determined by a liquid scintillation counter (LS7000; Beckman, Fullerton, CA).

Rubisco Protein Determination

The crude extract (see Rubisco activity assay above) was incubated in presence of 20 mM $NaHCO_3$ for 10 min at room temperature. Then ^{14}C-CABP (specific activity 94 dpm $pmol^{-1}$, made from reaction of ^{14}C-KCN and RuBP; Collatz et al., 1979) was added to the mixture and was incubated for 45 min at room temperature. The proteins were then precipitated in the presence of 20% (w/v) polyethylene glycol 4000 (in 100 mM Bicine, pH 8.0, and 25 mM $MgCl_2$), incubated for 10 min at room temperature, and then centrifuged for 5 min at 15,000*g*. The pellet was washed once with 20% (w/v) polyethylene glycol 4000 containing 20 mM $MgCl_2$. The pellet was resolved in a solution containing 100 mM Bicine, pH 8.0, and 10 mM $MgCl_2$. Ten milliliters of scintillation cocktail was then added and the radioactivity was determined by a liquid scintillation counter (LS7000; Beckman).

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