Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" rbcS

IV. Impact on photosynthesis in conditions of altered nitrogen supply

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Abstract. The effect of nitrogen supply during growth on the contribution of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco; EC 4.1.1.39) to the control of photosynthesis was examined in tobacco (*Nicotiana tabacum* L.). Transgenic plants transformed with antisense rbcS to produce a series of plants with a progressive decrease in the amount of Rubisco were used to allow the calculation of the flux-control coefficient of Rubisco for photosynthesis (C_R). Several points emerged from the data: (i) The strength of Rubisco control of photosynthesis, as measured by C_R, was altered by changes in the short-term environmental conditions. Generally, C_R was increased in conditions of increased irradiance or decreased CO_2. (ii) The amount of Rubisco in wild-type plants was reduced as the nitrogen supply during growth was reduced and this was associated with an increase in C_R. This implied that there was a specific reduction in the amount of Rubisco compared with other components of the photosynthetic machinery. (iii) Plants grown with low nitrogen and which had genetically reduced levels of Rubisco had a higher chlorophyll content and a lower chlorophyll *a/b* ratio than wild-type plants. This indicated that the nitrogen made available by genetically reducing the amount of Rubisco had been re-allocated to other cellular components including light-harvesting and electron-transport proteins. It is argued that there is a "luxury" additional investment of nitrogen into Rubisco in tobacco plants grown in high nitrogen, and that Rubisco can also be considered a nitrogen-store, all be it one where the opportunity cost of the nitrogen storage is higher than in a non-functional storage protein (i.e. it allows for a slightly higher water-use efficiency and for photosynthesis to respond to temporarily high irradiance).

Key words: *Nicotiana* (photosynthesis) – Nitrogen – Photosynthesis (control analysis) – Ribulose-1,5-bisphosphate carboxylase-oxygenase – Transgenic plant

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Abbreviations: C_R = flux control coefficient of Rubisco for photosynthesis; rbcS = gene for the Rubisco small subunit; Rubisco = ribulose-1,5-bisphosphate carboxylase-oxygenase

Introduction

Rodermel et al. (1988) have recently produced a series of tobacco plants that exhibit a range of reduced activities of the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) using antisense mRNA to the gene for the small subunit of Rubisco (rbcS). These plants provide an ideal experimental system to examine the control exerted by Rubisco on the rate of photosynthesis (Quick et al. 1991a) and plant growth (Quick et al. 1991b). Using these plants we were able to quantify the flux-control coefficient of Rubisco for photosynthesis (C_R) in the ambient growth environment and in a variety of other short-term environmental conditions (Stitt et al. 1991). This work demonstrated that for tobacco grown in optimum environmental conditions the amount of Rubisco in a leaf could be reduced by more than one-third before any significant effect on the rate of photosynthesis or plant growth was detected, i.e. C_R was small. Several factors were shown to compensate for the loss of Rubisco in the transgenic plants; firstly the activation state of Rubisco was increased from about 55% in wild-type plants to almost 100% (Quick et al. 1991a) and secondly, several morphological features were altered; the specific leaf area and shoot/root ratio were increased. These factors served to both increase Rubisco use-efficiency and maximise photosynthetic leaf area (Quick et al. 1991b).

The calculated value of C_R for tobacco was altered dramatically, in the short term, by changes in the environmental conditions (Stitt et al. 1991) and showed general agreement with the predictions of mechanistic models of photosynthesis (Farquhar and von Caemmerer 1982; Woodrow 1986; Petterson and Rhyde-Petterson 1988; Sharkey 1989). Less is known concerning the extent to which the control exerted by Rubisco on the rate of photosynthesis depends on the longer-term environmental growth conditions. The amount of Rubisco found in a mature leaf is dynamic and fluctuates over relatively short time scales (1–5 d) according to the environmental conditions of light and CO_2, the supply of nitrogen and the plant demand for carbohydrate (Wong 1979; Clough et al. 1981; Vu et al. 1983; von Caemmerer and Farquhar 1984). A priori it is difficult to predict the affect of altered growth environments on the control exerted by Rubisco on the rate of photosynthesis and plant growth.

C_3 plants have adapted to the relative catalytic inefficiency of Rubisco by producing large amounts of the protein. Consequently, Rubisco can account for up to 50% of leaf protein and this requires a considerable investment of plant nitrogen. The supply of nitrogen during plant growth is known to affect profoundly biomass production. Generally, there is a good correlation between leaf nitrogen content and the rate of photosynthesis (Evans 1989) and this is also reflected by large changes in the amount of Rubisco protein (Evans 1983; Sage 1987). The partitioning of biomass between photosynthetic and non-photosynthetic parts of the plant is also known to be affected by nitrogen supply: more biomass is allocated to the roots and the photosynthetic capacity on a leaf dry-weight or area basis is reduced (e.g. Rufty et al. 1984; Hirose 1986; Cure et al. 1988). These data indicate that in conditions of restricted nitrogen supply there is a selective reallocation of nitrogen away from the photosynthetic apparatus. In this paper we have used these transgenic plants to test directly the consequences of reduced Rubisco activity for plant photosynthesis and growth in conditions of altered nitrogen supply. From the data we have estimated C_R values for a variety of growth conditions.

Materials and methods

The selfed progeny of transformants 3 and 5 of tobacco (*Nicotiana tabacum* L. SR1) described by Rodermel et al. (1988) were used in the following experiments. Seeds were germinated on moist filter paper at $20°$ C, transferred to 500-cm^3 pots filled with acid-washed quartz sand and grown under a 20/17° C, 12/12h day/night regime (300 μmol quanta $\cdot$ m^{-2} $\cdot$ s^{-1} during the day) and at 65% relative air humidity. Plants were watered daily with 200 cm^3 of Hoagland's nutrient solution with altered levels of NH_4NO_3: 5.0 mM – high nitrogen; 0.7 mM – medium nitrogen; 0.1 mM – low nitrogen. Plants were used for experimentation and then harvested after 59, 46, and 38 d growth at low, medium and high nitrogen supply, respectively. The harvesting date allowed for growth of sufficient plant material and also ensured plants were studied prior to the onset of flowering. Gas exchange was measured as described previously (Quick et al. 1991a) using a portable porometer (Zimmerman et al. 1988) connected to a remote leaf chamber located inside the growth chamber. The environmental conditions of the leaf inside the porometer were maintained as in the growth room. The irradiance was reduced using neutral-density filters or was supplemented using a standard light-projector to obtain light-saturated rates of photosynthesis. Carbon dioxide was altered by mixing CO_2-free air and pure CO_2 which was controlled by two mass-flow controllers. Assimilation of CO_2, stomatal conductance to water vapour, transpiration and the internal CO_2 partial pressure of the leaf were calculated as in von Caemmerer and Farquhar (1981).

Chlorophyll *a* fluorescence and photosynthetic oxygen evolution were measured using a PAM-fluorimeter (Walz, Effeltrich, FRG) connected to a leaf-disc electrode (Hansatech, King's Lynn, Norfolk, UK) as described in Quick et al. (1989) and using leaf discs (3 cm^2) taken from the first fully expanded leaves. Fluorescence quenching parameters were calculated as described in Quick and Stitt (1989). In some experiments the photosynthetic incorporation of $^{14}CO_2$ into starch (insoluble fraction) and sucrose (soluble and neutral fraction) was determined by the addition of $NaH^{14}CO_3$ to the CO_2 buffer in the oxygen electrode as described in Quick et al. (1989).

Extracts for Rubisco determination were made in the light from material which had been illuminated for at least 1 h and using the same areas of leaf previously used for gas-exchange measurements. Extraction and radiometric assay of Rubisco were as described previously (Quick 1991a). The amount of Rubisco was also determined by Rocket-immune electrophoresis in all extracts (Quick et al. 1991a); this allowed us to check that the V_{max} activity was linearly related to the amount of protein (data not shown, but see Quick et al. 1991a).

Results and discussion

Photosynthetic rate and Rubisco activity. Tobacco plants were grown at 5.0, 0.7 and 0.1 mM NH_4NO_3. Figure 1 shows the rate of photosynthesis (A, D, G), leaf transpiration (B, E, H) and the calculated intercellular CO_2 partial pressure (C, F, I), as determined under ambient growth conditions, plotted against Rubisco activity measured in the same leaf. Data are shown for tobacco plants grown with different supplies of nitrogen. The Rubisco activity measured in wild-type plants (solid symbols) was dependent on the nitrogen supply and the average value increased with increasing nitrogen supply (15, 31, 43 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} Rubisco activity for 0.1, 0.7, 5.0 mM NH_4NO_3, respectively). There was also a variation in Rubisco activity found in wild-type plants and this probably reflected either natural variation or slight differences in the developmental stage of the leaf material used. Plants containing the antisense *rbc*S gene, in general had a reduced Rubisco activity and the extent of reduction has been previously shown to roughly correlate with the copy number of the inserted gene (Rodermel et al. 1988). Some transgenic plants show no reduction in Rubisco activity,; these represent pseudo-wild-type plants obtained after self pollination of the heterozygous F1 generation.

The dependence of the rate of photosynthesis on Rubisco activity was dependent on the level of nitrogen supplied during growth. Plants grown with 5.0 mM NH_4NO_3 (Fig. 1A) showed a biphasic response; initially when the Rubisco activity was reduced from the average wild-type activity (43 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) to the first group of antisense plants (average activity of 22
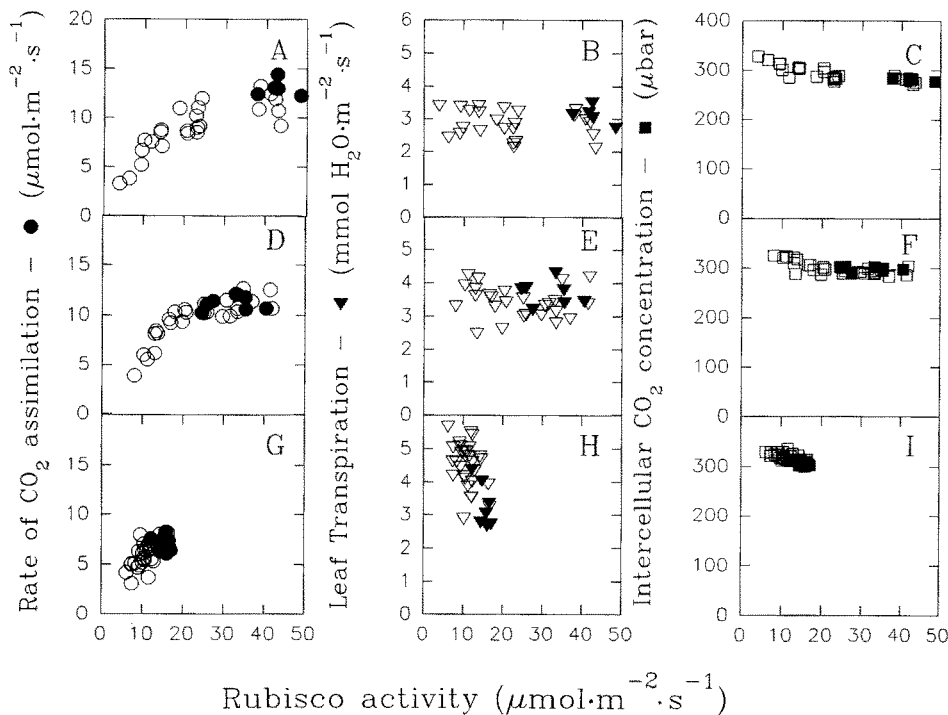
Fig. 1A–I. Variation of the rate of photosynthesis (**A, D, G**) leaf transpiration (**B, E, H**) and the intercellular CO_2 partial pressure (**C, F, I**) as a function of the Rubisco content of tobacco leaves. Photosynthesis was measured under ambient growth conditions (20° C, 300 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} light, 330 μbar CO_2, 70% relative humidity) in plants with altered Rubisco activity. During growth, plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: **A–C** 5.0 mM, **D–F** 0.7 mM, **G–I** 0.1 mM. Each symbol represents a single masurement obtained from an individual plant and *solid* symbols indicate wild-type plants

Rubisco activity (μmol$\cdot$m$^{-2}\cdot$s^{-1})

μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) there was only a slight reduction in the rate of CO_2 fixation (12.6 to 10.8 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}). Further reductions in the activity of Rubisco reduced the rate of CO_2 fixation more severely. These results are in close agreement with those published previously (Quick et al. 1991a; Stitt et al. 1991). A similar biphasic response was also observed in plants grown with a reduced (0.7 mM NH_4NO_3) supply of nitrogen (Fig. 1D). Here the average wild-type Rubisco activity was reduced from 43 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} to 31 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} and the ambient rate of photosynthesis was reduced from an average 22 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} to 18 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} (Fig. 1D). Plants grown with a low (0.1 mM NH_4NO_3) nitrogen supply (Fig. 1G) had substantially reduced activities of Rubisco even in wild-type plants (average wild type is 15 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) and showed a steep, largely monophasic, dependence of the rate of CO_2 fixation on the activity of Rubisco. Interestingly, the slope of this graph is similar to that observed in Figs. 1A and 1D when the activity of Rubisco was reduced to similar values in antisense plants (see below).

The rate of leaf transpiration increased dramatically (Fig. 1H) as the Rubisco activity decreased in plants grown in low (0. 1 mM) nitrogen (correlation coefficient of linear regression = 0.793, $P < 0.01$). At a higher nitrogen supply however, the rate of transpiration was largely unaffected by the Rubisco content (Fig. 1B, E). The reason for this large increase in transpiration observed in plants grown in low NH_4NO_3 is not clear. A common symptom of plant nutrient deficiency (particularly nitrogen) is a reduced rate of leaf transpiration and this is typically associated with elevated levels of abscisic acid (Karmoker et al. 1991; Chapin et al. 1988; Mizrahi and Richmond 1972). The increased rate of transpiration measured in plants with reduced Rubisco could reflect an

increased availability of nitrogen in the leaves of these plants (see below). Leaf nitrate concentration was also observed to increase in the leaves of plants as the amount of Rubisco was reduced (data not shown, but see Quick et al. 1991b).

The estimated internal CO_2 partial pressure increased slightly as the Rubisco content of the leaves declined for all levels of NH_4NO_3 supply. This increase largely resulted from a decline in the rate of photosynthesis, rather than changes in stomatal conductance, at 5.0 mM and 0.7 mM NH_4NO_3 supply.

Previous experiments had demonstrated that the dependence of the rate of CO_2 fixation on the Rubisco content was affected by changes in the short-term environmental conditions (Quick et al. 1991a; Stitt et al. 1991). Figure 2 shows the effect of high (1000 μbar) and low (100 μbar) CO_2 partial pressures on the rate of photosynthesis for the same plants used in Fig. 1, determined at the same irradiance but during the following photoperiod. Note the x-axis is expanded in subsequent figures, compared with Fig. 1, for plants grown with reduced nitrogen supply to allow full expansion of the data. For ease of comparison the ambient-photosynthesis data of Fig. 1 are also shown (open triangles). All plants showed increased rates of CO_2 fixation in high CO_2 compared with ambient conditions. This result is expected since Rubisco oxygenase activity will be suppressed and carboxylase activity correspondingly enhanced. However, plants grown with a low NH_4NO_3 supply (Fig. 2C) showed a far larger stimulation of photosynthesis in high CO_2; the average wild-type value was increased by 60% compared with 22% and 30% for medium and high NH_4NO_3 supply, respectively. The dependence of the rate of CO_2 fixation on Rubisco content was reduced at 1000 μbar CO_2 compared
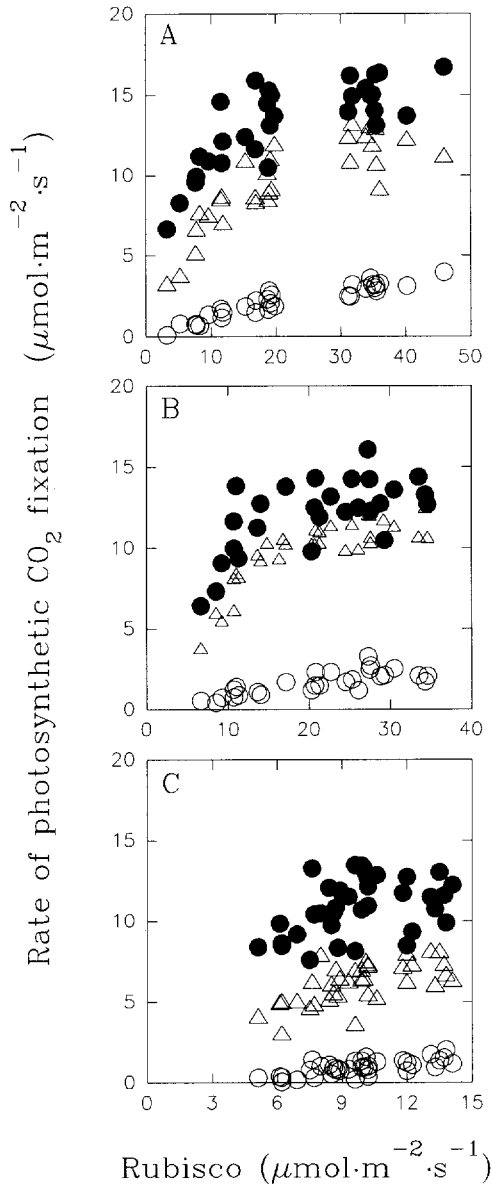
Fig. 2A–C. Variation of the rate of photosynthesis as a function of the Rubisco content of tobacco leaves when measured at 1000 μbar (●), 330 μbar (△), and 100 μbar (○) of CO_2. Photosynthesis was otherwise measured in the ambient growth conditions (20° C, 300 μmol · m^{-2} · s^{-1} light, 70% relative humidity) in plants with altered amounts of Rubisco. During growth plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: **A** 5.0 mM, **B** 0.7 mM, **C** 0.1 mM

Fig. 3A–C. Variation of the rate of photosynthesis as a function of the Rubisco content of tobacco leaves when measured at irradiances of 1000 μmol · m^{-2} · s^{-1} (●) and 100 μmol · m^{-2} · s^{-1} (△). Photosynthesis was otherwise measured in the ambient growth conditions (20° C, 330 μbar CO_2, 70% relative humidity) in plants with altered amounts of Rubisco. During growth plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: **A** 5.0 mM, **B** 0.7 mM, **C** 0.1 mM

with 330 or 100 μbar CO_2. Plants with only 10–15 μmol · m^{-2} · s^{-1} Rubisco activity (Fig. 2A, B) still had photosynthetic rates similar to wild-type plants. Plants grown with a low NH_4NO_3 supply (Fig. 2C) again showed almost no decrease of photosynthesis at 1000 μbar CO_2 when Rubisco was decreased (Fig. 1G). In conditions of low CO_2 (100 μbar) the rate of CO_2 fixation was almost linearly related to the Rubisco activity measured in the leaf for all nitrogen treatments.

Figure 3 shows how the rate of CO_2 fixation was affected by short-term changes of irradiance. These re-
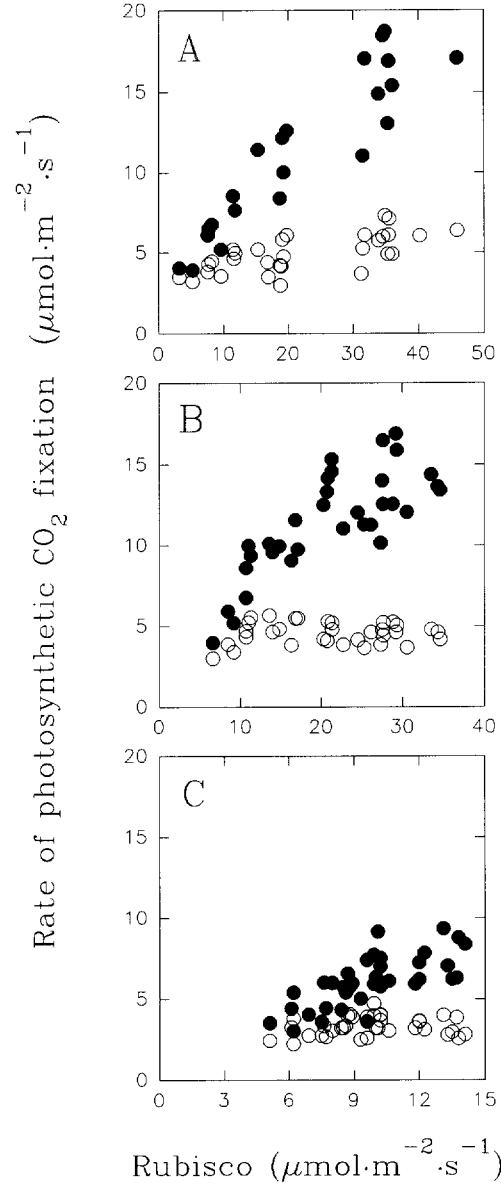
sults were obtained from the same plants used for Fig. 1 and 2. All plants were largely light-saturated at a photon fluence rate of 1000 μmol · m^{-2} · s^{-1}. At this high irradiance there was a strong dependence of the rate of CO_2 fixation on Rubisco activity at all three nitrogen supplies. However, the extent to which the rate of CO_2 fixation was stimulated by high light (compared with ambient light) depended on the nitrogen supply: for high NH_4NO_3 (Fig. 3A) the average wild-type rate was increased by 27% (12.6 to 15.9 μmol · m^{-2} · s^{-1}), for medium NH_4NO_3 (Fig. 3B) it was 20% (11.0

to 13.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and with low NH_4NO_3 (Fig. 3C) the stimulation was only 4.0% (7.00 to 7.29 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

When photosynthesis was measured in low light, again the rate of CO_2 fixation depended weakly but positively on Rubisco activity for plants grown with a high nitrogen supply (Fig. 3A). However, for plants grown with a lower nitrogen supply, the dependence was reversed (Fig. 3B, C): photosynthesis at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance actually rose as Rubisco was decreased for both 0.7 mM and 0.1 mM NH_4NO_3 supplies.

Allocation of nitrogen. The data described so far demonstrate that the extent to which the rate of photosynthesis is controlled by Rubisco depends on the supply of nitrogen to the plant. Firstly, reduced nitrogen supply lowers the amount of Rubisco to a level whereby it has a much stronger control on the rate of CO_2 fixation (see below for more details). Secondly, the increased sensitivity of photosynthesis to CO_2 partial pressure in wild-type plants grown in low nitrogen is also consistent with Rubisco exerting more control over the rate of photosynthesis in these conditions (see the mechanistic models of Farquhar and von Caemmerer 1982; Woodrow 1986; Petterson and Rhyde-Petterson 1988; Sharkey 1989 which predict that Rubisco-limited photosynthesis is more sensitive to alteration of the CO_2 partial pressure than is photosynthesis limited by ribulose-1,5-biphosphate regeneration). Thirdly, the lack of stimulation by light in plants grown with low nitrogen supply (compare Fig. 1G and Fig. 3C) is also consistent with a major role for Rubisco in controlling the rate of photosynthesis.

When nitrogen is readily available Rubisco levels are increased substantially, but changes of Rubisco content in the wild-type provide only a marginal increase in the rate of photosynthesis as measured in the ambient growth conditions. The ambient rate of photosynthesis for wild-type plants grown with a high or medium NH_4NO_3 supply is therefore only marginally dependent on the Rubisco activity (Fig. 1A, D). Clearly, ambient photosynthesis is largely restricted by other factors in high-NH_4NO_3-grown plants: when the NH_4NO_3 supply

was increased from 0.7 mM to 5.0 mM, Rubisco activity increased 40%, from 31.5 to 44.0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ whereas the average rate of CO_2 fixation, for wild-type plants, increased 5%, from 13.0 to 13.7 $\mu mol \cdot m^{-2} \cdot s^{-1}$. This extra Rubisco in high-NH_4NO_3-grown plants did, however, allow photosynthesis to proceed much faster at high irradiances and this could confer an enhanced ability to respond to upwardly fluctuating irradiances that can occur in natural environments.

Estimation of flux-control coefficients. The extent to which Rubisco controls the rate of CO_2 fixation can be quantified by calculating C_R. This is determined from the slope of the plot of photosynthesis versus Rubisco; for any given value of Rubisco the C_R can be calculated from the tangent to the normalised curve at that point. Table 1 shows approximate values of C_R for the average wild-type plant calculated from the photosynthesis data presented in Figs. 1, 2, and 3. As the Rubisco activity was reduced in and below the wild-type range, the rate of photosynthesis usually exhibited a biphasic response: an initial relatively linear decline of CO_2 fixation followed by a steeper decrease as Rubisco activity was reduced further. The values of C_R were obtained only from the first phase (in effect only from data where the Rubisco activity was reduced by no more than 45% from the average wild-type value) by calculating the slope of these data using linear regression analysis (significance levels, P, of the linear regression analysis are shown in Tables 1 and 2). The values of C_R shown are therefore only approximations and, depending on the curvature of the actual response, will tend to over-estimate the true value of C_R (see Stitt et al. 1991 for further discussion). The data of Table 1 show that the strength of Rubisco control of CO_2 fixation in the ambient growth conditions was 0.29 for both a high and medium supply of NH_4NO_3 but was increased to 0.58 when the nitrogen supply was further reduced to 0.1 mM.

Interestingly, the value of C_R was the same for both 5.0 and 0.7 mM NH_4NO_3 supply despite the fact that the average wild-type Rubisco activity was reduced by 28%. This indicates that the decrease of Rubisco in plants grown in low 0.7 mM NH_4NO_3 is accompanied by a

Table 1. Apparent strengths of Rubisco control of photosynthesis (C_R values) for tobacco plants grown under three different nitrogen regimes. Values are determined in conditions of altered actinic irradiance and CO_2 concentration. Exchange of CO_2 was measured using infrared gas analysis at 20° C. Values of C_R were calculated from the data shown in Fig 1. The slope of the graph was calculated using a linear regression of the data from the average wild-type value. Data obtained from plants where Rubisco was reduced by more than 40% were not included in the regression analysis. Significance levels of the linear regression analysis obtained from the correlation coefficients are shown (* $P \le 0.01$; ** $P \le 0.02$; *** $P \le 0.05$).

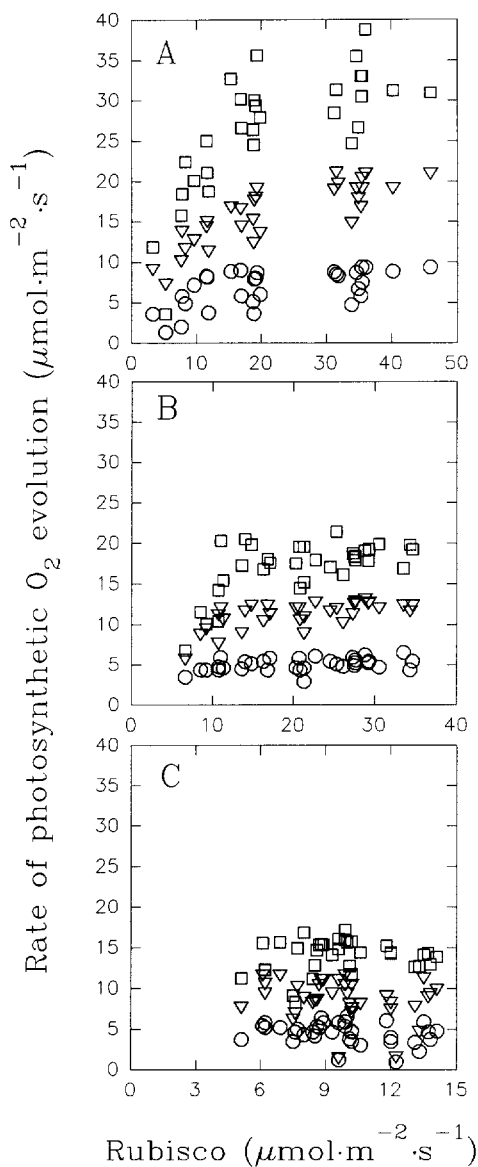| NH_4NO_3 (mM)	Irradiance ($\mu mol \cdot m^{-2} \cdot s^{-1}$)				
	1000	300	100	300	300
	CO_2 partial pressure (μbar)				
	330	330	330	1000	100
5.00	0.78*	0.29*	0.15*	0.05**	0.89*
0.70	0.60*	0.29*	−0.02**	0.06***	0.84*
0.10	0.68*	0.58*	−0.18***	0.14**	0.84***

Fig. 4A–C. Variation of the rate of photosynthetic oxygen evolution as a function of the Rubisco content of the tobacco leaf when measured in an oxygen electrode at 20° C and irradiances of 950 μmol · m^{-2} · s^{-1} (□), 250 μmol · m^{-2} · s^{-1} (△), and 100 μmol · m^{-2} · s^{-1} (○). During growth plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: A 5.0 mM, B 0.7 mM, C 0.1 mM

co contents, was only 0.56. This means that although Rubisco exerts more control on photosynthesis in low-nitrogen-grown plants (see above), other proteins have also decreased. Hence, ambient rates of photosynthesis in the wild-type do not become solely dependent on Rubisco.

The highest values of C_R were observed in conditions of low CO_2 or high irradiance for all nitrogen treatments. Previous work has shown that these are the conditions when Rubisco would be expected to be fully activated (Sage et al. 1988; Sage 1990) and we have previously shown a good correlation between the activation state of Rubisco and C_R (Stitt et al. 1991). In conditions of low light the C_R of Rubisco was low as would be predicted. However, in conditions of reduced nitrogen supply there is evidence for negative values of C_R (i.e. decreased Rubisco leads to an increased rate of photosynthesis). This would indicate that, in some conditions, reduced Rubisco activity may actually enhance the rate of photosynthesis.

Control coefficients at saturating CO_2 concentrations. Figure 4. shows how the rate of photosynthetic oxygen evolution measured in leaf discs was affected by the Rubisco content of the leaf. The high CO_2 concentration in an oxygen electrode (5%) completely saturates Rubisco with CO_2. In these conditions control by Rubisco is likely to be a reduced and the rate of photosynthesis will be controlled to a greater extent by other processes. In general, the data confirm the reduced dependence of photosynthesis on Rubisco activity, this being particularly apparent in conditions of high irradiance (950 μmol · m^{-2} · s^{-1}). The data also demonstrate the absence of major pleiotropic effects associated with reduced Rubisco activity in the transformed plants. One striking feature of these data is the almost complete lack of response of photosynthesis to Rubisco activity when the NH_4NO_3 supply is reduced (Fig. 4B). Indeed, in plant material grown at 0.1 mM NH_4NO_3 the rate of photosynthesis in antisense plants with lower Rubisco actually rose slightly compared with the wild type (Fig. 4C).

The values for C_R obtained from the data of Fig. 4 are shown in Table 2. The values of C_R were reduced con-

decline of other proteins which maintain the balance between Rubisco and other components of the photosynthetic machinery in ambient conditions. However, when the nitrogen supply was further reduced to 0.1 mM this balance was not maintained. A similar conclusion is reached if we compare plants where Rubisco was reduced to similar levels either genetically or by reducing the nitrogen supply during growth: whereas plants grown at high NH_4NO_3 and with 12–15 μmol · m^{-2} · s^{-1} Rubisco activity, had a C_R value close to unity (there was a direct linear relation between Rubisco and photosynthesis in this region, see Fig. 1a, b), the C_R value for wild-type plants grown in low NH_4NO_3, which had similar Rubis-

Table 2. Apparent strengths of Rubisco control of photosynthesis (C_R values) for tobacco plants grown under three different nitrogen regimes. Values are determined for three actinic irradiances. Photosynthesis was measured in a leaf-disc oxygen electrode at 20° C. Values of C_R were calculated from the data shown in Fig. 4. The slope of the graph was calculated using a linear regression of the data from the average wild-type value. Data obtained from plants where Rubisco was reduced by more than 40% were not included in the regression analysis. Significance levels of the linear regression analysis obtained from the correlation coefficients are shown (* $P \le 0.05$; ** $P \le 0.10$)

NH_4NO_3 (mM)	Irradiance (μmol · m^{-2} · s^{-1})		
	100	280	950
5.0	0.15*	0.21*	0.27*
0.7	0.00*	0.11*	0.19*
0.1	−0.29*	−0.20**	−0.12**

siderably in high CO_2 compared with those in Table 1, especially in conditions of high light. This adds further support to the suggestion that in these conditions factors other than Rubisco, including the rate of end-product synthesis, exert more control on the rate of photosynthesis (Sharkey 1985; Walker and Sivak 1986). The values of C_R were also further reduced at all irradiances by lowering the NH_4NO_3 supply. This is the opposite of the result obtained at non-saturating CO_2 (Table 1).

Negative values of C_R were observed with 0.1 mM NH_4NO_3 even at high light, becoming more negative at lower irradiance. The data again show that the rate of photosynthesis can be increased in plants grown with a low NH_4NO_3 supply by reducing the amount of Rubisco. The increasingly negative values of C_R in low light indicate that increased light-harvesting and electron-transport capacity in the antisense plants may be involved. It is possible than genetically reducing the amount of Rubisco may allow nitrogen, normally allocated to Rubisco, to be redistributed to other processes.

Another factor operative at high CO_2 might be the rate of end-product synthesis. This is generally thought to contribute to the control of photosynthetic rate in saturating CO_2 (Sharkey 1985; Walker and Sivak 1986). Comparison of Fig. 4 with Fig. 3 emphasises that photosynthetic capacity (the maximum rate of photosynthesis in saturating light and CO_2) is far higher than light-saturated photosynthesis in ambient CO_2 in high-nitrogen-grown material (94%) whereas this stimulation is much smaller in material grown in limiting nitrogen (68% and 59% for 0.7 mM and 0.1 mM NH_4NO_3 respectively). These results are opposite to the response to increased CO_2 under ambient light (Fig. 2), and indicate that a CO_2-insensitive process, such as end-product synthesis, may indeed contribute to the control of photosynthetic capacity in plants grown with a limiting nitrogen supply. It might also be noted that these low-nitrogen-grown plants contain very high levels of carbohydrate (Fichtner et al. 1992) which could directly and-or indirectly depress the rate of end-product synthesis. This effect might be ameliorated in antisense plants, firstly, because the lower rate of ambient photosynthesis restores the balance between photosynthesis and growth and hence decreases the accumulation of stored carbohydrate in the leaf (see following paper) and, secondly, because more nitrogen is available for allocation to the enzymes of end-product synthesis.

Chlorophyll fluorescence. Figure 5 shows chlorophyll fluorescence data measured in parallel to the photosynthesis data of Fig. 4. The chlorophyll fluorescence-quenching parameters, photochemical (q_P), a measure of the reduction state of PSII, and nonphotochemical (q_N), indicative of the transthylakoid proton gradient, were measured when photosynthesis had achieved a steady-state rate. The values of q_P (Fig. 5A–C) show that PSII remains largely oxidised at low (150 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) and medium (250 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) irradiances independent of the NH_4NO_3 supply during growth or the Rubisco activity of the leaf (plants with very low Rubisco show some reduction of PSII). At saturating irradiances, PSII
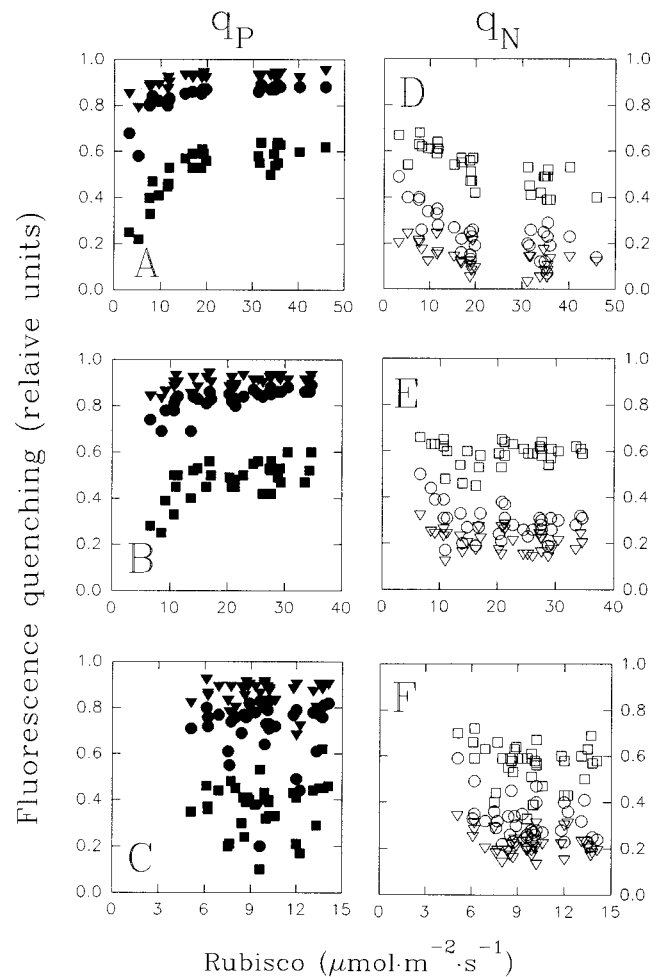

Fig. 5A–F. Effect of Rubisco content of tobacco leaves on the steady-state chlorophyll fluorescence-quenching parameters, q_P (photochemical; **A, B, C**) and q_N (nonphotochemical; **D, E, F**). Chlorophyll fluorescence was measured in an oxygen electrode at 20° C and irradiances of 950 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} (■, □) 250 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} (●, ○) 100 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} (▲, △). During growth plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: **A, D** 5.0 mM; **B, E** 0.7 mM; **C, F** 0.1 mM

is more reduced and this is increased further in plants grown with a reduced nitrogen supply. At a given irradiance, PSII reduction is always higher in plants grown with reduced nitrogen, which indicates that they become light-saturated at lower irradiances, as also shown in Fig. 3. In conditions of high light and high NH_4NO_3 supply, PSII becomes more reduced as Rubisco activity is reduced (Fig. 5A), in close agreement with the rate of photosynthesis (Fig. 4A). The reduction state of PSII is largely unaffected by Rubisco activity in low-nitrogen grown plants, resembling the rate of photosynthesis in saturating CO_2 (Fig. 4C).

Nonphotochemical quenching increased as the Rubisco activity decreased in conditions of high NH_4NO_3 supply (Fig. 5D). This is in agreement with previous work where it was shown that a high q_N was associated with an increased ATP/ADP ratio and an increased activation state of Rubisco (Quick et al. 1991). The in-
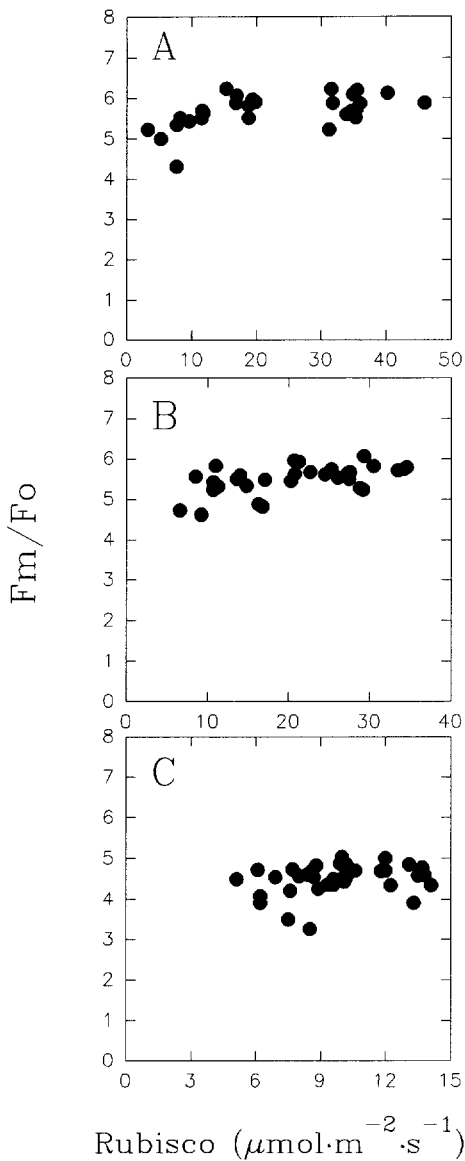
Fig. 6A–C. Effect of Rubisco content of tobacco leaves on the ratio of initial to maximum chlorophyll fluorescence (Fm/Fo). Leaf discs were darkened for 60 min prior to measurement in an oxygen electrode. During growth plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: **A** 5.0 mM, **B** 0.7 mM, **C** 0.1 mM

creased activation of Rubisco was previously shown to be the primary factor that allowed transformed plants to compensate for reduced Rubisco. However, at reduced nitrogen supply, q_N did not increase as Rubisco activity was decreased (Fig. 5E, F), indicating that neither the ATP/ADP ratio nor Rubisco activation were changing in response to reduced Rubisco and therefore there was no restriction on electron transport.

Several studies have shown that photosynthetic capacity rather than quantum yield are reduced in plants grown with limiting nitrogen (Evans 1983; Evans 1989) and this can lead to photoinhibition (Khamis et al. 1990). The ratios of initial to maximum fluorescence yield (Fm/Fo) ratio determined from chlorophyll fluorescence measure-

ments made on plants that were darkened for 1 h are shown in Fig. 6. Plants grown with either 5.0 or 0.7 mM NH_4NO_3 show a high Fm/Fo ratio, indicative of non-stressed healthy plants, and only in plants with severely reduced Rubisco activity was any decline apparent in the Fm/Fo ratio. Plants grown with a low NH_4NO_3 supply showed a reduced Fm/Fo ratio (4.6 compared with 5.6) which was also largely independent of Rubisco activity and indicates that some photoinhibition had occurred. It has previously been shown that plants grown with a low nitrogen supply have high levels of zeaxanthin compared with violoxanthin, a situation which is associated with a reduced Fm/Fo ratio, is was thought to serve as a protection against photoinhibition (Khamis et al. 1990).

Chlorophyll content. The chlorophyll content of the leaf and the chlorophyll a/b ratio are shown in Fig. 7. The amount of chlorophyll found in a leaf of wild-type plants decreased dramatically when the NH_4NO_3 supply was reduced (470, 300, 135 mg $\cdot$ m^{-2} for 5.0, 0.7 and 0.1 mM NH_4NO_3 respectively) in agreement with other studies (see Evans 1989). The amount of chlorophyll was also affected by the Rubisco activity of the leaf. In plants grown with a high NH_4NO_3 supply (Fig. 7D) the amount of chlorophyll in the leaf remained largely constant until the Rubisco activity was reduced by more than 50% when it declined steeply (see also Quick et al. 1991). In plants grown with medium NH_4NO_3 (Fig. 7E) the chlorophyll
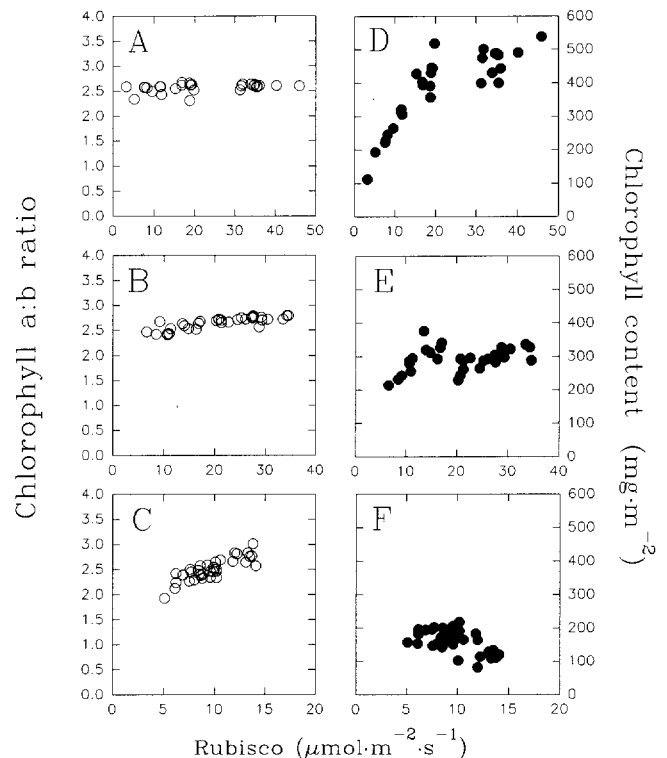

Fig. 7A–F. Effect of Rubisco content of tobacco leaves on the chlorophyll content (●) and the chlorophyll a/b ratio (○). During growth plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: **C, D** 5.0 mM; **B, E** 0.7 mM, **C, F** 0.1 mM
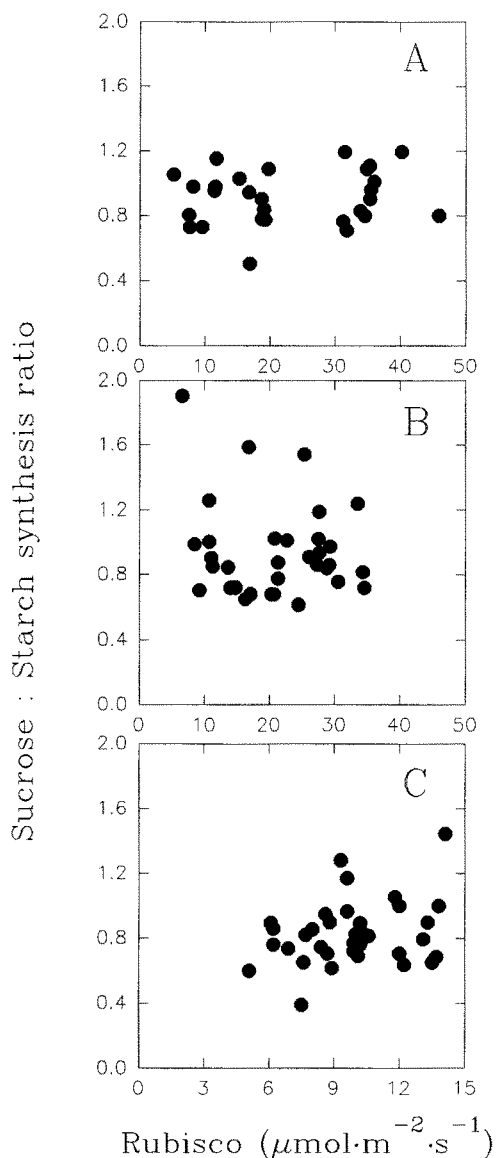
Fig. 8A–C. Variation of the ratio of $^{14}CO_2$ incorporated into starch (insoluble fraction) or sucrose (soluble neutral fraction) during photosynthesis as a function of the Rubisco content of the leaf when measured in an oxygen electrode at 20° C and 950 μmol · m^{-2} · s^{-1} light. During growth plants were supplied with nutrient solution containing different concentrations of NH_4NO_3: **A** 5.0 mM, **B** 0.7 mM, **C** 0.1 mM

content remained relatively constant, independent of the Rubisco activity of the leaf. Plants grown in low NH_4NO_3 showed a pronounced increase in chlorophyll content as Rubisco activity was reduced (Fig. 7F). This increase in chlorophyll agrees with the increase in photosynthetic rate that was observed in these plants when the Rubisco activity was reduced and provides further support for our suggestion that the nitrogen made available when Rubisco decreases has been reallocated to the electron-transport chain. The chlorophyll a/b ratio was largely unaltered in wild-type plants grown with different amounts of NH_4NO_3 (Fig. 7A, B, C) and was also largely unaffected by the Rubisco content of the leaf for plants grown with medium or high nitrogen

(Fig. 7A, B). However, plants grown in low nitrogen showed a decline in the chlorophyll a/b ratio as the Rubisco activity decreased (Fig. 7C). A decrease in the chlorophyll a/b ratio indicates that the ratio of light-harvesting to reaction-centre proteins is increased. This explains why plants with reduced Rubisco activity and grown with a low nitrogen supply had higher rates of photosynthesis in low light compared with wild-type plants (Fig. 3C, 4C).

Plants grown in low nitrogen show a large accumulation of carbohydrate in their leaves (Fichtner et al. 1992). In order to examine how this large accumulation of carbohydrate affected the partitioning of photosynthate we measured the short-term incorporation of $^{14}CO_2$ into starch and sucrose (Fig. 8). The photosynthetic rates measured in these samples (data not shown) were similar to those measured earlier in a different set of samples (Fig. 4). Incorporation of $^{14}CO_2$ into ionic fractions represented 19, 13 and 16% of total radioactive counts for plants grown with 5.0, 0.7, and 0.1 mM NH_4NO_3 supply, respectively, and this was not affected by Rubisco activity (data not shown). The partitioning of $^{14}CO_2$ between sucrose (soluble neutral) and starch (insoluble) is shown as a ratio in Fig. 8. For wild-type plants the average partitioning value was 0.95, 0.94, and 0.84 for plants grown with 5.0, 0.7, and 0.1 mM NH_4NO_3 supply, respectively. This was also not affected by Rubisco activity. Despite large changes in the carbohydrate content of the leaves brought about either genetically (reduced Rubisco) or by the supply of nitrogen (Fichtner et al. 1992), very little effect on the short-term partitioning of CO_2 was observed, indicating that any feed-back inhibition of photosynthesis is affecting both starch and sucrose synthesis in a broadly parallel manner.

Conclusions. Four main points arise from these data:

1. The strength of Rubisco control of photosynthesis (C_R) is altered by changes in the short-term environmental conditions. Generally, C_R is increased in conditions of increased irradiance or decreased CO_2.

2. The amount of Rubisco in wild-type plants is reduced as the nitrogen supply during growth is reduced. Using plants with genetically manipulated Rubisco activity, we have shown that C_R is increased in low-nitrogen-grown plants. This implies that there is a specific reduction in the amount of this protein compared with other components of the photosynthetic machinery.

3. In this sense the allocation of nitrogen to Rubisco is optimised. When a plant is grown in limiting nitrogen, the relative allocation of nitrogen to Rubisco is decreased, such that just enough Rubisco is produced to avoid a one-sided limitation of photosynthesis. The "luxury" additional investment of nitrogen in plants grown in high nitrogen means that, in these conditions, Rubisco can also be considered a nitrogen-store, all be it one where the opportunity cost of the nitrogen storage is higher than in a non-functional storage protein (e.g. it allows for a slightly higher water-use efficiency and for photosynthesis to respond to temporarily high irradiance).

4. Plants grown with low nitrogen and genetically reduced levels of Rubisco have a higher chlorophyll content and a lower chlorophyll a/b ratio than wild-type plants. This indicates that the nitrogen made available by genetically reducing the amount of Rubisco has been re-allocated to other components, including light-harvesting and electron-transport proteins. This allowed genetically manipulated plants to have higher rates of photosynthesis (negative values of C_R) than the equivalent wild-type plants in some conditions (high CO_2 or low irradiance).

An intriguing possibility remains that genetically decreasing Rubisco activity may be of benefit to plants grown in low light; experiments are in currently in progress to test this hypothesis.

References

Chapin, F.S., Walter, C.H.S., Clarkson, D.T. (1988) Growth response of barley and tomato to nitrogen stress and its control by abscisic acid, water relations and photosynthesis. Planta 173, 352–366

Clough, J.M., Peet, M.M., Kramer, P.J. (1981) Effect of high atmospheric CO_2 and sink size on rates of photosynthesis of a soybean cultivar. Plant Physiol. 67, 1007–1010

Cure, J.D., Israel, D.W., Rufty, T.W. (1988) Nitrogen stress affects growth and seed yield of non-nodulated soybean exposed to elevated carbon dioxide. Crop Sci. 28, 671–677

Evans, J.R. (1983) Nitrogen and photosynthesis in the flag leaf of wheat (Triticum aestivum L.). Plant Physiol. 72, 297–302

Evans, J.R. (1989) Photosynthesis and nitrogen relationships in leaves of C3 plants. Oecologia 78, 9–19

Farquhar, G.D., von Caemmerer, S. (1982) Modelling of photosynthetic response to environmental conditions. In: Encyclopedia of plant physiology, vol. 12B: Physiological plant ecology II, pp. 549–587, Lange, O.L., Osmond, C.D., Ziegler, H., eds. Springer, Berlin, Heidelberg, New York

Fichtner, K., Quick, W.P., Schulze, E.-D., Mooney, H.A., Rodermel, S.R., Bogorad, L., Stitt, M. (1992) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" rbcS. V. Relationship between photosynthetic rate, storage strategy, biomass allocation and vegetative plant growth at three different nitrogen supplies. Planta in press

Hirose, T. (1986) Nitrogen uptake and plant growth. II. An empirical model of vegetative growth and partitioning. Ann. Bot. 58, 487–496

Karmoker, J.L., Clarkson, D.T., Saker, L.R., Joy, M., Rooney, M., Purves, J.V. (1991) Sulphate deprivation depresses the transport of nitrogen to the xylem and the hydraulic conductivity of barley roots. Planta 185, 269–278

Khamis, S., Lamaze, T., Lemoine, Y., Foyer, C.H. (1990) Adaptation of the photosynthetic apparatus in maize leaves as a result of nitrogen limitation. Plant Physiol. 94, 1436–1443

Mizrahi, Y., Richmond, A.E. (1972) Abscisic acid in relation to mineral deprivation. Plant Physiol. 50, 667–670

Petterson, G., Ryde-Petterson, U. (1988) A mathematical model of the Calvin photosynthesis cycle. Eur. J. Biochem. 175, 661–672

Quick, W.P., Stitt, M. (1989) An examination of factors contributing to non-photochemical quenching of chlorophyll fluorescence in barley leaves. Biochim. Biophys. Acta 977, 287–296

Quick, W.P., Neuhaus, H.E., Stitt, M. (1989) Increased pyrophosphate is responsible for a restriction of sucrose synthesis after supplying fluoride to spinach leaf discs. Biochim. Biophys. Acta 973, 263–271

Quick, W.P., Schurr, U., Scheibe, R., Schulze, E.-D., Rodermel, S.R., Bogorad, L., Stitt, M. (1991a) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" rbcS. I. Impact on photosynthesis in ambient growth conditions. Planta 183, 542–554

Quick, W.P., Schurr, U., Fichtner, K., Schulze, E.-D., Rodermel, S.R., Bogorad, L., Stitt, M. (1991b) The impact of decreased Rubisco on photosynthesis and plant growth, allocation and storage in tobacco plants which have been transformed with antisense rbcS. Plant J. 1, 51–58

Rodermel, S.R., Abbott, M.S., Bogorad, L. (1988) Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell 55, 673–681

Rufty, T.W., Raper, D.C., Huber, S.C. (1984) Alterations in internal partitioning of carbon in soybean plants in response to nitrogen stress. Can. J. Bot. 62, 501–508

Sage, R.F. (1987) The effect of temperature on the occurrence of CO_2 and O_2 insensitive photosynthesis in field grown plants. Plant Physiol. 84, 658–664

Sage, R.F., Sharkey, T.D., Seeman, J.R. (1988) The in-vivo response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO_2 in Phaseolus vulgaris. Planta 174, 407–416

Sage, R.F. (1990) A model describing the regulation of Rubisco, electron transport and triose phosphate use, in response to light intensity and CO_2 in C3 plants. Plant Physiol. 94, 1728–1734

Sharkey, T.D. (1985) Photosynthesis in intact leaves of C3 plants: physics, physiology and rate limitations. Bot. Rev. 51, 53–106

Sharkey, T.D. (1989) Evaluating the role of Rubisco activation in photosynthesis of C3 plants. Philos. Trans. R. Soc. London Ser. B 323, 435–448

Stitt, M., Quick, W.P., Schurr, U., Fichtner, K., Schulze, E.-D., Rodermel, S.R. and Bogorad, L. (1991) Decreased ribulose-1,5-bisphoshate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" rbcS. II. Flux-control coefficients for photosynthesis in varying light, CO_2 and air humidity. Planta 183, 555–566

von Caemmerer, S., Farquhar, G.D. (1984) Effects of partial defoliation, changes in irradiance during growth, short term water stress and growth at enhanced $p(CO_2)$ on the photosynthetic capacity of leaves of Phaseolus vulgaris L. Planta 160, 320–329

von Caemmerer, S., Farquhar, G.D. (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153, 376–387

Vu, C.V., Allen, L.H., Bowes, G. (1983) Effects of light and elevated CO_2 on the Rubisco activity and RUBP level of soybean leaves. Plant Physiol. 73, 729–734

Walker, D.A., Sivak, M.N. (1986) Photosynthesis and phosphate: a cellular affair? Trends Biochem. Sci. 11, 176–179

Wong, S.C. (1979) Elevated atmospheric partial pressure of CO_2 and plant growth. I. Interactions of nitrogen nutrition and photosynthetic capacity in C3 and C4 plants. Oecologia 44, 68–74

Woodrow I.E. (1986) Control of the rate of photosynthetic carbon dioxide fixation. Biochim. Biophys. Acta 851, 181–192

Zimmerman, R., Oren, R., Schulze, E.-D., Werk, K.S. (1988) Performance of two Picea abies L. Karst. stands at different stages of decline. II. Photosynthesis and leaf conductance. Oecologia 76, 513–516

Note added in proof: Paper III in this series is Quick et al. (1991b)