Effects of Ambient CO$_2$ Concentration on Growth and Nitrogen Use in Tobacco (Nicotiana tabacum) Plants Transformed with an Antisense Gene to the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase

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Growth of the R$_1$ progeny of a tobacco plant (Nicotiana tabacum) transformed with an antisense gene to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was analyzed under 330 and 930 μbar of CO$_2$, at an irradiance of 1000 μmol quanta m$^{-2}$ s$^{-1}$. Rubisco activity was reduced to 30 to 50% and 13 to 18% of that in the wild type when one and two copies of the antisense gene, respectively, were present in the genome, whereas null plants and wild-type plants had similar phenotypes. At 330 μbar of CO$_2$ all antisense plants were smaller than the wild type. There was no indication that Rubisco is present in excess in the wild type with respect to growth under high light. Raising ambient CO$_2$ pressure to 930 μbar caused plants with one copy of the DNA transferred from plasmid to plant genome to achieve the same size as the wild type at 330 μbar, but plants with two copies remained smaller. Differences in final size were due mostly to early differences in relative rate of leaf area expansion (m$^2$ m$^{-2}$ d$^{-1}$) or of biomass accumulation (g g$^{-1}$ d$^{-1}$): within less than 2 weeks after germination relative growth rates reached a steady-state value similar for all plants. Plants with greater carboxylation rates were characterized by a higher ratio of leaf carbon to leaf area, and at later stages, they were characterized also by a relatively greater allocation of structural and nonstructural carbon to roots versus leaves. However, these changes per se did not appear to be causing the long-term insensitivity of relative growth rates to variations in carboxylation rate. Nor was this insensitivity due to feedback inhibition of photosynthesis in leaves grown at high partial pressure of CO$_2$ in the air ($p_a$) or with high Rubisco activity, even when the amount of starch approached 40% of leaf dry weight. We propose that other intrinsic rate-limiting processes that are independent of carbohydrate supply were involved. Under plentiful nitrogen supply, reduction in the amount of nitrogen invested in Rubisco was more than compensated for by an increase in leaf nitrate. Nitrogen content of organic matter, excluding Rubisco, was unaffected by the antisense gene. In contrast, it was systematically lower at elevated $p_a$ than at normal $p_a$. Combined with the positive effects of $p_a$ on growth, this resulted in the single-dose antisense plants growing as fast at 930 μbar of CO$_2$ as the wild-type plants at 330 μbar of CO$_2$ but at a lower organic nitrogen cost.

Photosynthetic CO$_2$ fixation provides the organic substrate for plant growth, and many workers in the photosynthetic field have taken the view that improvements in the photosynthetic performance of a plant will inevitably lead to an increase in growth rate and biomass. Despite this reasonable premise, the relationships between photosynthetic rate, both on a whole plant and leaf area basis, and plant growth have not been clear. In fact, a causal, direct link between genetic improvement of photosynthesis by classical breeding and improvement of yield has proven difficult to demonstrate unequivocally (Evans, 1992). However, a clear example of how improved photosynthetic properties may influence the performance of one species can be found when plants are grown at elevated CO$_2$. In many ways, this treatment is equivalent to equipping the plant with an improved Rubisco activity, conferring lower photorespiration and a higher CO$_2$ fixation rate per unit leaf area. With C$_3$ plants, increasing $p_a$ above current ambient levels generally leads to greater biomass accumulation at the whole plant or canopy level (Cure and Acock, 1986; Poorter, 1993; Rogers and Dahlman, 1993). Although these results can be used to infer a positive relationship between photosynthetic rate and growth performance, a closer examination of data indicates that improved carboxylation rate induced by increased $p_a$ usually does not translate into a steady proportional increase in relative growth rate, especially in the long term (scale of weeks) (Badger, 1992; Wong et al., 1992).

The relationships between the properties of photosynthesis and plant growth need to be investigated in more detail if we are to define steps to improve growth performance in the

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Abbreviations: a, leaf area; A, CO$_2$ assimilation rate per unit leaf area; $A_w$, CO$_2$ assimilation rate on a leaf mass basis; $A'$, CO$_2$ assimilation rate averaged over all leaves and the whole photoperiod; $A''$, whole plant net CO$_2$ assimilation rate; CABP, 2'-carboxy-D-arabinitol-1,5-bisphosphate; $g_c$, internal conductance to CO$_2$; $\phi$, proportion of $A$ respired by the shoot at night and by the roots day and night; $p_{aw}, p_a, p_r$, partial pressure of CO$_2$ in the air, the intercellular spaces, and the chloroplasts, respectively; $r_a$ and $r_w$, relative rate of leaf area expansion and of biomass accumulation, respectively; ribS, gene encoding the small subunit of Rubisco; $p$ and $p_t$, ratio of the carbon mass invested in the whole plant or in the leaves, respectively, to the leaf area; RubBP, D-ribulose-1,5-bisphosphate; T-DNA, DNA transferred from plasmid to plant genome; $W, W_t,$ and $W_w$, whole plant, shoot, and root mass, respectively.
future. The engineering of plants with altered photosynthetic enzymes provides a powerful means to do this. In particular, the engineering of transgenic tobacco (Nicotiana tabacum) plants with reduced Rubisco activities, using antisense RNA to the Rubisco small subunit gene (Rodermel et al., 1988; Hudson et al., 1992), presents a unique opportunity to examine the limitation imposed by Rubisco itself on photosynthetic rate and thereby on growth, independently of variation in other photosynthetic attributes normally associated with it, such as electron transport capacity and stomatal conductance (Hudson et al., 1992). Under low light (340 μmol quanta m⁻² s⁻¹) and moderate temperature (20°C) photosynthesis was only marginally reduced until Rubisco activity was decreased to nearly half that present in the wild type (Quick et al., 1991b). Plant size at the end of the 7- to 10-week experiment was also significantly affected, causing Quick et al. (1991a) to conclude that the plant “overinvests” about 15% more protein in Rubisco than is needed. However, when measured under higher light conditions (1050 μmol quanta m⁻² s⁻¹) photosynthesis was limited by Rubisco activity in all antisense plants (Stitt et al., 1991). Hudson et al. (1992) reported the same result with another set of similar antisense plants grown and analyzed at high light.

Our aim was to analyze the limitation to growth imposed by Rubisco level and photosynthetic rate and to assess the extent to which this limitation may be offset by increasing the external carbon supply through an increase in pₚ. One particular emphasis was on the dynamics of growth and carbon partitioning, starting as early in the life of the plant as growth parameters could be accurately assessed. In the context of increasing CO₂ levels, a subsidiary question was whether the engineering of plants with reduced Rubisco content may be a way of improving nitrogen-use efficiency while maintaining photosynthetic and growth performance.

**MATERIALS AND METHODS**

**Plant Material**

We used the R₁ selfed progeny of tobacco (Nicotiana tabacum var W38) S₇ transformant described by Hudson et al. (1992) which, compared to the wild type, showed an 80% reduction in the A at 350 μbar of CO₂, 1000 μmol quanta m⁻² s⁻¹, 25°C, and 11 mbars of leaf-to-air vapor pressure difference, whereas stomatal conductance and electron transport capacity were not significantly affected. Primary transformants were not used directly for several reasons. First, we wanted to avoid the complications introduced by possible somatic variation during tissue culture and by after-effects of the period in tissue culture. Second, although some growth parameters may be measured in situ (see below), others, like the rate of dry-matter accumulation, require destruction of part or all of the plant. In this latter case, relationships between growth performance and Rubisco content are necessarily statistical relationships, which implies that several plants of the same genotype, defined in this case by the number of copies of the antisense gene that are present in the genome and their position, are needed to provide replicates. Third, several plants of different genotypes but originating from seeds with the same history, as is provided by a segregating progeny, were necessary to unambiguously separate the effects of genetic versus nongenetic variation in Rubisco content.

**Genetic Characterization of the Primary Transformant and R₁ Progeny**

**Molecular Analysis**

The primary transformant was created by transformation with a T-DNA containing a kanamycin-resistance gene and the antisense RNA gene (Hudson et al. 1992). The number of T-DNA inserts present in the genome was assayed by an inverse polymerase chain reaction protocol (Does et al., 1991). Only one T-DNA right border could be detected in the genome of the primary transformant (data not shown).

**Inheritance of the Antisense T-DNA**

The inheritance of the kanamycin-resistance gene linked to the antisense gene was assayed by germinating 100 surface-sterilized R₄ seeds on Murashige and Skoog agar plates containing 100 μg mL⁻¹ of kanamycin. All seeds germinated; 77% of the seedlings grew, and 23% bleached and died. This is not significantly different (using a χ² test) from a 3:1 ratio of resistant to sensitive seedlings, corresponding to the expected mendelian segregation ratio of a single gene, and allowed us to infer that a single, segregating, antisense gene was also present in the genome of transformant S₇.

Our study required the genotype of each single R₁ plant used for physiological analysis to be unambiguously characterized genetically as homozygous, heterozygous, or null (wild type) with respect to the rbcS antisense gene. Because the two first categories cannot be readily distinguished by DNA blotting or the polymerase chain reaction, a preliminary experiment was conducted to assess whether this could be achieved indirectly by scoring differences in phenotype. About 30 wild-type seeds and 300 R₁ seeds were germinated on a 2:1:1 sand:perlite:peat moss mixture in a growth chamber at a day/night temperature of 26/20°C, 5 mbar of air vapor pressure deficit, low light (80 μmol quanta m⁻² s⁻¹), and a 12-h thermo- and photoperiod. As soon as the first pair of leaves started to expand (total leaf area 1 cm²) there were obvious visual differences in seedling size and color. Eighty R₁ seedlings and 15 wild-type ones were chosen at random and transplanted into pots containing the same mixture as above and acclimated over a 6-d period to an irradiance of 1000 μmol quanta m⁻² s⁻¹. The soil was flushed every 2 d with full-strength Hewitt and Smith (1975) solution and with water on the alternate day. On four occasions during the next 2 weeks (d 4, 11, 14, 17) these seedlings were scored for Rubisco activity and Chl fluorescence (see below). Measurements were done on the third youngest visible leaf; on d 8 measurements were also done on all leaves of a few plants of each genotype to determine the magnitude of ontogenetic effects.

**Assay of Activated Rubisco Activity**

As soon as the fluorescence readings were stable, recording was terminated, and a leaf disc (0.58 cm²) was punched from
was quickly ground in 250 μL of ice-cold extraction buffer (100 mM Epps/KOH [pH 7.1], 2 mM EDTA, 10 mM DTT, 1% w/v) PVP 40, 1% casein, 20 mM sodium ascorbate, 1 mM sodium diethyldithiocarbamate, 20 mM MgSO₄, 20 mM NaHCO₃, and 20 mM K₂HPO₄. Samples were centrifuged for 5 min in an Eppendorf microcentrifuge at 4°C. Triplicate samples of the supernatant (20 μL) were incubated at 25°C for 5 min in 250 μL of 100 mM Epps/KOH (pH 8.0), 20 mM MgCl₂, 15 mM [14C]NaHCO₃. The reaction was initiated by addition of RuBP to a 0.4 mM final concentration and terminated after 2 min by addition of formic acid.

**Fluorescence Measurements**

Chl fluorescence was measured between 4 and 8 h after the beginning of the light period with a fluorometer (FAM-101; H. Walz, Effeltrich, Germany) set at the door of the chamber. The probe was immediately positioned directly on the area that had been probed for fluorescence. The leaf disc (0.58 cm²) was immediately taken at the same position on the leaf as where fluorescence was measured, and another one was taken on the other side of the midrib in a symmetrical position for biochemical assays. The two discs were snap-frozen in liquid nitrogen and stored at −80°C until analysis. The shoot was cut from the roots, and the leaves were separated from the stem to measure their area and fresh weight. Dry weights of leaves, stem, and roots were measured separately after oven drying for 48 h at 80°C.

**Rubisco Activity and Chl Concentration**

One disc was used to measure Rubisco activity and Chl concentration using the extraction procedure described above. Part of the disc was used for Chl analysis in 80% acetone (Porra et al., 1989), and the remaining homogenate was centrifuged and assayed for Rubisco as described above.

**Rubisco Content and Carbamylation Level**

Rubisco catalytic site concentration and carbamylation levels were determined on a subsample of plants of each genotype by the method reported by Butz and Sharkey (1989) with the following modification: 20% PEG (4000–6000 kD) was used to precipitate the Rubisco-[14C]CABP complexes, with BSA (2.5 mg mL⁻¹) as carrier. [14C]CABP was prepared by the method described by Collatz et al. (1978). The Rubisco catalytic site concentration was determined by measuring the [14C]CABP-binding capacity of extracts in which Rubisco had been fully carbamylated by saturating CO₂ and Mg⁺⁺ concentrations. Rubisco content was calculated assuming eight binding sites per 550,000-kD holoenzyme. The catalytic turnover rate of activated Rubisco was calculated by dividing the activated Rubisco activity by the total number of sites. Soluble protein content in the same extract was estimated by the method of Bradford (1976) using BSA as a standard.

**Mineral and Carbohydrate Composition**

Total nitrogen concentration in both leaf and root tissue was measured chromatographically on oven-dried ground material with a Carlo Erba model 1108 elemental analyzer. The contribution of Rubisco to total leaf nitrogen was estimated by assuming Rubisco is 16% nitrogen. Nitrate concentration in leaves and roots was estimated from nitration of salicylic acid according to the method of Cataldo et al. (1975). Mineral elements present above trace levels (other than nitrogen) were measured by x-ray spectrometry with correction for interelement effects (Hutton and Norrish, 1977; Norrish and Hutton, 1977). Total soluble sugars and starch were extracted with boiling water for 15 min and measured enzymically according to the method of Jones et al. (1977) and Wong (1990).
Gas Exchange Measurements

Rates of transpiration and CO₂ assimilation were measured on a subsample of three to four plants per treatment on d 20 to 27, i.e. during the week following the third harvest. Measurements were made in an open gas exchange system (Brugnoli et al., 1988, as modified by Hudson et al., 1992). Measurements were made at an irradiance of 1000 μmol quanta m⁻² s⁻¹, a leaf temperature of 26 to 27°C, and a leaf-to-air vapor pressure difference of 11 to 13 mbar, i.e. at conditions similar to growth conditions. The first measurement was made at growth pₚ; then pₚ was varied to examine the dependence of CO₂ assimilation rate on pₚ. Calculation of gas exchange parameters was done as reported by von Caemmerer and Farquhar (1981).

RESULTS

Segregation of Rubisco Activity and Chl Fluorescence

The in vitro activities of activated Rubisco were measured on 15 wild-type plants and an R₁ population of 80 plants on d 14, and their relationship to the in vivo electron transport rates was estimated from measurements of Chl fluorescence on the same leaf punch (see “Materials and Methods”) was examined. The two parameters co-varied and defined a hyperbolic curve, as expected (Genty et al., 1989). Individual values in the R₁ population were distributed into three groups clearly separated on the two parameters (Table I). These three groups of plants remained stable throughout the experiment: 19 plants were similar to the wild type and had the highest enzymic activities and Chl fluorescence values; 36 plants showed reduced, intermediate photosynthetic performance; and 25 plants had much lower Rubisco activity and electron transport rate (Table I). The number of plants in each group was not significantly different (using a χ² test) from that expected for progeny segregating for a single gene. This confirmed the conclusion drawn from molecular analysis and the screen on kanamycin and left little doubt that the primary transformant carried only one copy of the antisense gene, which segregated in a mendelian fashion, producing heterozygous plants with one copy of the gene, and homozygous plants with two copies or no copy. Measurements of Chl fluorescence and Rubisco activity done on several occasions from d 4 to 27 on the two next batches of seedlings used for growth analysis (see “Materials and Methods”) showed similar ranges of values and similar distributions of these two parameters as described above and allowed unambiguous identification of the genotype of most plants.

Rubisco Activity and Carbamylation Level

Rubisco activity per unit leaf area or mass was markedly reduced by the antisense gene (Fig. 1). It was comparatively less affected by growth pₛ. However, when expressed on a mass basis Rubisco activity was always significantly lower (P = 0.01) in leaves grown at 930 μbar compared to 330 μbar CO₂ (Fig. 1), reflecting the increased mass of carbon laid down per unit area in these leaves (see below).

The in vivo carbamylation levels of the enzyme measured on d 15 were little affected by either antisense gene or growth pₛ. The ratio of the in vivo activity to the activity of the activated enzyme determined by CABP-binding assay and averaged across genotypes was 0.62 ± 0.01 and 0.67 ± 0.03 in leaves grown at 330 and 930 μbar of CO₂, respectively. Therefore, differences in Rubisco content (Table I) reflect relative differences in the amount of active enzyme.

Plant Size at Harvests

Total plant dry weight at harvest was significantly greater at 930 μbar of CO₂ than at 330 μbar for all three genotypes (Fig. 2a). At 330 μbar genotypic differences were also all significant (P = 0.01); plant mass was positively correlated with Rubisco activity per unit leaf area over the whole range analyzed (Fig. 2a), which is in contrast to the observations of Quick et al. (1991a) in lower light. However, the difference between null plants and single-copy antisense plants was no longer significant at 930 μbar of CO₂. Rubisco activity and growth pₛ also affected leaf area per plant (Fig. 2b) but to a lesser extent than dry weight accumulation. Final leaf area was similar for null and single-copy antisense plants at both pₛ’s, and there was no significant effect of pₛ on the leaf area of wild-type plants. Although the relationship between Rubisco activity per unit mass and per unit area was affected by growth pₛ (see above, Fig. 1), the conclusions derived from Figure 2 still held when Rubisco activity was expressed on a mass rather than area basis.

Table I. Comparison of Rubisco activity and Chl fluorescence (parameter fPSII, see “Materials and Methods") for the three groups of plants constituting the R₁ progeny of primary transformant S₁ (see text) and for wild-type plants

<table>
<thead>
<tr>
<th>No. of Plants</th>
<th>Rubisco Activity</th>
<th>μmol of CO₂</th>
<th>fPSII</th>
<th>Inferred No. of T-DNA Copies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>± SE</td>
<td>m⁻² s⁻¹</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>R₁ progeny</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>61.2 ± 2.4</td>
<td>115</td>
<td>0.43 ± 0.007</td>
<td>98</td>
</tr>
<tr>
<td>36</td>
<td>20.6 ± 2.6</td>
<td>39</td>
<td>0.32 ± 0.01</td>
<td>72</td>
</tr>
<tr>
<td>25</td>
<td>7.8 ± 0.4</td>
<td>15</td>
<td>0.17 ± 0.01</td>
<td>39</td>
</tr>
<tr>
<td>Wild type</td>
<td>53 ± 7.0</td>
<td>100</td>
<td>0.44 ± 0.01</td>
<td>100</td>
</tr>
</tbody>
</table>

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Growth Limitation by Rubisco in Transgenic Tobacco

Carbohydrate Levels at Harvests

The amount of starch stored in the leaves in the middle of the photoperiod increased with increasing Rubisco level and with increased \( p_a \) (Fig. 3a), representing up to 35% of the leaf dry weight in null plants at 930 \( \mu\)bar of \( CO_2 \) as opposed to 7 to 10% in double-copy antisense plants at 330 \( \mu\)bar of \( CO_2 \). The concentration of reducing sugars (Fig. 3b) followed a similar trend but remained low in absolute concentrations (2% leaf dry weight). It is interesting that concentrations of both starch and reducing sugars remained very low in roots and were of comparable magnitude. Sugar concentrations in roots were somewhat higher at 930 \( \mu\)bar of \( p_a \) (Fig. 3b), whereas there was no obvious effect of \( p_a \) on starch.

The leaf starch concentrations shown in Figure 3 are very high. However, they are consistent with those measured in tobacco by Fichtner et al. (1993), who used a different analytical method. In their study in which growth was limited by light and nitrogen, starch represented up to 40% of leaf dry weight. Such high values have also been reported in other \( CO_2 \)-enrichment experiments with other species (Wong, 1990; Wong et al., 1992).

The increased accumulation of starch with increasing \( p_a \) and increasing Rubisco activity did not totally account for the differences in total mass shown in Figure 2a. At 330 \( \mu\)bar of \( CO_2 \) the structural mass of both leaves and roots (mass corrected for content in starch and reducing sugars) was significantly reduced (\( P = 0.01 \)) by the presence of one and two copies of the antisense gene (Fig. 4), and as shown in Figure 2, increasing \( p_a \) to 930 \( \mu\)bar overcame the effects of up to 50% reduction in Rubisco levels in heterozygous antisense plants.

Figure 1. Activity of fully activated Rubisco measured at successive harvests expressed per unit leaf area (x axis) and per mol of leaf structural carbon (y axis). Symbols represent the mean of five to eight plants, and the horizontal bars represent the SE on the x axis. The SE on the y axis are not shown because they were similar. Circles and squares denote plants grown at 330 and 930 \( \mu\)bar of \( CO_2 \), respectively. Null plants are represented by closed symbols, single-copy antisense plants are represented by half-shaded symbols, and double-copy antisense plants are represented by open symbols. The digit (1, 2, or 3) beside the symbols denotes harvest date (d 11, 15, and 19, respectively).

Figure 2. Plant mass (a) and leaf area (b) at final harvest (d 19) plotted against the Rubisco activity measured on a leaf disc sampled from the third youngest leaf just before harvest. Circles and squares denote individual plants grown at 330 and 930 \( \mu\)bar of \( CO_2 \), respectively; closed symbols represent null plants, half-shaded symbols represent single-copy antisense plants, and open symbols represent double-copy antisense plants. At each growth \( p_a \), the data were fitted by a function \( y = a \left(1 - \exp(bx)\right) \), where \( a \) and \( b \) are fitted parameters. The equality of the regression functions for plants grown at 330 and 930 \( \mu\)bar of \( CO_2 \) (dashed and solid lines, respectively) was tested and rejected for both plant weight and leaf area (\( P = 0.01 \)) by using an \( F \) statistic on the error sum of the squares (Neter and Wasserman, 1974). The same analysis and conclusions were achieved when relationships were examined with respect to Rubisco activity per unit leaf mass instead of per unit leaf area.
Carbon Partitioning

The earliest detectable variations in carbon partitioning were in the amount of carbon invested per unit area of leaf (\(a\)) (Fig. 5). At the first harvest on d 11 \(p_1\) was significantly lower at 330 than 930 \(\mu\text{bar}\) of \(\text{CO}_2\) in all three genotypes and was lower in the antisense plants except at 930 \(\mu\text{bar}\) of \(\text{CO}_2\), when only one copy of the antisense gene was present. These variations reflected changes in the amount of both structural and nonstructural carbon. The relative allocation of carbon between roots and leaves was initially mostly unaffected (see similarities of the root-to-shoot ratios in Fig. 4). However, by d 19 root-to-shoot ratios were higher at 930 compared with 330 \(\mu\text{bar}\) of \(\text{CO}_2\) and, at 330 \(\mu\text{bar}\), were higher in wild-type than in antisense plants.

Dynamics of Growth

Figure 6 shows the rates of structural dry-matter accumulation, \(r_s\), between harvests, expressed in relative terms: \(r_s = (1/W)\frac{dW}{dt}\). Remarkably, all rates were similar, regardless of genotype and growth \(p_0\), as were the rates calculated on a total mass basis (data not shown). This implies that differences in weight at the second and third harvests (d 15 and 19, respectively) had largely been determined by what happened before the first harvest (d 11). The relative rates of leaf area expansion, \(r_a = (1/a)\frac{da}{dt}\), calculated for individual plants from the in situ measurements of leaf area for successive periods starting from d 1, confirm that implication (Fig. 7). Before d 8, leaf expansion rate was clearly lowest in the homozygous antisense plants, especially at 330 \(\mu\text{bar}\) of \(\text{CO}_2\), in a manner totally consistent with the ranking in leaf area seen at later harvests (Fig. 2b). However, this difference progressively disappeared (compare a–d in Fig. 7), and by d 13 (at 330 \(\mu\text{bar}\) of \(\text{CO}_2\)) or d 8 (at 930 \(\mu\text{bar}\) of \(\text{CO}_2\)), all plants were expanding at about the same rate.

Photosynthetic Limitation to Growth

To analyze growth responses to changes in Rubisco levels or in atmospheric \(\text{CO}_2\) levels, it is useful to express the relative
rate of growth, \( r_w \), in terms of rate of CO2 fixation using the identity (Masle et al., 1990)

\[
r_w = \bar{A}(1 - \phi)/\rho
\]

where \( \bar{A} \) is the rate of CO2 assimilation per unit leaf area averaged over all leaves and the whole photoperiod, \( \phi \) is the proportion of \( \bar{A} \) that is respired by the shoot at night and by the roots during the day and night, \( l \) is the length of the photoperiod as a proportion of a 24-h day, and \( \rho \) is the ratio of the amount of carbon invested in the whole plant to the leaf area.

It is difficult to measure \( \phi \), but the product \( A' = \bar{A}(1 - \phi) \) can be calculated from the growth measurements made at successive harvests as

\[
A' = \frac{(W_2 - W_1)(\ln a_2 - \ln a_1)}{l(t_2 - t_1)(a_2 - a_1)}
\]

where \( W \) denotes plant mass (in mol of carbon), \( a \) denotes leaf area (m²), \( l \) is as defined in Equation 1, and subscripts 1 and 2 refer to values measured at time \( t_1 \) and \( t_2 \) (s). \( A' \) is equivalent to the term \( A(1 - \phi) \) in Equation 1. The average \( A' \) values between harvests were significantly lower in antisense than in wild-type plants except, again, for the single-copy antisense plants grown at 930 \( \mu \)bar of CO2. The mean values of \( A' \) between d 11 and 19, for example, were 5.1, 9.4, and 13.3 \( \mu \)mol of carbon m⁻² s⁻¹ at 330 \( \mu \)bar \( p_a \) and 8.8, 18, and 16 \( \mu \)mol of carbon m⁻² s⁻¹ at 930 \( \mu \)bar \( p_a \) for double-copy, single-copy, and null antisense plants, respectively.

The ranking of whole plant average net assimilation rates was confirmed by conventional gas exchange measurements. The CO2 dependence of the assimilation rate, \( A \), of individual, rapidly expanding leaves was examined for three to four plants per treatment at the end of the experiment (d 20–27). The CO2 assimilation rate at 330 \( \mu \)bar \( p_a \) increased with Rubisco activity over the whole range measured (data not shown). All curves could be well described by the biochemical model of Farquhar et al. (1980) (Fig. 8) where steady-state CO2 assimilation is limited either by the RuBP-saturated rate of Rubisco or by the rate of RuBP supply. The CO2 assimilation rate in wild-type and null transgenic plants closely followed the theoretical line set by Rubisco kinetics, up to a \( p_a \) of 300 to 550 \( \mu \)bar, depending on the plant and the value assumed for the \( g_i \) (see legend to Fig. 8). At higher CO2 pressures \( A \) declined below that line and was well described by the theoretical relationship set by the rate of RuBP regeneration. The \( p_a \) at which the shift from Rubisco limitation to RuBP regeneration limitation occurred varied slightly from plant to plant. The values were similar to or greater than the operating internal pressures at 330 \( \mu \)bar \( p_a \) but were always lower than the operating pressures at 930 \( \mu \)bar \( p_a \) (see examples in Fig. 8). In contrast, in all antisense plants \( A \) was consistent with Rubisco-limited kinetics (Fig. 8) up to high \( p_a \) beyond the \( p_a \) prevailing in plants grown at 930 \( \mu \)bar \( p_a \). The \( A \) values were always lower than the corresponding \( A' \) values, especially for the biggest plants (plants with highest Rubisco content or grown at highest \( p_a \)) because they describe the assimilation of a young leaf measured at 1000 \( \mu \)mol quanta m⁻² s⁻¹, i.e. under the irradiance that only the horizontal and nonshaded leaves of the plant would have intercepted in the growth chamber, and also because \( A' \) values are diminished by the respiration term.

## Nitrogen Use

Total nitrogen concentration in leaves, expressed on a total dry-weight basis, was greater in the double-copy antisense
plants than in the wild type (Fig. 9a). Concentrations in the single-copy plants were initially (first harvest, d 11) intermediate. They remained so at 330 μbar of CO₂, whereas at 930 μbar they became similar to those measured in the wild type (see d 19). At all times and for all genotypes leaf nitrogen concentrations were greater in low- than in high-CO₂-grown leaves (Fig. 9a). The above differences were partly due to differences in nitrate levels (Fig. 9a), and, given the variations in starch previously mentioned and also of ash-constituting minerals (mainly P, K, Ca, and Si; data not shown), nitrogen use is better analyzed when its concentrations are expressed on an organic matter basis (corresponding to total dry mass minus stored carbohydrates and minerals, minus nitrates). On that basis, at 330 μbar Pₐ concentrations of organic nitrogen were lower in double-copy antisense plants than in wild type, and differences were mostly accounted for by reduction in Rubisco levels (Fig. 9b). For plants grown at 930 μbar of CO₂ the pattern was more complicated, with organic nitrogen concentrations being higher in the wild type on d 11 but lower by the time of the last harvest (d 19), despite greater Rubisco levels.

A similar pattern was seen for total Chl concentrations (data not shown). When organic nitrogen was expressed on a leaf area basis (Fig. 9c), concentrations were consistently lower in double-copy antisense plants than in the wild-type plants at either Pₐ, with concentrations in the single-copy antisense plants being intermediate (at 330 μbar Pₐ) or similar to those in null plants (at 930 μbar Pₐ). Total Chl and organic nitrogen (excluding Rubisco) varied in parallel (data not shown), indicating that the partitioning of nitrogen between proteins associated with the light-harvesting complex and other proteins was mostly unaffected by alteration in Rubisco gene expression or growth pₐ. At the whole plant level, the amount of nitrate-free nitrogen invested per unit of structural matter was little affected by genotype but was always significantly lower at 930 μbar than at 330 μbar of CO₂. The values were 44.0, 43.4, and 48.6 mg of nitrogen g⁻¹ of dry matter in double-copy antisense plants, single-copy antisense plants, and wild-type plants, respectively, at 330 μbar of CO₂ compared with 33.9, 29.5, and 28.4 mg of nitrogen g⁻¹ of dry matter, respectively, at 930 μbar of CO₂.

**DISCUSSION**

**Effects of Antisense RNA and Growth CO₂ on Rubisco Activity**

Plants with the antisense gene directed toward rbcS mRNA showed a 50 to 87% reduction in Rubisco activity compared with the wild type. The reduction mostly depended on how many copies of the antisense gene were present in the genome. In contrast to earlier reports with lower light (Quick et al., 1991a, 1991b), at high light we found no evidence that the proportion of carbamylated enzyme in the antisense plants was increased, even in the plants with two copies of the antisense gene. Under the 340 μmol quanta m⁻² s⁻¹ (at the top of the plants) used by Quick et al. (1991a, 1991b) the
especially in plants with greater Rubisco content (Lane et al., 1993) also in variation in carbamylation in the light was also found to be insensitive to consistent with those of other in vivo studies in which experiment did not appear to be significantly affected by pressure of 0.95 bar) for \( g_0 \), infinite, and \( K_r \), pressure of 258 mbar, CO2 compensation point in the absence of dark respiration \( (\Gamma^*) = 36.9 \) mbar (at Canberra's atmospheric pressure of 0.95 bar) for \( g_0 \), infinite, and \( K_r = 258 \) mbar, \( K_o = 171 \) mbar and \( I^* = 38.6 \) mbar for \( g_0 = 0.3 \). Best-fit estimates were obtained using a Simplex procedure (Nelder and Mead, 1976). The assumption on \( g_0 \), influenced the value of \( p_I \), for which the model indicated a switch from Rubisco limitation to RuBP limitation, but the position of that value with respect to the operating \( p_0 \), under 330 and 930 mbar \( \rho_a \), remained the same, i.e. the nature of the limitation that could be inferred from the fitted curves for the different genotypes was independent of the assumption on \( g_0 \). The fitted curves shown in the figure are, therefore, those obtained for the case of infinite \( \rho_0 \), i.e. \( \rho_0 = \rho \). The operating stomatal conductances to CO2 for the plants shown were 0.340, 0.358, and 0.344 mol of CO2 m\(^{-2}\) s\(^{-1}\) for double-dose antisense plants, single-dose antisense plants, and wild-type plants, respectively, at 930 mbar \( p_0 \).}

**Photosynthetic Limitation to Growth: In Its Effects on Growth, Loss of Rubisco Is Similar to Reduction in Ambient CO2 Pressure**

At 330 mbar \( p_0 \), reduction in Rubisco activity penalized growth whether expressed in terms of biomass accumulation or of leaf area expansion (Figs. 2 and 7). Quick et al. (1991a) and Fichtner et al. (1993) previously analyzed growth of other tobacco plants transformed with an antisense gene to \( rbcS \). Using low light, they found that growth under plentiful nutrient supply was reduced compared with the wild type only when Rubisco activity had been decreased by at least 50%. In our experiment at higher light, growth in mass was Rubisco limited in all antisense plants (Fig. 2). More impor-
Figure 9. Leaf nitrogen concentrations at harvests for each genotype and growth \( p_a \). Nitrogen concentrations are expressed in three ways: (a) total nitrogen \( \text{mg} \text{g}^{-1} \text{total dry matter} \) (mean and ss); height of column below the horizontal bar shows the level of nitrate-free nitrogen; (b) organic nitrogen in structural matter (i.e. nitrate-free nitrogen as a proportion of dry matter excluding starch and reducing sugars and ash-containing minerals); height of column below the horizontal bar marks the level of organic nitrogen excluding nitrogen-Rubisco; (c) non-Rubisco organic nitrogen per unit leaf area.


tant, we show that in most respects the effects on growth of genetically reduced Rubisco content could be compensated by increasing growth \( p_a \). A particularly intriguing feature of the growth response observed in the present experiment is that the reduction in plant size at 330 \( \mu \text{bar} \) \( p_a \), compared with 930 \( \mu \text{bar} \), or in antisense plants compared with wild-type plants, was mostly set very early in the life of the plant. Within 2 weeks after germination, all plants had indeed similar relative growth rates (Figs. 6 and 7). Such a response has been seen in other \( \text{CO}_2 \)-enrichment experiments with other species (\textit{Mimulus cardinalis} [Badger, 1992]; \textit{Eucalyptus} [Wong et al., 1992]; \textit{Trifolium subterraneum} L. [Morin et al., 1992]) and, in the latter experiment, also when plants were transferred to high light. We suggest that it is a fundamental growth response to changes in the rate of photosynthesis, whether caused by changes in the rate of carboxylation or of light reactions.

\textbf{Short-Term Response of Relative Growth Rate to Variations in Growth \( p_a \) or in Rubisco Activity}

The mechanisms involved in the short-term variations in relative growth rate, which took place during the first few days of the present experiment, are not clear. The fact that plant dry matter at harvest was more affected than leaf area (Fig. 2), even when allowing for differences in stored carbohydrates, implies that the initial response of \( r_a \) to changes in carboxylation rate was greater than that of \( r_a \). This differential response of the increase in area and in mass mainly reflected, in the short term, adjustments in leaf characteristics. Indeed, by d 11 the leaf carbon mass invested per unit area (Fig. 5) and leaf water content (data not shown) were already significantly different among all three genotypes and between plants grown at different \( p_a \). At the same time, there was no significant genotypic variation in root-to-shoot ratio, and an effect of growth \( p_a \) on that ratio was detectable only in double-copy antisense plants. These features may be characteristic of the short-term plant growth response to changes in carbohydrate supply in a range of species. In an experiment with wheat (Masle et al., 1990) \( r_a \) was unaffected by increasing \( p_a \), above current ambient pressures (480 and 690 \( \mu \text{bar} \)) for more than 2 weeks after germination, whereas the increase in mass was significantly enhanced, and the root-to-shoot ratio was initially insensitive to \( p_a \).

In the present experiment, plants grown under 330 \( \mu \text{bar} \) \( p_a \) or carrying the \textit{rbcS} antisense gene had fewer leaves at any time and flowered later than plants grown at 930 \( \mu \text{bar} \) or with no antisense insert (data not shown). These observations indicate that their slower growth rate may have been in part related to delays in timing of developmental events. This would be consistent with the enhancing effect of elevated \( p_a \) on leaf appearance rate in young vegetative wheat seedlings (J. Masle, G.T.S. Beemster, G.D. Farquhar, unpublished data) and also with the role of carbohydrate supply to the apical meristem on floral initiation, now demonstrated in a range of species (Bernier, 1988; King and Evans, 1991). Studies are in progress to examine effects of the \textit{rbcS} antisense gene and of variation in growth \( p_a \) on leaf ontogeny and on the rate of initiation of vegetative and floral meristems.

\textbf{Steady-State Relative Growth Rate: Compensatory Variations of \textit{CO}_2 Net Assimilation Rate and of Carbon Partitioning}

Large amounts of starch were found in leaves under 930 \( \mu \text{bar} \) of \textit{CO}_2 and correlated with Rubisco content (Fig. 3). One obvious possibility for the absence of long-term differences in \( r_a \) is, therefore, that photosynthetic rate had become insensitive to changes in Rubisco activity or \textit{CO}_2 supply. The leaf gas exchange measurements done at the end of the experiment (Fig. 8) allow us to exclude that interpretation. The \( A \) was greatly enhanced at 930 \( \mu \text{bar} \) of \textit{CO}_2 compared...
with $330 \, \mu\text{bar}$ and was reduced in antisense plants with lower Rubisco levels. The $A'$ calculated from growth measurements at harvests showed that this conclusion held at the whole plant level and throughout the experiment, including the period when $r_n$'s were similar.

The large increase in $A$ with $p_o$ or Rubisco levels shown in Figure 8 does not in itself mean that $A$ was completely free of inhibition. However, in assessing the different causes of photosynthesis feedback inhibition one can reasonably exclude three mechanisms that could occur: (a) Limitation by triose-P use (Sage and Sharkey, 1987; Sharkey, 1990). The shape of the $A:p_o$ curve was equally well described for all plants by the same theoretical equations (Fig. 8), which assume no limitation by triose-P use. (b) Deactivation of Rubisco (von Caemmerer and Edmonson, 1986; Sage et al., 1989). Rubisco activity did show a small decline through time in the present experiment (Fig. 1). However, this decline occurred in all plants and did not appear to be more pronounced in plants with higher photosynthetic rates and starch levels, indicating that it probably did not correspond to a direct regulatory mechanism of photosynthesis. Therefore, we conclude that some inhibition of photosynthesis may have occurred in wild-type plants and at $930 \, \mu\text{bar} \, p_o$ but if so, it was small and not responsible for the absence of long-term differences in $r_n$ or $r_d$.

Because growth $p_o$ and genetic manipulation of Rubisco had last effects on $A'$ and on $p_o$, the similarity of $r_n$ seen from at least d 11 implies that subsequent variations in $A'$ were offset by proportionally similar variations in $p_o$ (cf. $r_n = A'/p_o$). Leaves of plants carrying the antisense gene or grown at $330 \, \mu\text{bar}$ of CO$_2$ had a lower $p_o$ than plants with greater carboxylation rate (Fig. 5), with this effect becoming more marked through time. Their greater water content (even on a nonstarch basis, data not shown) suggests that this may have been partly achieved through an increase in mean cell size. Given these differences, it may, therefore, be more meaningful when analyzing relationships between photosynthesis and growth to express $A$ and $A'$ on a leaf dry-matter basis or carbon basis rather than on an area basis (Mooney et al., 1978; Poorter et al., 1990) and thus write $r_n$ as $r_n = A_n/(1 + W_s/W_l)$, where $A_n$ [mol of CO$_2$ (mol of carbon)$^{-1}$ (s daylight)$^{-1}$] = $A/p_o$, and $W_s/W_l$ is the ratio of root carbon mass to shoot carbon mass. Until d 15, when $r_n$ values were already all similar, $W_s/W_l$ was not significantly different between genotypes, with the exception of the double-copy antisense plants grown under low $p_o$, which were characterized by a lower root-to-shoot ratio (Fig. 4). This implies that $A_n$ was only lowered in this latter group of plants: in the other groups the changes in $p_o$ were such that on a mass basis all leaves were fixing CO$_2$ at a similar rate. By the time of the last harvest (d 19), however, there was more genetic variation in $W_s/W_l$ and consistent $p_o$ effects on $W_s/W_l$ in all antisense plants but, remarkably, $r_n$ still remained similar for all plants (Fig. 6). The fact that $r_n$ values were similar for plants with contrasted and changing patterns of carbon allocation indicates that changes in the amounts of stored starch and in carbon partitioning per se were not causing the long-term insensitivity of the relative growth rate to changes in carboxylation rate. This conclusion is consistent with the observations of Morin et al. (1992), who analyzed in clover the effects on plant carbohydrate status and growth kinetics of increasing whole plant CO$_2$ assimilation rate through increased $p_o$ or increased irradiance. Both treatments induced only a short-term stimulation of the relative growth rate, despite starch levels being much increased only under high $p_o$ conditions.

One possible interpretation of our data is that after a transient where $r_n$ was strongly dependent on carboxylation rate, whole plant $r_n$ was constrained to a single value set by overriding processes that are independent of carbohydrate supply. The increase in stored carbohydrates in plants with greater carboxylation rate would, therefore, have occurred as a result of these limitations. The challenge now lies in identifying these rate-limiting processes and unravelling their controls. One possibility that we can readily exclude is that the long-term insensitivity of the relative growth rate to variations in carboxylation rate was caused by factors related to root-pot interactions as seen in other CO$_2$-enrichment studies (Arp, 1991; Thomas and Strain, 1991). There was no significant difference in rate of leaf area expansion between plants in small and big pots at least until d 10 (data not shown), i.e. until after wild-type plants and single-copy antisense plants were already expanding at about the same rate and differences in $r_n$ over the last 8 d of the experiment were independent of root mass.

Another possibility is that the lack of variation of $r_n$ with $p_o$ or Rubisco activity was only apparent due to the fact that null plants or plants grown at $330 \, \mu\text{bar}$ of CO$_2$ were at a given time bigger and more advanced phenologically than antisense plants and those grown at $330 \, \mu\text{bar} \, p_o$. This per se could cause their $r_n$ to be lower (Dijkstra and Lambers, 1989; Poorter and Pothman, 1992) and offset the advantage for growth of increased carboxylation rate. One can exclude such an interpretation in the present experiment because $r_n$ was remarkably independent of plant mass (Fig. 10). Consistent with this observation, the relative decline of $r_n$ between d 13 and 19 (Fig. 7) and the resulting absolute value of $r_n$ were similar for all groups of plants with the exception of the double-copy antisense plants at $330 \, \mu\text{bar}$ of CO$_2$.

**Nitrogen Economy**

The total nitrogen cost of growth (total nitrogen/total mass) was not reduced in antisense plants but rather was increased because the reduction in the amount of nitrogen invested in Rubisco was more than compensated by an increase in vacuolar nitrate, especially at $330 \, \mu\text{bar}$ of CO$_2$ (Fig. 9a). Nitrate accumulated in the leaves as reported by Quick et al. (1991a). In contrast, nitrate concentrations in the roots remained similar for all treatments, like starch concentrations, which suggests that active processes were involved in keeping nonreduced nitrogen and sugars in the leaves. Because nitrogen concentrations in roots remained low, the rates of nitrogen
uptake and unloading to the xylem were maintained. It is interesting that the most obvious response to reduced expression of Rubisco genes was to increase the concentration of the nitrogen substrate necessary for synthesizing the enzyme. At 330 μbar of CO₂, genetic manipulation of Rubisco had no effect on nitrogen concentration in organic matter after allowance was made for reduced Rubisco levels (Fig. 9b), but this matter was spread over a much larger area in antisense plants, especially when carrying two copies of the antisense gene, than in the wild type (Fig. 5). A similar conclusion applies to plants grown at 930 μbar p₀, except in the last few days of the experiment, when there was a change in the elemental composition of organic matter (increased carbon to nitrogen ratio due to decreased protein content) in single-copy antisense and wild-type plants. This change, which did not occur in double-copy antisense plants, may in part reflect ontogenetic effects associated with faster leaf tissue aging in the most advanced plants.

At the whole plant level, the investment of nitrogen per unit of organic matter was significantly reduced under higher p₀. Combined with the increased A and overall rₑ at 930 μbar compared with 330 μbar of CO₂, this effect resulted in the single-copy antisense plants achieving similar growth at 930 μbar p₀ to that of the wild type at 330 μbar but at a 40% lower organic nitrogen cost. However, on a total nitrogen basis, this nitrogen saving was offset by the increased nitrate accumulation.

The present data were obtained under conditions in which nitrogen supply was plentiful. Were nitrogen supply limiting and, therefore, presumably nitrate levels no longer increased in antisense plants, we calculate that, under high-light conditions (i.e. when CO₂ assimilation rate is set by the kinetics of Rubisco), about 6% of the amount of nitrogen that is invested in wild-type plants at 330 μbar of CO₂ could be saved, with no loss of growth performance, by growing antisense plants with half less Rubisco at 930 μbar p₀. In other words one would expect that, in nitrogen-limited environments, single-copy antisense plants grown at 930 μbar p₀ would be about 6% heavier than the wild type under present day p₀. This calculation is approximate because it ignores other possible effects of nitrogen limitation on root-to-shoot ratio, for example, or on the partitioning of nitrogen between different photosynthetic proteins (Quick et al., 1992). However, it shows that reducing the investment of nitrogen in Rubisco may be a worthwhile means of improving nitrogen-use efficiency in a future world with increased air CO₂ concentrations and would most likely confer a selective advantage for nitrogen-limited environments. This advantage may, however, be difficult to demonstrate by conventional growth analysis.

CONCLUSIONS

(a) Under 330 μbar of CO₂, Rubisco is not present in excess in wild-type tobacco plants with respect to growth at high light. CO₂ levels would have to increase by at least 280% before a 50% reduction in leaf Rubisco could be envisaged without incurring a growth penalty. (b) Were that to happen nitrogen-use efficiency would only be improved in nitrogen-limited environments. (c) Growth responses to decreased carbon supply via genetic manipulation of Rubisco level or via decrease in ambient CO₂ pressure were similar. The most intriguing feature of both responses is that rₑ was affected only for a few days early in development, rapidly reaching a steady-state value similar for all plants. The long-term insensitivity of rₑ to carbohydrate supply was not directly caused by changes in carbon partitioning between roots and leaves. Nor was it due to end product feedback inhibition of photosynthesis. Further work is needed to understand what appears as a fundamental response to changes in photosynthetic rate: what is the nature of the short-term stimulation of relative growth rate by increased carboxylation rate, and what factors control the steady state value?

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