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Photosynthetic Acclimation to Elevated CO_2 Occurs in Transformed Tobacco with Decreased Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Content[1]

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Inhibition of net carbon assimilation rates during growth at elevated CO_2 was studied in transgenic tobacco (*Nicotiana tabacum* L.) plants containing zero to two copies of antisense DNA sequences to the small subunit polypeptide (*rbcS*) gene of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). High- and low-Rubisco tobacco plants were obtained from the selfed progeny of the original line 3 transformant (S.R. Rodermel, M.S. Abbott, L. Bogorad [1988] Cell 55: 673–681). Assimilation rates of high- and low-Rubisco tobacco plants increased 22 and 71%, respectively, when transferred from 35- to 70-Pa CO_2 chamber air at 900 μmol m^{-2} s^{-1} photon flux density. However, CO_2-dependent increases of net carbon assimilation rates of high- and low-Rubisco plants virtually disappeared after 9 d of growth in elevated CO_2 chamber air. Total above-ground dry matter production of high- and low-Rubisco plants was 28 and 53% greater, respectively, after 9 d of growth at 70 Pa compared with 35 Pa CO_2. Most of this dry weight gain was due to increased specific leaf weight. Rubisco activity, Rubisco protein, and total chlorophyll were lower in both high- and low-Rubisco plants grown in enriched compared with ambient CO_2 chamber air. Soluble leaf protein also decreased in response to CO_2 enrichment in high- but not in low-Rubisco tobacco plants. Decreased Rubisco activities in CO_2-adapted high- and low-Rubisco plants were not attributable to changes in activation state of the enzyme. Carbonic anhydrase activities and subunit levels measured with specific antibodies were similar in high- and low-Rubisco tobacco plants and were unchanged by CO_2 enrichment. Collectively, these findings suggested that photosynthetic acclimation to enriched CO_2 occurred in tobacco plants either with or without transgenically decreased Rubisco levels and also indicated that the down-regulation of Rubisco in CO_2-adapted tobacco plants was related to decreased specific activity of this enzyme.

Carbon assimilation rates of almost all terrestrial plants are increased by brief exposures to elevated atmospheric CO_2 (Kimball, 1983; Cure and Acock, 1986). However, photosynthetic rates of perennial and annual species possessing the C_3 carbon reduction pathway frequently acclimated to elevated CO_2 environments after a period of days or weeks (Peet et al., 1986; Sage et al., 1989). Photosynthetic adjustment to CO_2 enrichment varies extensively on an interspecific basis. Acclimation to elevated CO_2 usually results in a down-regulation of CO_2 fixation, although long-term positive changes in carbon assimilation rate also have been reported (e.g. Campbell et al., 1990; Ziska et al., 1990). Negative photosynthetic acclimation was severe in tobacco (*Nicotiana tabacum*) (Raper and Peedin, 1978) and cabbage (Sage et al., 1989) but almost negligible in soybean (Campbell et al., 1988) and potato (Sage et al., 1989). Variation in photosynthetic adjustment also has been observed within the same species (i.e. Clough et al., 1981; Campbell et al., 1990; Bunce, 1992). It will not be possible to predict long-term effects of elevated CO_2 on plant growth and development without an improved understanding of the underlying mechanisms responsible for photosynthetic acclimation.

Biochemical factors that induce photosynthetic acclimation during plant growth in elevated CO_2 are poorly understood. A number of investigators have reported that Rubisco activity was decreased in species exhibiting negative photosynthetic acclimation (Wong, 1979; Peet et al., 1986; Sage et al., 1989; Yelle et al., 1989b). Decreased Rubisco activity resulted from a decline in leaf enzyme content in some species (Sage et al., 1989; Besford et al., 1990; Rowland-Bamford et al., 1990) and a change in enzyme carbamylation state in others (Sage et al., 1989). It was not determined if decreased carboxylase capacity contributed directly to the suppression of photosynthesis in the majority of studies performed to date (Stitt, 1991).

Transgenically modified plants offer a novel approach to the study of photosynthetic metabolism and the physiological responses of plants to environmental change (Sonnewald and Willmitzer, 1992). Rodermel et al. (1988) genetically transformed tobacco plants with antisense DNA sequences to *rbcS*. The transformants synthesized mRNA in the antisense orientation and exhibited decreased Rubisco protein and activity. Moreover, whole-plant growth rates were inversely proportional to the number of antisense DNA copies present. The transformed plants had altered photosynthetic properties, although under some conditions up to 50% of the Rubisco could be removed from the leaf without affecting net CO_2 assimilation rates (Quick et al., 1991b; Stitt et al., 1991).

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Abbreviations: *rbcS*, nuclear gene encoding the small subunit of Rubisco; SLW, specific leaf weight.

The objective of the current study was to assess the relationships between net carbon assimilation rate, leaf Rubisco content, leaf Rubisco activity, and photosynthetic adjustment in normal and transformed tobacco plants. Carbonic anhydrase activity also was examined, because of its potential involvement in photosynthetic acclimation (Porter and Grodzinski, 1984) and because decreased levels of this enzyme have been observed in transformed tobacco with lowered Rubisco levels (Hudson et al., 1992).

MATERIALS AND METHODS

Plant Materials

Experiments were conducted in facilities of the Climate Stress Laboratory at Beltsville, MD, beginning on April 6, 1992. Studies were performed using the selfed progeny of transformant line 3 of tobacco (*Nicotiana tabacum* L. cv W38) as described previously (Rodermel et al., 1988). The selfed line 3 germplasm segregated into large, medium, and small plants in a 1:2:1 ratio having either 0, 1, or 2 copies of *rbcS* antisense DNA sequences, respectively (Quick et al., 1991a, 1991b).

Seeds were germinated on the surface of 1.5-L plastic pots containing water-saturated Jiffy-mix[2] and vermiculite in a 1:1 ratio. The pots were covered with one or two layers of clear plastic wrap and seeds were allowed to germinate for 1 week in controlled environment chambers at 27°C with a 14-h light/10-h dark cycle. The plastic wrap was removed and the seedlings were grown for an additional 2 weeks at an irradiance of 450 μmol m^{-2} s^{-1} PPFD provided by a mixture of 24 1.5-A fluorescent tubes (F96T12CW/VHO, GTE-Sylvania, Danvers, MA) and 10 52-W incandescent bulbs (11441/WM, General Electric Co., Louisville, KY). Single seedlings were transplanted to individual 3.0-L pots essentially as described above. Plants were irrigated from the bottom with full-strength mineral nutrient solution (Robinson, 1984), and supplemental CO_2 (Potomac Air Gas, Inc., Linthicum, MD) was injected into the chamber to maintain a minimal CO_2 partial pressure of 35 Pa, as previously described (Bunce, 1992).

The segregating population was divided into high- and low-Rubisco plants 2 weeks after transplanting. Putative high- and low-Rubisco tobacco plants were preliminarily identified based on differences in plant height, vigor, and anthesis date (Rodermel et al., 1988). Subsequently, leaf Rubisco protein levels of each tobacco plant were quantified directly (see below), and low-Rubisco plants were identified as having less than 3 g m^{-2} Rubisco (see Rodermel et al., 1988; Quick et al., 1991b). Eight high- and eight low-Rubisco plants were transferred to high-irradiance (900 ± 25 μmol m^{-2} s^{-1} PPFD) controlled-environment chambers 1 week prior to initiating elevated CO_2 treatments. High-irradiance chambers were equipped with a mixture of high-pressure sodium and multivapor high-intensity discharge lamps (models LU400 and MVR400/I/U, respectively, General Electric Co., Cleveland, OH). Ambient (35 ± 2 Pa) and elevated CO_2 treatments (70 ± 3 Pa) were imposed using matching controlled environment chambers after 6 weeks of growth when the largest members of the segregating population attained a height of 15 to 20 cm. Plants were about 90 cm tall when the studies were terminated 9 d later. All experiments were repeated at least once.

[2] Mention of a trademark, proprietary product, or vendor neither implies an endorsement nor constitutes a warranty of the product by the U.S. Department of Agriculture.

Photosynthesis Measurements

The high- and low-Rubisco plants were about 2 and 5 d prior to anthesis, respectively, when the CO_2 treatments and photosynthetic measurements were initiated. Net CO_2 exchange rates were determined on the most recently expanded leaf on each plant. The selected leaf usually was the sixth from emergence and was over 80% of final size at the start of CO_2 enrichment. Gas-exchange measurements were performed on the proximal half of the leaf using a portable, closed photosynthesis system (model 6200, Li-Cor, Inc., Lincoln, NE) equipped with a 1-L clamp-on leaf cuvette and having an exposed area of 10 cm^2. Light, temperature, humidity, and CO_2 levels were adjusted to the current controlled environment settings for plant growth. Measurements were performed on leaves that had been in light for a minimum of 4 h and were repeated until two consecutive measurements varied by less than 10%. Immediately after the net CO_2 assimilation rate was determined, four to six 0.8-cm^2 leaf discs were removed from the distal portion of the leaf. Leaf samples were instantly frozen in liquid N_2 and stored at −80°C until extraction. Dry matter distribution was determined following the last photosynthesis measurement for each experiment (Bunce, 1992).

Rubisco Measurements

Rubisco assays were performed as described previously (Perchorowicz et al., 1981; Quick et al., 1991b). Individual frozen leaf discs were extracted in ground-glass tissue homogenizers at 0°C in 1 mL of buffer containing 50 mM Bicine-NaOH, pH 8.2, 10 mM $MgCl_2$, 10% (v/v) glycerol, 5 mM DTT, and 0.01% Triton X-100. Extracts were centrifuged for 3 min at 4°C in a microcentrifuge (model B, Beckman, Fullerton, CA), and the supernatants were immediately transferred to liquid N_2. Enzyme assays were performed immediately after thawing the samples on ice. Initial, or unactivated Rubisco activity, was determined radiochemically in stoppered vials containing 40 mM Bicine-NaOH, pH 8.2, 8 mM $MgCl_2$, 0.6 mM ribulose-1,5-bisP (Sigma), and 10 mM [^{14}C]$NaHCO_3$ (0.35 Ci mol^{-1}). Assays were initiated with 0.02 mL of sample and were terminated after 30 s at 30°C with 0.2 mL of 0.5 N HCl. Total, or fully activated Rubisco activity, was determined similarly except that samples were preincubated in the assay buffer for 6 to 8 min at 30°C before initiating the reactions with ribulose-1,5-bisP. An aliquot of each leaf extract also was used to measure soluble protein (Bradford, 1976). One leaf disc from each set of samples was extracted with 80% (v/v) acetone and used for Chl determinations (Vernon, 1960).

Rubisco protein levels were measured using a dye-binding method as described by Makino et al. (1986). Briefly, two

tobacco leaf discs (1.6 cm^2) were homogenized with 0.75 mL of buffer containing 50 mM Bicine-NaOH, pH 8.6, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, and 40 mM 2-mercaptoethanol, and the samples were spun in a microcentrifuge (model B, Beckman) for 2 min at full power to remove insoluble debris. Supernatant fractions were diluted with an equal volume of 100 mM Tris-HCl buffer (pH 6.8) containing 3.3% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, and 33% (v/v) glycerol, and soluble proteins were denatured at 100°C for 30 s. After SDS-PAGE at 200 V for 55 min in a mini-gel apparatus (Bio-Rad) the gels were washed briefly in distilled H_2O and stained for 1 h with 0.25% (w/v) Coomassie brilliant blue-R in 45% (v/v) methanol and 10% (v/v) acetic acid. The gels were then destained overnight in 20% methanol and 7% (v/v) acetic acid with constant agitation in the presence of activated charcoal. The large subunit of Rubisco (55 kD) was excised from each lane and the stained bands were extracted with 1 mL of formamide for 6 h at 50°C in the dark. A_{595} was measured and the Rubisco concentration was quantified using standard curves prepared with purified tobacco Rubisco isolated by repeated crystallization and washing according to Servaites (1985).

Carbonic Anhydrase

Carbonic anhydrase activity was measured as the rate of CO_2 hydration using a pH-dependent assay (Keys and Parry, 1990). Single tobacco leaf discs were extracted at 0°C as described above with 1 mL of buffer containing 100 mM Tris-H_2SO_4, pH 8.3, and 1 mM EDTA. Enzyme activity was determined at 0°C by mixing 1 mL of CO_2-saturated water with 1 mL of 20 mM Hepes-NaOH, pH 8.3, containing 0.002% (w/v) bromthymol blue and 10 to 20 μL of leaf extract. Enzyme activity units were calculated according to the formula $2(T_o/T - 1)$ where T_o = reaction time without enzyme and T = reaction time with enzyme, as described by Shiraiwa and Miyachi (1983).

RESULTS

Plant Growth and Dry Matter Distribution

The effects of CO_2 enrichment on dry matter allocation of high- and low-Rubisco tobacco plants are shown in Table I. Total above-ground dry weights of high- and low-Rubisco plants increased 19.1 and 9.8 g, respectively, in response to doubling the ambient CO_2 partial pressure from 35 to 70 Pa. Increased leaf dry weight constituted 60 and 90% of the total difference in plant dry weight gain of CO_2-enriched high- and low-Rubisco tobacco plants, respectively. Total leaf areas of ambient CO_2-grown high- and low-Rubisco tobacco plants were not significantly different, although leaf areas of high-Rubisco tobacco plants were 17% greater ($P < 0.05$) following 9 d of growth in CO_2-enriched compared with ambient CO_2 chamber air. By comparison, total leaf areas of low-Rubisco plants measured on the last sampling date were unaffected by CO_2 treatment ($P > 0.05$). The SLW (g m^{-2}) of low-Rubisco plants was lower than that of the high-Rubisco plants in ambient CO_2 chamber air ($P < 0.01$). This difference was not observed after 9 d of growth in elevated CO_2 chamber air.

Net CO_2 Exchange Rates

Maximum carbon assimilation rates of high- and low-Rubisco tobacco plants grown and measured in ambient CO_2 chamber air were 26.3 ± 0.8 and 19.1 ± 0.5 μmol m^{-2} s^{-1} (n = 4), respectively, at 900 μmol m^{-2} s^{-1} PPFD (Fig. 1, A and B). Peak rates were observed 3 d after the experiment was initiated. On the final measurement, net assimilation rates of ambient CO_2-grown high- and low-Rubisco tobacco plants were 35 and 25% below these peak rates, respectively. In agreement with earlier reports (Quick et al., 1991b; Hudson et al., 1992), net carbon assimilation rates of high- and low-Rubisco tobacco plants initially increased 22 and 71%, respectively, when the chamber air CO_2 partial pressure was doubled. Mean photosynthesis values of high- and low-Rubisco plants were not significantly different ($P > 0.05$) over 9 d of growth in CO_2-enriched chamber air. The CO_2-dependent stimulation of net carbon assimilation rates of both high- and low-Rubisco tobacco plants disappeared after 9 d of growth in CO_2-enriched chamber air, relative to photosynthesis rates of plants grown and assayed in ambient CO_2 chamber air.

Rubisco Activity

Both initial and total Rubisco rates were measured for each sample at 30°C. Mean Rubisco activities of tobacco leaf extracts were not significantly different ($P > 0.05$) when measured before and after Mg^{2+}- and CO_2-dependent activation. Initial and total Rubisco rates of high-Rubisco plants grown in ambient CO_2 were 143 ± 10.2 and 141 ± 11.0 μmol m^{-2} s^{-1} (n = 18), respectively, averaged over the 9-d study. Under the same conditions, initial and total Rubisco rates of low-Rubisco plants were 56 ± 3.0 and 50.1 ± 2.3 μmol m^{-2}

Table I. *Effects of CO_2 enrichment on growth and dry matter distribution in a segregating population of tobacco plants transformed with antisense cDNA sequences to the rbcS gene*

Leaf area, leaf weight, and total plant dry weight (DW) of high- and low-Rubisco tobacco plants (i.e. zero to two copies of antisense cDNA) were measured after 9 d of growth in ambient (35 Pa) or in elevated (70 Pa) CO_2 chamber air.

Sample	n	CO_2	Total DW	Leaf DW	Leaf Area	SLW
		Pa	*g*	*g*	*m^2*	*g m^{-2}*
High Rubisco	9	35	37.1 ± 1.6	17.0 ± 0.9	0.30 ± 0.01	57.4 ± 2.4
	8	70	56.2 ± 1.4	28.4 ± 0.7	0.35 ± 0.02	82.7 ± 5.3
Low Rubisco	5	35	30.6 ± 1.1	16.2 ± 0.3	0.33 ± 0.01	49.4 ± 1.1
	4	70	40.4 ± 0.5	25.1 ± 0.2	0.32 ± 0.01	79.2 ± 1.3

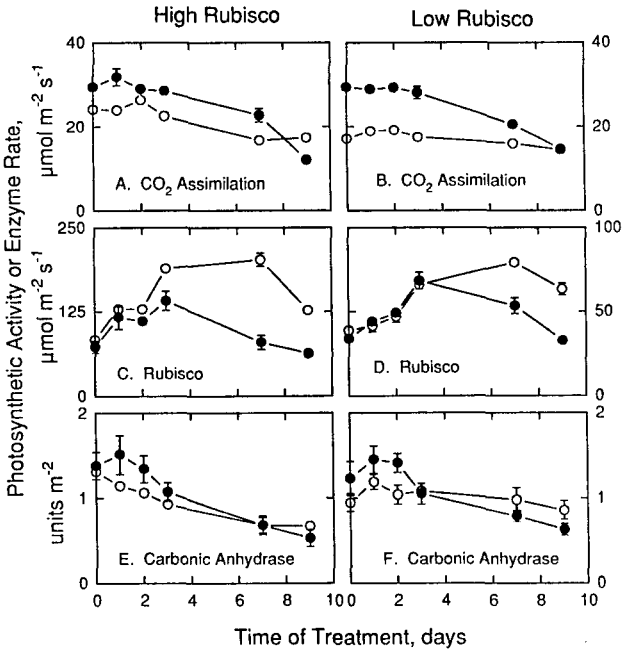

Figure 1. Photosynthetic responses and enzyme activities in leaves of transgenic tobacco plants during growth in ambient and elevated CO_2. Net CO_2 assimilation rates (A and B), total Rubisco activities (C and D, note scale change), and extractable carbonic anhydrase activities (E and F) of high- (A, C, and E) and low- (B, D, and F) Rubisco tobacco plants grown in ambient (O, 35 Pa) and enriched (●, 70 Pa) CO_2 chamber air are shown. Values are means ± SE for $n = 4$ to 8.

s^{-1} ($n = 30$), respectively. No change in Rubisco activation state of either high- or low-Rubisco plants was observed upon acclimation to enriched CO_2 (data not shown).

Rubisco activities in extracts of high- and low-Rubisco tobacco plants grown in ambient CO_2 were maximal (i.e. 202 and 79 μmol m^{-2} s^{-1}, respectively, at 30°C) between 3 and 7 d of treatment (Fig. 1, C and D). Thus, tobacco plants transformed with one to two copies of antisense *rbcS* DNA had about 60% less Rubisco activity on average than the high-Rubisco controls. Rubisco activities of high- and low-Rubisco plants grown in ambient CO_2 decreased 20 to 40% below these peak rates after 9 d of treatment. Rubisco activity measurements of high- and low-Rubisco tobacco plants were unaffected by CO_2 enrichment during the first 2 to 3 d of treatment. However, after 7 to 9 d of growth in CO_2-enriched chamber air, Rubisco rates of high- and low-Rubisco tobacco plants were 40 to 60% less than the corresponding activities of the ambient CO_2-grown plants.

Carbonic Anhydrase Activity

Carbonic anhydrase activities of leaf extracts from high- and low-Rubisco tobacco plants grown in ambient CO_2 and CO_2-enriched chamber air are shown in Figure 1, E and F. Mean carbonic anhydrase activities of high- and low-Rubisco tobacco plants were between 1.1 and 1.3 enzyme units m^{-2} initially. Enzyme rates of both genotypes grown in ambient CO_2 chamber air decreased about 40% between the first and last harvest. Carbonic anhydrase activities of high- and low-Rubisco tobacco plants, averaged over all sampling dates, were not significantly different ($P > 0.05$). Furthermore, carbonic anhydrase activities of both high- and low-Rubisco plants were unaffected by 9 d of CO_2 enrichment ($P > 0.05$).

Amounts of carbonic anhydrase protein in tobacco leaf extracts also were quantified by scanning western blots and analyzing the digital images by computer (data not shown). One major reactive polypeptide band with a molecular mass of 29 kD was observed when blots of tobacco leaf extracts were probed with rabbit antiserum prepared against purified spinach leaf carbonic anhydrase (Fawcett et al., 1990). In agreement with the enzyme rate measurements reported above, similar image densities attributable to carbonic anhydrase were observed in high- and low-Rubisco samples on d 0 of CO_2 enrichment. Moreover, carbonic anhydrase protein levels of both high- and low-Rubisco samples either were unchanged or decreased only slightly after 9 d of growth in either ambient CO_2 or in CO_2-enriched chamber air.

Leaf Chl

Amounts of Chl in high- and low-Rubisco tobacco plants are shown in Figure 2, A and B, respectively. Maximum Chl levels in both the high- and low-Rubisco plants grown in ambient CO_2 chamber air were about 0.6 g m^{-2}. Quick et al. (1991b) also reported that Chl levels were unchanged in transgenic tobacco plants containing 50% or more of the wild-type Rubisco activity. Both high- and low-Rubisco leaf Chl concentrations were 20 to 40% lower ($P < 0.05$) in plants grown in enriched rather than in ambient CO_2.

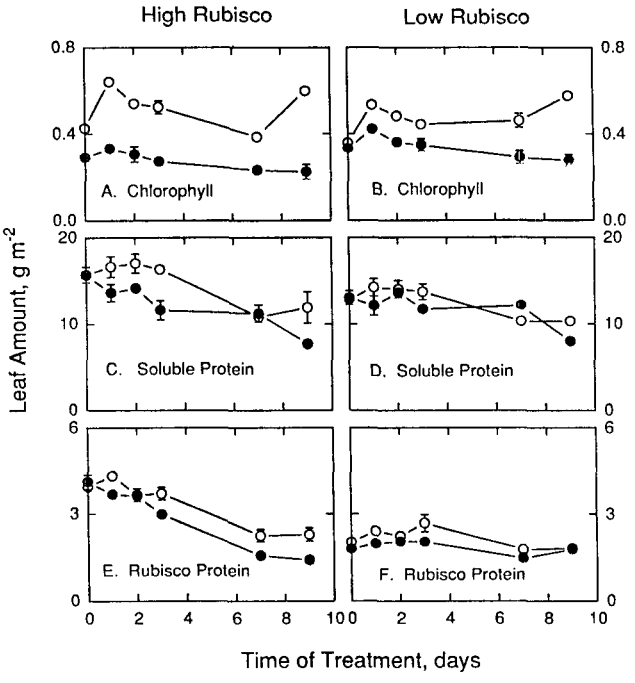

Figure 2. Effects of CO_2 enrichment on Chl and leaf protein levels in transgenic tobacco plants. Experimental details and symbols are as in Figure 1 except that total Chl (A and B), soluble leaf protein (C and D), and Rubisco protein (E and F) are shown.

Buffer-Soluble Protein

Mean soluble leaf protein concentrations were 2.0 ± 0.7 g m^{-2} greater ($P < 0.05$) in high- than in low-Rubisco tobacco plants on the first sampling (Fig. 2, C and D). This result was in agreement with earlier findings (Rodermel et al., 1988; Quick et al., 1991b; Hudson et al., 1992) showing that antisense *rbcS* DNA decreased the leaf protein content of transformed tobacco plants. Soluble leaf protein levels of high- and low-Rubisco plants in both ambient CO_2 and CO_2-enriched chamber air remained high during the first few harvests and then decreased between 25 and 35% by the end of the experiment. Mean soluble leaf protein levels in high-Rubisco tobacco plants were 14% greater ($P < 0.05$) in ambient CO_2 than in CO_2-enriched chamber air averaged over the 9-d study period. However, mean protein levels in the low-Rubisco plants were not significantly different ($P > 0.05$) with respect to CO_2 treatment.

Rubisco Protein

Leaf Rubisco concentrations of high- and low-Rubisco tobacco plants were 4.0 ± 0.2 and 1.9 ± 0.1 g m^{-2}, respectively, prior to the start of CO_2 enrichment (Fig. 2, E and F). These measurements compared favorably with published values obtained with alternate quantification methods (Quick et al., 1991b). The Rubisco content of high-Rubisco tobacco plants decreased approximately 50% after 9 d of growth in ambient CO_2. Surprisingly, there was little change in the Rubisco content of ambient CO_2-grown low-Rubisco tobacco plants between the first and last sampling. Excluding d-0 measurements, mean Rubisco concentrations of high- and low-Rubisco plants were both 15% lower ($P < 0.05$) in enriched-CO_2 compared with ambient-CO_2 environments. Note that leaf Rubisco concentrations of high- and low-Rubisco tobacco plants were similar by the end of the experiment.

DISCUSSION

The impact of decreased Rubisco concentrations on net carbon assimilation rates of tobacco plants transformed with antisense *rbcS* DNA sequences has been analyzed extensively elsewhere (Quick et al., 1991a; Stitt et al., 1991; Hudson et al., 1992). Results of these prior studies indicated that tobacco plants with up to one-half of the Rubisco removed from the leaf had normal CO_2 assimilation rates in low light and ambient CO_2. Moreover, Rubisco imposed even less of a limitation on CO_2 fixation under high light and elevated CO_2. The photosynthesis measurements reported in this study were in broad agreement with these earlier conclusions. In contrast to what was reported by Quick et al. (1991b), a greater degree of Rubisco activation state was not observed in low- compared with high-Rubisco plants (see also Hudson et al., 1992). This discrepancy may have resulted from the low PPFD levels employed in the study by Quick et al. (1991b).

In the present experiments, negative photosynthetic acclimation was observed in tobacco plants after 7 to 9 d of elevated CO_2 treatment. Evidence for this conclusion was based on the changes in net assimilation rates of both high- and low-Rubisco tobacco plants exposed to elevated atmospheric CO_2. The long-term suppression of net CO_2 exchange rates during growth in CO_2-enriched chamber air was readily assayed in the high-CO_2 environment. Therefore, negative photosynthetic adjustment in tobacco, both in this and in an earlier study (Raper and Peedin, 1978), was similar to that reported for soybean, cabbage, and eggplant (Sage et al., 1989; Bunce, 1992). We further conclude that initial leaf Rubisco concentrations were not a determinant in the onset of photosynthetic adjustment.

The presence of *rbcS* antisense DNA sequences decreased SLW of tobacco grown in ambient CO_2 chamber air (Quick et al., 1991a). In agreement with earlier findings using soybean and tomato (Clough et al., 1981; Yelle et al., 1989a), elevated CO_2 treatment produced a dramatic increase in SLW of tobacco and also eliminated the observed differences in SLW between the high- and low-Rubisco plants. Changes in SLW during growth in elevated CO_2 occurred with little or no increase in total leaf area. Most of the increased photosynthate formed as a consequence of CO_2 enrichment in soybean was retained in the leaf starch fraction and was not exported into the phloem (Huber et al., 1984). This finding supported the suggestion that photosynthetic acclimation could be induced by an imbalance between sink and source organs on the plant (Neales and Incoll, 1968; Stitt, 1991). According to this hypothesis, negative photosynthetic adjustment to elevated CO_2 develops when plant growth is limited by the capacity of sink organs to utilize assimilates. A sink limitation would elevate leaf carbohydrates and result in feedback-inhibited photosynthesis. A source/sink imbalance could be of particular importance to tobacco, which is an annual plant that lacks major vegetative and reproductive storage organs for accumulating excess carbohydrate.

Diminished Rubisco activity has been observed in several plant species during growth in CO_2-enriched atmospheres (Wong, 1979; Peet et al., 1986; Yelle et al., 1989b). This was also true for the transgenic tobacco used here. Biochemical mechanisms responsible for down-regulating Rubisco activity in CO_2-acclimated plants are variable (Bowes, 1991). In contrast to the present results, Sage et al. (1989) reported that Rubisco activation state decreased in response to elevated CO_2 in all five species they examined. Decreased leaf Rubisco protein content also has been observed after prolonged elevated CO_2 treatment in *Chenopodium*, cabbage, rice, and tomato (Sage et al., 1989; Rowland-Bamford et al., 1990; Yelle et al., 1991b). Some of the diminished Rubisco activity in tobacco could be attributed to lowered leaf Rubisco content. However, the overall correlation between Rubisco activity and leaf Rubisco content in tobacco was poor. For instance, specific activities of Rubisco determined at 30°C after 7 d of treatment were 52 and 30 mol CO_2 fixed (mol Rubisco)$^{-1}$ s^{-1} for both ambient CO_2- and elevated CO_2-grown high-Rubisco tobacco plants, respectively. Also, decreases of Rubisco activity in low-Rubisco tobacco plants exposed to 9 d of CO_2 enrichment were not accompanied by comparable changes in Rubisco protein levels.

It is not possible to conclude from the present results that the decreased Rubisco activity of CO_2-adapted transgenic tobacco plants was a causal factor in the suppression of photosynthesis. Because negative photosynthetic adjustment

occurred in both high- and low-Rubisco plants acclimated to elevated CO_2, decreased Rubisco activity in CO_2-adapted versus ambient CO_2-grown high-Rubisco plants is probably not a response to an over-production of this enzyme.

Soluble leaf protein and Chl also were lower in enriched CO_2- compared with ambient CO_2-grown high-Rubisco plants. Thus, growth in elevated CO_2 may have affected the N status and the C:N ratio of tobacco leaves. Sage et al. (1989) did not observe a consistent response of leaf Chl or leaf N to growth in high-CO_2 based on the results of a comparative study employing five different plant species. However, both species that exhibited decreased leaf Rubisco content during growth in elevated CO_2 also exhibited decreased Chl levels. Collectively, these results suggest that growth in elevated CO_2 may have had a broad impact on the protein complement of tobacco leaves rather than a specific effect on leaf Rubisco protein.

The activities and amounts of carbonic anhydrase in leaves of high- and low-Rubisco plants were similar in the present study. This result was not in agreement with the prior observations of Hudson et al. (1992). One possible explanation for these conflicting results might be that Rubisco levels in the tobacco plants used in the earlier study were significantly lower than those of the line 3 transformants used here. There also was no direct evidence of decreased carbonic anhydrase activity in CO_2-enriched tobacco plants. Therefore, we conclude that the principal isoform of this enzyme in tobacco was not involved in negative photosynthetic adjustment to elevated CO_2.

In summary, down-regulation of Rubisco in CO_2-adapted tobacco plants was accompanied by an unexplained decrease in the specific activity of this enzyme. Rowland-Bamford et al. (1990) also observed a lower specific activity of Rubisco in a previous study of photosynthetic acclimation in rice.

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LITERATURE CITED

Besford RT, Ludwig LJ, Withers AC (1990) The greenhouse effect: photosynthesis and ribulose 1,5-bisphosphate carboxylase protein. J Exp Bot **41:** 925–931

Bowes G (1991) Growth at elevated CO_2: photosynthetic responses mediated through Rubisco. Plant Cell Environ **14:** 795–806

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Bunce JA (1992) Light, temperature and nutrients as factors in photosynthetic adjustment to an elevated concentration of carbon dioxide. Physiol Plant **86:** 173–179

Campbell WJ, Allen LH Jr, Bowes G (1988) Effects of CO_2 concentration on Rubisco activity, amount, and photosynthesis in soybean leaves. Plant Physiol **88:** 1310–1316

Campbell WJ, Allen LH Jr, Bowes G (1990) Responses of soybean canopy photosynthesis in CO_2 concentration, light, and temperature. J Exp Bot **41:** 427–433

Clough JM, Peet MM, Kramer PJ (1981) Effects of high atmospheric CO_2 and sink size on rates of photosynthesis of a soybean cultivar. Plant Physiol **67:** 1007–1010

Cure JD, Acock B (1986) Crop response to carbon dioxide doubling: a literature survey. Agric For Meteorol **38:** 127–145

Fawcett TW, Browse JA, Volokita M, Bartlett SG (1990) Spinach carbonic anhydrase primary structure deduced from the sequence of a cDNA clone. J Biol Chem **265:** 5414–5417

Huber SC, Rogers HH, Mowry EL (1984) Effects of water stress and photosynthesis and carbon partitioning in soybean [*Glycine max* (L.) Merr.] plants grown in the field at different CO_2 levels. Plant Physiol **76:** 224–249

Hudson GS, Evans JR, von Caemmerer S, Arvidsson YBC, Andrews TJ (1992) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic plants. Plant Physiol **98:** 294–302

Keys AJ, Parry MAJ (1990) Ribulose bisphosphate carboxylase/oxygenase and carbonic anhydrase. *In* PJ Lea, ed, Enzymes of Primary Metabolism: Methods in Plant Biochemistry, Vol 3. Academic Press, London, pp 1–14

Kimball BA (1983) Carbon dioxide and agricultural yield: assemblage and analysis of 430 prior observations. Agron J **75:** 779–788

Makino A, Mae T, Ohira K (1986) Colorimetric measurement of proteins stained with Coomassie brilliant blue R on sodium dodecylsulfate polyacrylamide gel electrophoresis by eluting with formamide. Agric Biol Chem Tokyo **50:** 1911–1912

Neales TF, Incoll LD (1968) The control of leaf photosynthesis rate by the level of assimilate in the leaf. Bot Rev **34:** 431–454

Peet MM, Huber SC, Patterson DT (1986) Acclimation to high CO_2 in monoecious cucumbers. II. Carbon exchange rates, enzyme activities, and starch and nutrient concentrations. Plant Physiol **80:** 63–67

Perchorowicz JT, DA Raynes, Jensen RG (1981) Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proc Natl Acad Sci USA **78:** 2985–2989

Porter MA, Grodzinski B (1984) Acclimation to high CO_2 in bean. Carbonic anhydrase and ribulose bisphosphate carboxylase. Plant Physiol **74:** 413–416

Quick WP, Schurr U, Fichter K, Schulze E-D, Rodermel SR, Bogorad L, Stitt M (1991a) The impact of decreased Rubisco on photosynthesis, growth, allocation and storage in tobacco plants which have been transformed with antisense *rbcS*. Plant J **1:** 51–58

Quick WP, Schurr U, Schiebe R, Schulze E-D, Rodermel SR, Bogorad L, Stitt M (1991b) 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

Raper CD Jr, Peedin GF (1978) Photosynthetic rate during steady-state growth as influenced by carbon dioxide concentration. Bot Gaz **139:** 147–149

Robinson JM (1984) Photosynthetic carbon metabolism in leaves and isolated chloroplasts from spinach plants grown under short and intermediate photosynthetic periods. Plant Physiol **75:** 397–409

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

Rowland-Bamford AJ, Baker JT, Allen LH Jr, Bowes G (1990) Acclimation of rice to changing atmospheric carbon dioxide concentration. Plant Cell Environ **14:** 577–583

Sage RF, Sharkey TD, Seemann JR (1989) Acclimation of photosynthesis to elevated CO_2 in five C_3 species. Plant Physiol **89:** 590–596

Servaites JC (1985) Crystalline ribulose bisphosphate carboxylase/oxygenase of high integrity and catalytic activity from *Nicotiana tabacum*. Arch Biochem Biophys **238:** 154–160

Shiraiwa Y, Miyachi S (1983) Factors controlling induction of carbonic anhydrase and efficiency of photosynthesis in *Chlorella vulgaris* 11h cells. Plant Cell Physiol **24:** 919–923

Sonnewald U, Willmitzer L (1992) Molecular approaches to source-sink interactions. Plant Physiol **99:** 1267–1270

Stitt M (1991) Rising CO_2 levels and their potential significance for

carbon flow in photosynthetic cells. Plant Cell Environ **14:** 741–762

Stitt M, Quick WP, Schurr U, Rodermel SR, Bogorad L (1991) Decreased ribulose 1,5-bisphosphate 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

Vernon LP (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal Chem **32:** 1144–1150

Wong SC (1979) Elevated atmospheric partial pressure of CO_2 and plant growth. I. Interactions of nitrogen nutrition and photosynthetic capacity in C_3 and C_4 plants. Oecologia **44:** 68–74

Yelle S, Beeson RC, Trudel MJ, Gosselin A (1989a) Acclimation of two tomato species to high atmospheric CO_2. I. Sugar and starch concentrations. Plant Physiol **90:** 1465–1472

Yelle S, Beeson RC, Trudel MJ, Gosselin A (1989b) Acclimation of two tomato species to high atmospheric CO_2. II. Ribulose-1,5-bisphosphate carboxylase/oxygenase and phospho*enol*pyruvate carboxylase. Plant Physiol **90:** 1473–1477

Ziska LH, Drake BG, Chamberlain S (1990) Long-term photosynthetic response in single leaves of a C_3 and C_4 salt marsh species grown at elevated CO_2 *in situ*. Oecologia **83:** 469–472