Plant Physiol. (1993) 103: 629–635

Use of Transgenic Plants with Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Antisense DNA to Evaluate the Rate Limitation of Photosynthesis under Water Stress[1]

Dhammika Gunasekera and Gerald A. Berkowitz*

Plant Science Department, Cook College, Rutgers-The State University of New Jersey, New Brunswick, New Jersey 08903

The biochemical lesion that causes impaired chloroplast metabolism (and, hence, photosynthetic capacity) in plants exposed to water deficits is still a subject of controversy. In this study we used tobacco (*Nicotiana tabacum* L.) transformed with "antisense" ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) DNA sequences to evaluate whether Rubisco or some other enzymic step in the photosynthetic carbon reduction cycle pathway rate limits photosynthesis at low leaf water potential (Ψ_w). These transformants, along with the wild-type material, provided a novel model system allowing for an evaluation of photosynthetic response to water stress in near-isogenic plants with widely varying levels of functional Rubisco. It was determined that impaired chloroplast metabolism (rather than decreased leaf conductance to CO_2) was the major cause of photosynthetic inhibition as leaf Ψ_w declined. Significantly, the extent of photosynthetic inhibition at low Ψ_w was identical in wild-type and transformed plants. Decreasing Rubisco activity by 68% did not sensitize photosynthetic capacity to water stress. It was hypothesized that, if water stress effects on Rubisco caused photosynthetic inhibition under stress, an increase in the steady-state level of the substrate for this enzyme, ribulose 1,5-bisphosphate (RuBP), would be associated with stress-induced photosynthetic inhibition. Steady-state levels of RuBP were reduced as leaf Ψ_w declined, even in transformed plants with low levels of Rubisco. Based on the similarity in photosynthetic response to water stress in wild-type and transformed plants, the reduction in RuBP as stress developed, and studies that demonstrated that ATP supply did not rate limit photosynthesis under stress, we concluded that stress effects on an enzymic step involved in RuBP regeneration caused impaired chloroplast metabolism and photosynthetic inhibition in plants exposed to water deficits.

Despite the recent controversy regarding the possibility of "patchy" stomatal closure under water deficits (Sharkey and Seeman, 1989; Gunasekera and Berkowitz, 1992; Lauer and Boyer, 1992; Wise et al., 1992), results of current studies support the contention that impairment of chloroplast metabolism can contribute significantly to the overall photosynthetic inhibition evidenced in plants exposed to water deficits (Graan and Boyer, 1990; Santakumari and Berkowitz, 1991). The characterization of this lesion in chloroplast function under stress is of obvious importance, especially in light of the possibility that adaptation to water stress is facilitated by acclimation of chloroplast metabolism to low leaf Ψ_w (Santakumari and Berkowitz, 1991).

A plethora of work using a wide range of experimental systems (intact chloroplast to whole plant) has been undertaken with the aim of identifying which lesion in chloroplast metabolism rate limits photosynthetic capacity under water deficits (Kaiser, 1987). Much of this work has focused on Rubisco. No definitive conclusions, however, can be made based on this research. Many researchers (Huffaker et al., 1970; O'Toole et al., 1976; Heitholt et al., 1991; Gimenez et al., 1992) have found no significant impairment of Rubisco activity in leaves of water-stressed plants. However, evidence can be found even in the current literature (Vapaavuori, 1986; Vu et al., 1987; Castrillo and Calcagno, 1989; Dreesman et al., 1991; Majumdar et al., 1991) that suggests that Rubisco activity is depressed in plants subjected to water stress and that this lesion may contribute to overall photosynthetic inhibition. One reason, among others, for this apparent discrepancy in the pertinent literature could be due to the complex issue of identifying a metabolic perturbation as a causal factor (to stress inhibition) rather than the repercussion of another, primary lesion. In the case of Rubisco, which can make up 30 to 50% of total leaf protein in a C_3 plant (Quick et al., 1991), a reduction in the level of this enzyme in plants subjected to water stress could be a consequence of impaired metabolism, which causes a depression in overall protein synthesis.

The objective of this study was to evaluate whether Rubisco activity contributes to the rate limitation of photosynthesis under plant water deficits. The recent development of transgenic tobacco with antisense Rubisco small subunit (rbcS) DNA sequences by Bogorod and coworkers (Rodermal et al., 1988) provides an ideal system for the examination of this question. The phenotypic result of this single-gene transformation is plants that have reduced levels of functional Rubisco. In our studies, we evaluated the photosynthetic response to in situ water deficits in transgenic tobacco plants that were near isogenic and differed only with regard to titer of functional Rubisco.

[1] New Jersey Agricultural Experiment Station publication No. 12149–2–93. This material is based on work supported by National Science Foundation grant No. DMB 8706240.

* Corresponding author; fax 1–908–932–9441.

Abbreviations: Ci, internal leaf CO_2 concentration; PGA, 3-phosphoglycerate; RuBP, ribulose 1,5-bisphosphate; Ψ_w, water potential.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* L.) seeds were obtained from selfed wild-type (SR1) and transformant line "5" plants described by Rodermal et al. (1988). Transformant line 5 plants contain multiple copies of the antisense Rubisco small subunit DNA integrated at various sites in the genome (Rodermal et al., 1988). The progeny of selfed line 5 plants contain a widely varying copy number of the antisense gene (Quick et al., 1991) and as a result display a broad range of functional Rubisco in mature leaves. Seeds were germinated in plastic trays containing 1:1 (v/v) peat:vermiculite. After emergence, seedlings were transferred to 4.8-L pots containing the same mixture and placed in a greenhouse that was maintained at approximately 27°C during the day and 18°C at night. Plants received natural sunlight; maximum daily irradiance was at least 375 μE m^{-2} s^{-1} and was typically greater than 550 μE m^{-2} s^{-1}. Pots were irrigated to runoff every day, with complete (Peter's Geranium Special macronutrient and STEM micronutrient) fertilizer added to the irrigation water every 4th d up to 4 weeks after planting and every 2nd d thereafter. Twice during the growth of the plants before their use for experiments, this fertilizer was supplemented with 3.5 g of $Ca(NO_3)_2$/pot. Plants were used after 7 to 8 weeks of growth. Young, nonsenescing, fully expanded leaves were used in all experiments.

Water stress was induced by withholding irrigation. During these periods of imposed stress, leaf Ψ_w declined to −1.2 MPa in approximately 10 d. Plants were grown with 2 cm of vermiculite placed over the potting mixture, which reduced soil evaporation during the periods of imposed water stress, resulting in a more gradual rate of leaf Ψ_w decline. The rate of stress imposition used in this study (≈0.8 MPa reduction during 10 d) is mild compared with that imposed in the original study showing stress-induced patchy stomatal closure (Sharkey and Seeman, 1989). Since that first report of water stress-induced patchy stomatal closure, work from this laboratory (Gunasekera and Berkowitz, 1992) and others (Wise et al., 1992) has shown that patchy stomatal closure occurs when plants are stressed more rapidly than that which occurred in our experiments. Patchy stomatal closure can induce artifacts in Ci calculations (Sharkey and Seeman, 1989). Because we did not evaluate patchy closure in this study, readers should view our Ci data with some caution. However, the current body of evidence suggests that the level and rate of stress used here does not typically result in patchy stomatal closure.

Water Relations

Leaf Ψ_w was measured using a pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA). For Ψ_w measurements the base of the leaf on either side of the midrib was cut away to allow the vascular system of the midrib to protrude out of the grommet of the pressure chamber. Three leaves were used as replicates for each measurement.

Photosynthesis

Gas exchange parameters of well-watered and water-stressed tobacco plants were measured with an ADC IRGA (PK Morgan Instruments, Andover, MA). The leaf chamber was illuminated by a 150-W incandescent floodlight; its distance from the chamber was adjusted to provide varying PAR values. Unless otherwise noted, all studies were done at saturating (850 μE m^{-2} s^{-1}) irradiances. Air at varying [CO_2] was supplied to the leaf chamber by mixing air with high and low concentrations of CO_2 supplied from compressed gas cylinders with an ADC GM 602 precision gas blender. Air temperature in the leaf cuvette was maintained between 23 and 25°C during all measurements by immersion of the aluminum heat exchanger of the leaf chamber in a water bath. Net photosynthesis, leaf conductance, and Ci were calculated using equations developed by von Caemmerer and Farquhar (1981). At least three leaves (usually four) were used for each measurement.

Leaf Illumination for RuBP and Rubisco Measurements

A Plexiglas chamber (9.5 × 20 × 13.5 cm) with an internal fan was used to illuminate leaves used for these measurements. Leaves were illuminated with saturating (850 μE m^{-2} s^{-1}) PAR provided by a 150-W incandescent floodlight. Air at approximately 350 μL L^{-1} of CO_2 and 50% RH was pumped into the chamber at 400 mL min^{-1}. Cool water circulated through an aluminum-finned heat exchanger that formed the base of the chamber, allowing the chamber atmosphere to be maintained at 25°C throughout the measurement period. Control experiments (not shown) demonstrated that leaves from both well-watered and water-stressed tobacco plants had reached steady-state photosynthesis after 10 min of illumination in the chamber. After a minimum of 15 min of illumination, a 2.27-cm^2 leaf disc was instantaneously (<1s after opening the chamber) frozen using a hand-held freeze clamp previously chilled in liquid N_2. The frozen disc was excised, covered with plastic wrap, and transferred into liquid N_2 until use for Rubisco and RuBP analyses. For each measurement three leaves were used as replicates. It should be noted that the time it took to freeze-clamp leaf tissue in our system had no measurable effect on our calculated Rubisco activity and RuBP values. Varying the time between opening the chamber and applying the freeze clamp had no significant effect on either measurement (data not shown). The replicate RuBP measurements shown in this report had low SE values, and our Rubisco activity and RuBP measurements matched those found by Quick et al. (1991) with this plant material when assay conditions were similar, further supporting this contention.

Rubisco Assay

The extraction procedure of Quick et al. (1991) was used to prepare leaf extracts for Rubisco measurements. Frozen leaf discs were transferred to a 1.5-mL microcentrifuge tube that was then dipped in liquid N_2. The leaf disc was thoroughly ground to a fine powder using a Teflon pestle that had been previously chilled in liquid N_2. Powdered leaf material was then homogenized (using the motorized Teflon

pestle) in a total volume of 1 mL of ice-cold extraction buffer containing 50 mM Tris HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10% (v/v) glycerol, 0.01% (v/v) Triton X-100, 1.5% (w/v) PVP, and 10 mM $NaHCO_3$. The DTT and $NaHCO_3$ stock solutions used to make the extraction buffer were mixed fresh daily. The homogenized leaf tissue was then centrifuged at 15,600*g* (i.e. maximum speed in an IEC Centra-M microcentrifuge) for 30 s at 4°C. Aliquots of the supernatant were transferred to 1.5-mL microcentrifuge tubes and immediately frozen in liquid N_2. For all experiments shown in this report, these tissue extracts were assayed for Rubisco activity the same day they were prepared. However, we noted no loss of activity after storage for several weeks at −80°C. Tubes containing tissue extracts were removed from liquid N_2 and allowed to thaw on ice (time for thawing was never more than 7 min) immediately before use.

Rubisco activity (initial and total) was determined by measuring the incorporation of $^{14}CO_2$ into acid-stable PGA as described by Quick et al. (1991). Total (i.e. fully activated) Rubisco activity was assayed by adding 50 μL of sample extract to incubation medium (480 μL final volume) containing 100 mM Tris HCl (pH 8.1), 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (0.135 μCi μmol^{-1}), and 5 mM DTT. After incubation for 30 min on ice with shaking, the sealed reaction vials were placed in a 30°C water bath for 4 to 5 min. After preincubation the reaction was initiated by adding 20 μL of RuBP (0.5 mM final) to the incubation medium and terminated after 60 s upon addition of 100 μL of 6 N formic acid. Control experiments (not shown) demonstrated that no loss of Rubisco activity occurred during the preincubation period. Initial Rubisco activity was assayed by adding the sample extract to the incubation medium, which contained 0.5 mM RuBP; there was no preincubation period before the initiation of the 60-s reaction period. For both initial and total Rubisco activity assays, vials were placed in a 60°C oven overnight after terminating the reaction. When the vials were dry, the precipitate was resuspended in 500 μL of distilled water before addition of scintillation cocktail and measurement of acid-stable product using a Beckman 3801 (Beckman, Somerset, NJ) counter. Three samples of each leaf extract were assayed, and the results were averaged.

RuBP Measurements

Leaf RuBP was measured as described by Servaites et al., 1987. A frozen leaf disc (tissue not stored before measurement) was transferred to a 1.5-mL microcentrifuge tube and immersed in liquid N_2. After the disc was ground to a fine powder with a chilled Teflon pestle, ice-cold 5% (v/v) perchloric acid (500 μL) was then added to the tube, and the contents were mixed to form a frozen slurry. After the extract was homogenized using a motorized Teflon pestle, the homogenate was centrifuged for 4 min at 4°C. The supernatant was collected, and the pellet was washed with 200 μL of 5% perchloric acid. The pooled supernatants were brought to 0.002% (w/v) bromocresol purple before neutralization with 2 N KOH, 0.4 M KCl, 0.4 M imidazole, and 50 mM EDTA. Addition of base was halted when the color of the extract turned bluish green (pH 6.5). The solution was then left for 10 min on ice to allow for precipitation of potassium perchlorate. After microcentrifugation of the solution for 4 min at 4°C, the clear extract was transferred to a 1.5-mL microcentrifuge tube and kept on ice. RuBP in this extract was determined by measuring $^{14}CO_2$ incorporation into PGA using Rubisco (Sigma, St. Louis, MO) as described by Servaites et al. (1987). The assay was carried out at room temperature in 500 μL of total volume by adding 100 μL of tissue extract to an assay solution containing 100 mM Tris HCl (pH 8.0), 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (1 μCi μmol^{-1}), 20 mM DTT, and 0.1 unit of Rubisco. Stock solutions of Rubisco, DTT, and $NaHCO_3$ were made fresh daily. Assays were run for 1 h at room temperature while the samples were shaking on a rotary shaker. The reaction was terminated by the addition of 100 μL of 6 N formic acid. $^{14}CO_2$ incorporated into acid-stable products was measured as indicated before for Rubisco assays. Three replicates of each leaf extract were assayed, and results were averaged. Blanks (assays run without leaf extract or with leaf extract but with formic acid present throughout the assay period) were typically less than 5% for both the RuBP and Rubisco assays.

General

Unless otherwise noted, all compounds were purchased from Sigma. $NaH^{14}CO_3$ was obtained from ICN Biochemicals (Irvine, CA). Data are presented in all tables and figures as means ± SE. In many cases one or both sets of error bars for a given data point are not visible because they are covered by the symbol representing the mean value for that treatment.

RESULTS AND DISCUSSION

In preliminary studies photosynthesis and Rubisco activity were monitored in large numbers of wild-type and transformed (well-watered) plants. Results of this work were similar to the data already reported by Quick et al. (1991) and Lauerer et al. (1993) and will, therefore, be presented here only in abbreviated fashion. Rubisco activity ranged between 50 and 80 $\mu mol\ m^{-2}\ s^{-1}$ in wild-type plants and from 10 to about 45 $\mu mol\ m^{-2}\ s^{-1}$ in transformed plants. For both wild-type and transformed plants, photosynthesis was maximal and did not change at Rubisco levels greater than 30 $\mu mol\ m^{-2}\ s^{-1}$ at both high (850 $\mu E\ m^{-2}\ s^{-1}$) and moderate (350 $\mu E\ m^{-2}\ s^{-1}$) PAR. As Rubisco level declined below 30 $\mu mol\ m^{-2}\ s^{-1}$ (presumably due to the presence in the genome of increasing copies of the antisense gene), photosynthesis was proportionately inhibited in transformed plants.

Further studies focused on comparing photosynthetic response to leaf Ψ_w decline in three different groupings of plants. A group of wild-type plants, which had an average Rubisco activity of 68.8 ± 2.0 $\mu mol\ m^{-2}\ s^{-1}$, were chosen for further study. Two groups of transformed plants, having average Rubisco activities of 41.8 ± 1.1 and 22.1 ± 0.6 $\mu mol\ m^{-2}\ s^{-1}$, were also used. These three groups of plants will subsequently be referred to as WT 69, TR 42, and TR 22, respectively. TR 42 and TR 22 plants had 60 and 32%, respectively, of the Rubisco activity of the WT 69 plants. WT 69, TR 42, and TR 22 plants were subjected to periods of in situ water deficits by withholding irrigation.

It should be noted that photosynthesis was monitored at

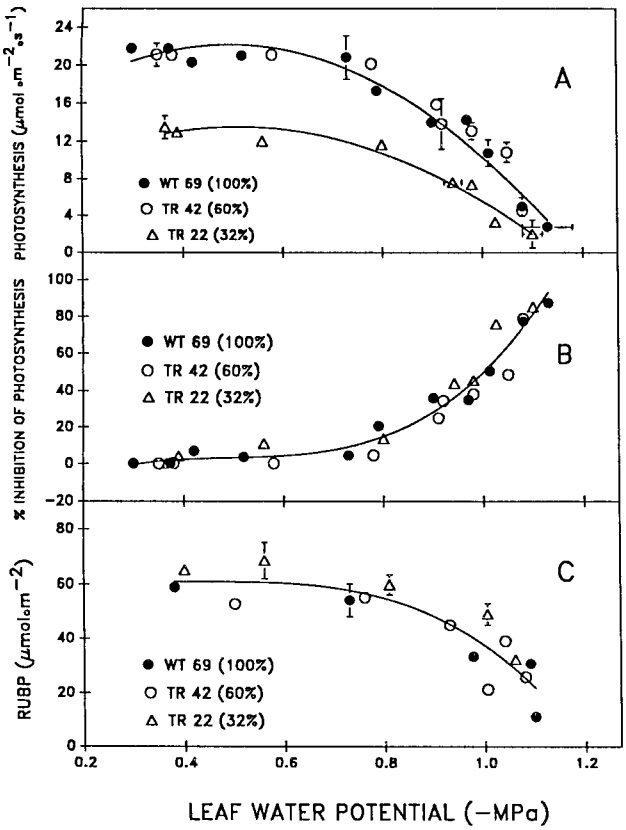

Figure 1. Photosynthesis and RuBP at declining leaf Ψ_w during an imposed water-stress episode in WT 69 (●), TR 42 (○), and TR 22 (△) tobacco plants. Absolute values of photosynthesis (A), percentage inhibition of photosynthesis from maximal values found in well-watered plants of that grouping (B), and steady-state levels of RuBP (C) are shown. Numbers in parentheses refer to the mean percentages of total (activated) Rubisco activity for that particular plant grouping as compared to the maximal activity demonstrated by wild-type plants. Measurements were made at an irradiance of 850 μE m^{-2} s^{-1} PAR and external [CO_2] of approximately 350 μL L^{-1}. Because data in B are presented as percentages, no error bars are shown. Computer-generated regression analysis using polynomial models was used for curve-fitting. When regression analysis indicated no significant difference between responses of the plant groupings, the data were pooled to generate the regressions represented as the curves. In A, one curve represents responses of WT 69 and WT 42 plants. In B and C, the curves represent the response of plants of all three groupings.

high (850 μE m^{-2} s^{-1}) light intensities during the period of imposed water stress. In prior studies (e.g. Vu et al., 1984), Rubisco was found to be 100% activated in tobacco leaves maintained under high light. We have also determined (data not shown) that Rubisco activation was 100% in all of the tobacco plants used for our studies under these (high light) assay conditions, even at low Ψ_w. At lower light intensities, differences between the in situ functional level of Rubisco in wild-type and transformed plants would not be as great. Quick et al. (1991) found that, at 340 μE m^{-2} s^{-1} PAR, transformed plants with a total Rubisco activity <40 μmol m^{-2} s^{-1} maintained activation state at 100%, but activation state in wild-type plants was about 60%.

Figure 1A shows the rate of photosynthesis as a function of declining leaf Ψ_w in the three groups of plants. Under well-watered conditions (at high Ψ_w), both WT 69 and TR 42 had similar and high rates of photosynthesis, i.e. 22.4 ± 0.3 and 20.9 ± 0.6 μmol of CO_2 m^{-2} s^{-1}, respectively. TR 22 had lower rates of photosynthesis (13.4 ± 0.5 μmol of CO_2 m^{-2} s^{-1}) at high Ψ_w. As leaf Ψ_w declined during a period of imposed water deficit, photosynthesis remained at maximal levels in all three groups until leaf Ψ_w declined to −0.8 MPa. Further decline in leaf Ψ_w was associated with inhibited photosynthesis in all three groups. When the percentage of inhibition of photosynthesis is plotted against declining Ψ_w (Fig. 1B), all three groups show a similar relationship; extent of low Ψ_w inhibition of photosynthesis was identical in all three groupings of plants despite widely differing levels of functional Rubisco.

Stomatal aperture effects on photosynthesis in plants of the three groups were also monitored during the periods of imposed water stress. The reduction in leaf conductance as Ψ_w declined was similar in WT 69, TR 42, and TR 22 plants (data not shown). A comparison of Ci in well-watered and stressed plants is shown in Table I. Under stress (−0.97 MPa leaf Ψ_w), Ci was maintained in WT 69 and TR 42 plants at levels close to that found in well-watered plants and declined by only approximately 30 μL L^{-1} in TR 22 plants. Leaf conductance and Ci measurements, then, demonstrate that any potential differences in response of chloroplast biochemical function to low Ψ_w in wild-type and transformed plants (with regard to impaired chloroplast metabolism effects on overall photosynthetic capacity) could not have been "masked" by differential response of leaf conductance to stress. The maintenance of Ci under stress at levels close to that found in well-watered plants of the three groups (Table I) suggests that photosynthetic decline under stress was not

Table I. *Photosynthesis and Ci of WT 69, TR 42, and TR 22 tobacco plants under well-watered conditions and after leaf Ψ_w declined to −0.97 MPa during a period of water deficit*

All measurements were made at 850 μE m^{-2} s^{-1} PAR and approximately 350 μL L^{-1} external [CO_2].

Group	Well-Watered Condition (−0.34 MPa)		Water-Stressed Condition (−0.97 MPa)	
	Photosynthesis	Ci	Photosynthesis	Ci
	μmol of CO_2 m^{-2} s^{-1}	μL L^{-1}	μmol of CO_2 m^{-2} s^{-1}	μL L^{-1}
WT 69	22.4 ± 0.3	263 ± 1	14.2 ± 0.1	254 ± 7
TR 42	21.0 ± 0.6	264 ± 1	13.9 ± 0.4	253 ± 6
TR 22	13.5 ± 0.5	295 ± 2	7.4 ± 0.3	263 ± 1

due primarily to reduction in leaf conductance at low Ψ_w but, rather, was caused by impaired chloroplast metabolism. This last assertion is further supported by the results shown in Figure 2. Photosynthesis:Ci curves are shown for WT 69 (Fig. 2A), TR 42 (Fig. 2B), and TR 22 (Fig. 2C) plants under well-watered and stressed (−1.0 MPa leaf Ψ_w) conditions. With plants of all three groups, photosynthesis at any Ci was substantially inhibited under stress. These data indicate that at low Ψ_w chloroplast metabolism was severely impaired and contributed substantially to the overall photosynthetic decline under stress in these tobacco plants.

Further studies focused on characterizing the nature of the lesion in chloroplast metabolism at low Ψ_w. Evidence in the current literature indicates that stress inhibition of photochemical production of ATP and NADPH does not contribute to impaired chloroplast metabolism in water-stressed plants until leaf Ψ_w declines to extremely low levels (Ben et al., 1987; Stuhlfauth et al., 1988; Ortiz-Lopez et al., 1991). Data presented in Figure 3 indicate that this is likely the case with these tobacco plants. With well-watered WT 69, TR 42, and TR 22 plants, photosynthesis was saturated at about 850 μE m^{-2} s^{-1} PAR (Fig. 3). Under stress, 850 μE m^{-2} s^{-1} PAR was again found to be saturating, and increasing light intensity (i.e. enhancing photochemical ATP production capacity) past this level did not reverse the photosynthetic inhibition evidenced in stressed plants. The assertion that impairment of ATP production was not the major lesion in chloroplast metabolism limiting photosynthesis in water-stressed plants at any Rubisco titer is more clearly demonstrated by the analysis shown in Table II. With WT 69, TR 42, and TR 22 plants, decreasing light intensity from saturating (850 μE m^{-2} s^{-1}) down to a moderate level of 350 μE m^{-2} s^{-1} or to a low level of 150 μE m^{-2} s^{-1} PAR did not cause any substantial increase in the stress inhibition of photosynthesis. If depressed ATP supply caused the photosynthetic inhibition under stress, then decreasing light intensity (whereby an ATP limitation of photosynthetic capacity would be more severe) should have increased the photosynthetic inhibition in stressed plants.

The main objective of this work was to determine whether

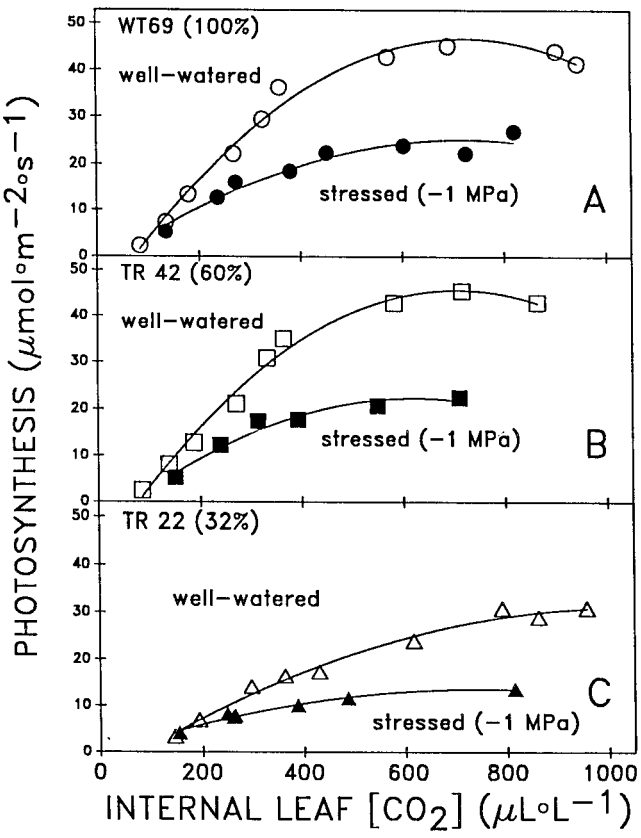

Figure 2. Photosynthesis of well-watered (open symbols) and water-stressed (closed symbols) tobacco plants at varying Ci. Results are shown for WT 69 (A), TR 42 (B), and TR 22 (C) plants. All measurements were made at an irradiance of 850 μE m^{-2} s^{-1} PAR. Numbers in parentheses refer to the mean percentages of total (activated) Rubisco activity for that particular plant grouping as compared to the maximal activity demonstrated by wild-type plants. Computer-generated regression analysis using polynomial models was used for curve-fitting.

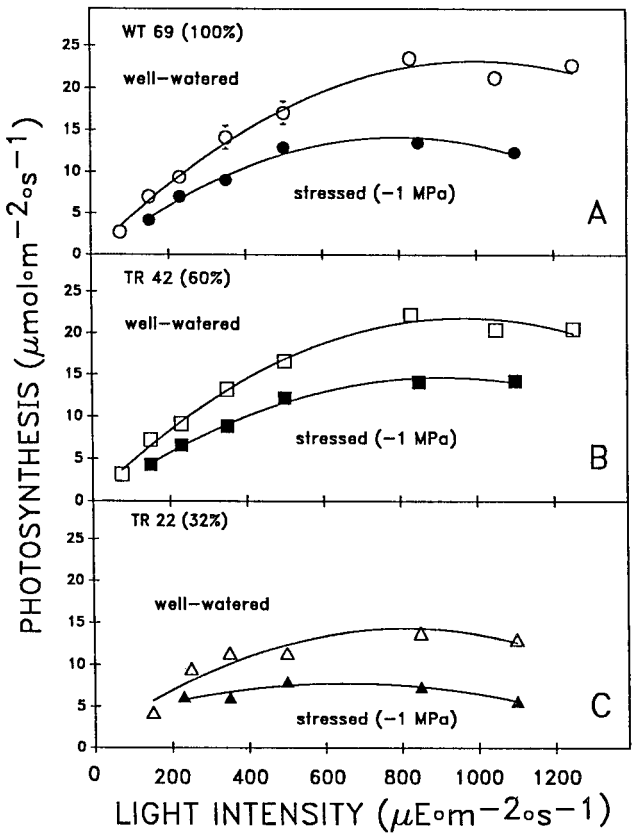

Figure 3. Photosynthesis of well-watered (open symbols) and water-stressed (closed symbols) tobacco plants at varying PAR. Results are shown for WT 69 (A), TR 42 (B), and TR 22 (C) plants. All measurements were made at an external $[CO_2]$ of approximately 350 μL L^{-1}. Numbers in parentheses refer to the mean percentages of total (activated) Rubisco activity for that particular plant grouping as compared to the maximal activity demonstrated by wild-type plants. Computer-generated regression analysis using polynomial models was used for curve-fitting.

Table II. *Photosynthetic inhibition due to water stress in WT 69, TR 42, and TR 22 tobacco plants at a range of PPFD values*

Photosynthetic inhibition was calculated by comparing the photosynthetic rate of water-stressed plants (−1.0 MPa leaf Ψ_w) to the rate of well-watered plants at a given irradiance. All measurements were made at an external [CO_2] of approximately 350 μL L^{-1}.

Irradiance	Percentage of Photosynthetic Inhibition		
	WT 69	TR 42	TR 22
$\mu E\ m^{-2}\ s^{-1}$			
850	42.6	36.7	46.6
350	36.6	33.1	46.9
150	40.8	40.8	NA[a]

[a] Not available.

Rubisco activity is the site of water stress inhibition of photosynthetic carbon assimilation. The finding that reducing Rubisco titer by as much as 68% did not enhance photosynthetic inhibition at low Ψ_w (Fig. 1B) suggests that Rubisco activity is not the main lesion in chloroplast metabolism under stress. More definitive evidence supporting this assertion is shown in Figure 1C. RuBP levels maintained during steady-state photosynthesis were monitored in WT 69, TR 42, and TR 22 plants as leaf Ψ_w declined during the imposed water-stress episode. It is interesting that RuBP levels declined in plants of the three groupings as leaf Ψ_w decreased during the stress (Fig. 1C). Photosynthesis of tobacco plants was little affected until leaf Ψ_w decreased below −0.8 MPa (Fig. 1B). As leaf Ψ_w declined below −0.8 MPa, RuBP levels declined precipitously (Fig. 1C). This decline was evident even in plants that contained only 32% of the functional Rubisco found in wild-type plants.

Data presented in Table I and Figure 2 indicate that decreased supply of one substrate, CO_2, to the carboxylation reaction was not the major factor responsible for photosynthetic inhibition at low Ψ_w. With regard to the carboxylation step in situ, therefore, the data presented in Figure 1 allow for a rather straightforward analysis of whether this step in the carbon assimilation pathway rate limited photosynthesis under water stress. The "product" of this enzymic step, i.e. CO_2 fixation into PGA, is reduced under water stress (evidence by inhibited photosynthesis; Fig. 1B). The inhibited rate of this reaction could only be due to an impairment in enzyme (Rubisco) function or a reduction in the steady-state supply of the substrates for the reaction. If impaired Rubisco function caused depressed CO_2 uptake under stress, then this would be evidenced by a buildup in the steady-state level of its substrate, RuBP. This hypothesized increase in RuBP level due to impaired PGA production would be similar to the RuBP increase occurring as Ci is lowered (Badger et al., 1984; Leegood and von Caemmerer, 1989). As shown in Figure 1C, RuBP did not build up or even remain constant as photosynthesis became inhibited at low Ψ_w but, rather, declined. Ci remained well above the compensation point in water-stressed wild-type and transformed tobacco plants (Table I). This stress-induced decline in RuBP (Fig. 1C) cannot be attributed, then, to the RuBP decline as Ci approaches the compensation point (Badger et al., 1984). These data, therefore, offer strong evidence that, even in plants with only 32% of the level of Rubisco found in wild-type plants, Rubisco does not rate limit photosynthesis at low Ψ_w.

The RuBP decline at low Ψ_w (Fig. 1C) suggests, conversely, that water-stress effects on the regenerative phase of the photosynthetic carbon reduction cycle (i.e. regeneration of RuBP) may cause the photosynthetic inhibition in water-stressed plants. Definitive evidence supporting this assertion would require the demonstration that RuBP levels declined under stress to a level below that which saturates the Rubisco-binding sites. As pointed out by Seeman and Sharkey (1986), experimental evidence suggests that RuBP levels limit photosynthesis when they decline below 1.8-fold of the Rubisco-binding sites. Rubisco-binding site levels were not monitored in the work reported here. However, the work of Lauerer et al. (1993) with this plant material does provide a basis for this analysis. They used immunological techniques to correlate Rubisco activity measurements with protein titer in this genetic material (approximately 27 μmol s^{-1} g^{-1} of Rubisco). Assuming a mol wt of 550,000 and 7.55 mol of binding sites mol^{-1} of Rubisco protein (Seeman and Sharkey, 1986), their data indicate that Rubisco activity of 69, 42, and 22 μmol m^{-2} s^{-1} correlates with 35, 21, and 11 μmol of Rubisco-binding sites m^{-2}, respectively. The decline in RuBP as leaf Ψ_w decreased below −1 MPa can, therefore, be seen as being theoretically causal to photosynthetic inhibition under stress in WT 69 and TR 42 plants (Fig. 1).

It should be noted that some investigators (Servaites et al., 1991) postulate that a tight feedback/feedforward relationship exists between Rubisco activity and RuBP levels. They suggest that reduced Rubisco activity may not necessarily be evidenced by a buildup of steady-state RuBP levels, because RuBP regeneration capacity can be "down-regulated" in response to decreased Rubisco activity. This could lead to the maintenance of constant steady-state RuBP at varying rates of carbon flow through the system. However, previous work with this plant material demonstrated no tight feedback/feedforward control. When Rubisco activity decreased in these tobacco plants, Quick et al. (1991) noted an increase in steady-state RuBP levels. We have also noted some (albeit not as great as that found by Quick et al., 1991) RuBP increase when Rubisco activity is reduced in these plants. For example, in one study, we found that wild-type plants had a RuBP level of 43.8 ± 0.01 μmol m^{-2}, whereas transformed plants with a Rubisco activity of 23.1 μmol m^{-2} s^{-1} maintained RuBP at 48.5 ± 0.01 μmol m^{-2}. In any case, under water stress RuBP levels were not maintained as photosynthetic rates were reduced but, rather, declined (Fig. 1).

The photosynthetic sensitivity and RuBP decline in tobacco plants with varying Rubisco are the critical data supporting the central conclusion of this report, that RuBP regeneration rather than Rubisco activity rate limits photosynthesis in water-stressed plants. Therefore, these data were collected during another experiment (undertaken with a different set of plants). In this second experiment, wild-type plants with a mean Rubisco activity of 70.4 ± 1.6 μmol m^{-2} s^{-1} were compared to transformed plants with a mean Rubisco activity of 23.1 ± 0.1 μmol m^{-2} s^{-1}. Photosynthetic sensitivity to low Ψ_w was again found to be identical in the wild-type and

transformed plants, and RuBP levels decreased as Ψ_w declined (data not shown).

In too many studies of photosynthesis in plants exposed to environmental stresses such as water deficit, rate limitation is evaluated by monitoring the in vitro activity of the pathway enzymes in leaf extracts under (typically optimized) substrate and cofactor concentrations. The enzymes are evaluated, therefore, under assay conditions that in no way match the milieu parameters that will limit the steady-state activity of the enzyme in situ, in the water-stressed chloroplast. An alternative approach, involving the comparison of stress effects on carbon flow through the cycle (i.e. photosynthetic rate) with effects on the in situ level of a photosynthetic metabolite (i.e. RuBP), was used in this study. With this type of analysis, a decline in product generation occurring concomitantly with a buildup of the substrate would identify a photosynthetic enzyme as the major stress-induced lesion site in the pathway. Clearly, the analysis of Rubisco undertaken in this study discounts any stress effects on this enzyme as significantly contributing to impaired chloroplast metabolism at low leaf Ψ_w, at least with this experimental system. In addition, data presented in Figure 3 and Table II demonstrate that decreased ATP-generating capacity (necessary for RuBP regeneration from PGA) was likely not the primary lesion limiting photosynthesis at low Ψ_w. The experimental results presented here point rather to an enzyme involved in RuBP regeneration as limiting photosynthesis under plant water deficits. This contention has not been widely focused on by researchers seeking to elucidate the main biochemical lesion limiting photosynthesis in water-stressed plants.

ACKNOWLEDGMENT

We wish to acknowledge and thank Dr. Lawrence Bogorad for his kind gift of SR1 wild-type and Rubisco antisense line 5 tobacco seeds and information concerning these lines.

Received April 30, 1993; accepted June 29, 1993.

LITERATURE CITED

Badger MR, Sharkey TD, von Caemmerer S (1984) The relationship between steady-state gas exchange of bean leaves and the levels of carbon-reduction-cycle intermediates. Planta **160:** 305–313

Ben G-Y, Osmond CB, Sharkey TD (1987) Comparisons of photosynthetic responses of *Xanthium strumarium* and *Helianthus annuus* to chronic and acute water stress in sun and shade. Plant Physiol **84:** 476–482

Castrillo M, Calcagno AM (1989) Effects of water stress and rewatering on ribulose-1,5-bis-phosphate carboxylase activity, chlorophyll, and protein contents in two cultivars of tomato. J Hort Sci Am **64:** 717–724

Dreesman DC, Harn C, Daie J (1991) Regulation of photosynthesis and expression of ribulose 1,5-bisphosphate carboxylase genes in water-stressed leaves of sugarbeet (abstract No. 617). Plant Physiol **96:** S-98

Gimenez C, Mitchell VJ, Lawlor DW (1992) Regulation of photosynthetic rate of two sunflower hybrids under water stress. Plant Physiol **98:** 516–524

Graan T, Boyer JS (1990) Very high CO_2 partially restores photosynthesis in sunflower at low water potentials. Planta **181:** 378–384

Gunasekera D, Berkowitz GA (1992) Heterogenous stomatal closure in response to leaf water deficits is not a universal phenomenon. Plant Physiol **98:** 660–665

Heitholt JJ, Johnson RC, Ferris DM (1991) Stomatal limitation to carbon dioxide assimilation in nitrogen- and drought-stressed wheat. Crop Sci **31:** 135–139

Huffaker RC, Radmin T, Kleinkopf GE, Cox EL (1970) Effect of mild water stress on enzyme nitrate reductase assimilation and of carboxylation phase of photosynthesis in barley. Crop Sci **10:** 471–473

Kaiser WM (1987) Effect of water deficit on photosynthetic capacity. Physiol Plant **71:** 142–149

Lauer MJ, Boyer JS (1992) Internal CO_2 measured directly in leaves. Abscisic acid and low leaf water potential cause opposing effects. Plant Physiol **98:** 1310–1316

Lauerer M, Saftic D, Quick WP, Labate C, Fichtner K, Schulze ED, Rodermel SR, Bogorad L, Stitt M (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' rbcS. VI. Effect on photosynthesis in plants grown at different irradiance. Planta **190:** 332–345

Leegood RC, von Caemmerer S (1989) Some relationship between contents of photosynthetic intermediates and the rate of photosynthetic carbon assimilation in leaves of *Zea mays* L. Planta **178:** 258–266

Majumdar S, Ghosh S, Glick BR, Dumbroff EB (1991) Activities of chlorophyllase, phosphoenolpyruvate carboxylase and ribulose 1,5-bisphosphate carboxylase in the primary leaves of soybean during senescence and drought. Physiol Plant **81:** 473–480

Ortiz-Lopez A, Ort DR, Boyer JS (1991) Photophosphorylation in attached leaves of *Helianthus annuus* at low water potentials. Plant Physiol **96:** 1018–1025

O'Toole JC, Crookston RK, Treharne KJ, Ozbun JL (1976) Mesophyll resistance and carboxylase activity. A comparison under water stress conditions. Plant Physiol **57:** 465–468

Quick WP, Schurr U, Scheibe R, Schulze ED, Rodermel SR, Bogorad L, Stitt M (1991) 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

Rodermal 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

Santakumari M, GA Berkowitz (1991) Chloroplast volume:water potential relationships and acclimation of photosynthesis to cellular water deficits. Photosyn Res **28:** 9–20

Seeman JR, Sharkey TD (1986) Salinity and nitrogen effects on photosyntesis, ribulose 1,5-bisphosphate carboxylase and metabolite pool size in *Phaseolus vulgaris*. Plant Physiol **82:** 555–560

Servaites JC, Shieh WJ, Geiger DR (1991) Regulation of photosynthetic carbon reduction cycle by ribulose bisphosphate and phosphoglyceric acid. Plant Physiol **97:** 1115–1121

Servaites JC, Tucci MA, Geiger DR (1987) Glyphosate effects on carbon assimilation, ribulose bisphosphate carboxylase activity, and metabolite levels in sugar beet leaves. Plant Physiol **85:** 370–374

Sharkey TD, Seemann JR (1989) Mild water stress effects on carbon-reduction-cycle intermediates, ribulose bisphosphate carboxylase activity, and spatial homogenicity of photosynthesis in intact leaves. Plant Physiol **89:** 1060–1065

Stuhlfauth T, Sultemeyer DF, Weinz S, Fock HP (1988) Fluorecense quenching and gas exchange in a water stressed C3 plant, *Digitalis lanata*. Plant Physiol **86:** 246–250

Vapaavuori FM (1986) Correlation of activity and amount of ribulose 1,5-bisphosphate carboxylase with chloroplast stroma crystals in water-stressed willow leves. J Exp Bot **37:** 89–98

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

Vu JCV, Allen LH, Bowes G (1984) Dark/light modulation of ribulose bisphosphate carboxylase activity in plants from different photosynthetic categories. Plant Physiol **76:** 843–845

Vu JCV, Leon H, Allen LH, Bowes G (1987) Drought stress and evaluated CO_2 effects on soybean ribulose bisphosphate carboxylase activity and canopy photosynthetic rates. Plant Physiol **83:** 573–578

Wise RW, Ortiz-Lopez A, Ort DR (1992) Spatial distribution of photosynthesis during drought in field-grown and acclimated and nonacclimated growth chamber-grown cotton. Plant Physiol **100:** 26–32