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Photosynthetic Gas Exchange and Discrimination against $^{13}CO_2$ and $C^{18}O^{16}O$ in Tobacco Plants Modified by an Antisense Construct to Have Low Chloroplastic Carbonic Anhydrase[1]

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The physiological role of chloroplastic carbonic anhydrase (CA) was examined by antisense suppression of chloroplastic CA (on average 8% of wild type) in *Nicotiana tabacum*. Photosynthetic gas-exchange characteristics of low-CA and wild-type plants were measured concurrently with short-term, on-line stable isotope discrimination at varying vapor pressure deficit (VPD) and light intensity. Low-CA and wild-type plants were indistinguishable in the responses of assimilation, transpiration, stomatal conductance, and intercellular CO_2 concentration to changing VPD or light intensity. At saturating light intensity, low-CA plants had lower discrimination against $^{13}CO_2$ than wild-type plants by 1.2 to 1.8‰. Consequently, tissue of the low-CA plants was higher in ^{13}C than the control plants. It was calculated that low-CA plants had chloroplast CO_2 concentrations 13 to 22 μmol mol^{-1} lower than wild-type plants. Discrimination against $C^{18}O^{16}O$ in low-CA plants was 20% of that of the wild type, confirming a role of chloroplastic CA in the mechanism of discrimination against $C^{18}O^{16}O$ ($\Delta C^{18}O^{16}O$). As VPD increased, stomatal closure caused a reduction in chloroplastic CO_2 concentration, and since VPD and chloroplastic CO_2 concentration act in opposing directions on $\Delta C^{18}O^{16}O$, no effect of VPD was seen on $\Delta C^{18}O^{16}O$.

The enzyme CA catalyzes the reversible hydration of CO_2 to form HCO_3^-. The uncatalyzed interconversion of CO_2 and HCO_3^- is often slow relative to photosynthetic processes. In some cases the requirement for CA activity in photosynthesis has been shown unequivocally. For example, in microalgae lacking an external CA, photosynthesis can be severely limited by the depletion of CO_2 outside of the cells under conditions of alkaline pH and high cell densities (Williams and Colman, 1995). In C_4 plants, CA is required in the cytosol of mesophyll cells to supply PEP carboxylase with HCO_3^- from CO_2 (Hatch and Burnell, 1990).

Although there is an abundance of CA activity within chloroplasts (Jacobsen et al., 1975; Tsuzuki et al., 1985), it has been difficult to show that CA has any significant involvement in photosynthesis in higher C_3 plants. Majeau et al. (1994) used antisense technology to reduce chloroplastic CA activity in primary transformed tobacco (*Nicotiana tabacum*) plants to as low as 1% of the wild type and yet could discern no difference in the CO_2 assimilation rate between the transformed and control plants. Price et al. (1994), using similar technology, also were unable to discern any difference in the assimilation rate between low-CA tobacco plants and wild-type plants. They did, however, observe a decrease in discrimination against $^{13}CO_2$ during short-term, on-line gas-exchange experiments. They calculated that the decline in ^{13}C discrimination was the result of a 15 μbar lower chloroplastic CO_2 partial pressure in the low-CA plants, a decrease that would result in only a 4.4% reduction in the assimilation rate. Although the change in assimilation rate would be difficult to detect using gas-exchange techniques, the reduction might have a significant impact on the overall fitness of the plant (Cowan, 1986; Price et al., 1994).

The results of Majeau et al. (1994) differed significantly from those of Price et al. (1994) with respect to the observation of changes in stomatal conductance. Majeau et al. (1994) observed a significantly higher stomatal conductance in plants with low-CA activity relative to the wild type. They interpreted this to mean that the plants were compensating for low chloroplastic CA activity by increasing stomatal conductance and thereby increasing intercellular CO_2 concentration. Price et al. (1994), however, observed no difference in stomatal conductance or intercellular CO_2 concentration between genotypes. The gas-exchange experiments in the two studies were performed under very different environmental conditions. Majeau et al. (1994) used a light intensity of 250 μmol m^{-2}

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Abbreviations: C_a, ambient CO_2 concentration in μmol mol^{-1}; C_c, chloroplastic CO_2 concentration in μmol mol^{-1}; C_i, intracellular CO_2 concentration in μmol mol^{-1}; CA, carbonic anhydrase; VPD, vapor pressure deficit.

s^{-1} and a relative humidity of 40 to 50%, presumably resulting in a VPD of approximately 1.5 to 2 kPa. Price et al. (1994) performed their experiments at a light intensity of 1000 μmol m^{-2} s^{-1} and a VPD of 1 kPa. The discrepancy between the two studies might be explained if low chloroplastic CA activity affects stomatal response to environmental conditions. Low-CA plants, for instance, may maintain high stomatal conductance at high VPDs. To fully evaluate the physiological requirement for CA, it is necessary, therefore, to perform gas-exchange experiments under varying environmental conditions.

Price et al. (1994) also observed that short-term, on-line discrimination against $C^{18}O^{16}O$ decreased dramatically in the low-CA tobacco plants. Qualitatively, this observation is consistent with the mechanistic model for $C^{18}O^{16}O$ discrimination developed by Farquhar and Lloyd (1993). Two main processes cause changes in the $^{18}O/^{16}O$ composition of CO_2 during photosynthetic gas exchange (Farquhar and Lloyd, 1993). Diffusional fractionation occurs because of the difference in mass between CO_2 molecules containing ^{18}O and ^{16}O. In addition, an oxygen isotope-exchange reaction occurs in the chloroplast between the oxygen in CO_2 and that in H_2O. During the isotope-exchange reaction, CO_2 becomes enriched in ^{18}O relative to that in chloroplast H_2O. The extent to which CO_2 becomes enriched in ^{18}O will depend on the degree to which equilibration between CO_2 and H_2O is achieved. The Farquhar and Lloyd (1993) model assumes that the presence of CA establishes near-complete isotopic equilibrium between CO_2 and H_2O. A portion of the CO_2 that enters the chloroplast and equilibrates with the chloroplast water will then diffuse back out of the leaf with an altered oxygen isotope ratio. The amount of CO_2 that escapes from the leaf depends on the partial pressure of CO_2 in the chloroplast and the resistances to diffusion along the path from the chloroplast to the atmosphere. The observation of a reduced discrimination against $C^{18}O^{16}O$ in low-CA plants (Price et al., 1994) is consistent with a requirement for CA to hydrate and dehydrate CO_2 and thus allow for isotopic equilibrium between oxygen in CO_2 and oxygen in chloroplast water. Although the results of Price et al. (1994) are consistent with the mechanistic model, to our knowledge a thorough examination of the effects of low chloroplastic CA on a comparison between predicted and observed $C^{18}O^{16}O$ discrimination has not been performed.

In addition to the oxygen isotope-exchange process and diffusional fractionation, $C^{18}O^{16}O$ discrimination also is influenced by the isotope composition of chloroplast water. The oxygen isotope ratio of chloroplast water is not constant but changes because of fractionation that occurs during transpiration (Craig and Gordon, 1965; Flanagan, 1993). The greater the VPD experienced by the leaf, the greater the enrichment of chloroplast H_2O with ^{18}O (Craig and Gordon, 1965; Flanagan, 1993). Whereas increased enrichment of ^{18}O in leaf water, due to a high VPD, will lead to greater enrichment of ^{18}O in CO_2, the higher VPD also will lead to a decrease in stomatal conductance. This in turn will cause C_c to decrease and cause a decline in $C^{18}O^{16}O$ discrimination by the plant (Farquhar et al., 1993). While a strong response of $C^{18}O^{16}O$ discrimination has been shown with increasing VPD (Flanagan et al., 1994), different responses to VPD might be expected between species if stomatal response to VPD is different.

The objectives of this study were 2-fold: (a) to compare the physiological response of low-CA plants to environmental stimuli (light and VPD) with those of wild-type plants, including an examination of $^{13}CO_2$ discrimination, and (b) to test assumptions of the mechanistic model of $C^{18}O^{16}O$ discrimination by plants (Farquhar and Lloyd, 1993).

MATERIALS AND METHODS

Plant Material and Growing Conditions

Nicotiana tabacum cv Carlson plants were transformed as described by Majeau et al. (1994). Plants were transformed using the plasmid vector pGA643; the wild-type plants were transformed with a control vector and the low-CA plants were transformed with an antisense vector. Plants produced from the seed of primary transformants were screened for low-CA activity as described by Majeau and Colman (1994), and a single plant with the lowest CA activity was chosen. Wild-type plants had a CA activity of $(2.04 \pm 0.37)10^6$ units m^{-2} (average $\pm$ SD, $n = 8$), whereas low-CA plants had a CA activity of $(0.17 \pm 0.08)10^6$ units m^{-2} (average $\pm$ SD, $n = 8$).

Ten plants of each genotype were propagated from apical cuttings. Five plants of each type were maintained in a controlled environment growth chamber (model E15, Conviron Products, Winnipeg, Manitoba, Canada) at 70% RH, 25°C, and a light intensity at the bottom of the chamber of 250 μmol m^{-2} s^{-1} for a photoperiod of 11 h and a dark period of 13 h. Five plants of each type also were maintained in a separate chamber with identical conditions, except that the light intensity was manipulated to expose the plants to 400 μmol m^{-2} s^{-1} (at the bottom of the chamber) for 30 min and 100 μmol m^{-2} s^{-1} for the next 30 min, a cycle that was repeated for 11 h before the lights were switched off for a 13-h dark period. The ^{13}C composition of organic tissue in plants grown under continuous or fluctuating light was determined (see below) in an effort to assess the possibility of there being differences in the transient stomatal response to light between genotypes, which might be manifest in the average integrated C_c.

Apical cuttings were rooted for approximately 2 weeks before being transferred to the growth chambers, after which they were grown for approximately 5 weeks before gas-exchange measurements were initiated. As the plants grew, they were continually trimmed to maintain a height of no more than 45 cm. Mature plants were watered twice per week and fertilized with complete nutrient solution once per week. The average size ($\pm$SE, $n = 49$) of the leaves used for the gas-exchange experiments was 28.4 ± 1.2 cm^2.

Gas-Exchange Measurements

Measurements of CO_2 and water vapor fluxes were carried out using an open gas-exchange system (MPH 1000 gas-exchange system, Campbell Scientific, Logan, UT; ADC

225-MK 3 infrared gas analyzer, Analytical Development, Hoddeson, Hertshire, UK). A leaf was clamped into the leaf chamber and maintained under controlled conditions of temperature, light, humidity, CO_2 (350 μmol mol^{-1} exiting the chamber), and O_2 (21%). To examine the effects of changes in light intensity on gas-exchange properties, one leaf from each of four plants of each genotype from each chamber (total of 16 plants) was subjected to three light intensities (150, 250, and 400 μmol m^{-2} s^{-1}). Leaves were illuminated by a 150-W quartz-halogen lamp filtered through a wide-band hot mirror (Optical Coating Laboratory, Santa Rosa, CA), and intensity was varied using a series of neutral density filters. To generate a VPD of 1 kPa the humidity was controlled. The following protocol was used to examine the effects of changes in VPD on gas-exchange properties: one leaf from a single plant was subjected to a single VPD; measurements were taken at a single VPD on three separate plants, and three different VPDs were used (1.1, 1.7, and 2.4 kPa; total of 18 plants). VPD was controlled by altering the flow rate of dry air through the leaf chamber, since all the water vapor present in the leaf chamber came from leaf transpiration. Light intensity was maintained at 1000 μmol m^{-2} s^{-1} in the VPD experiment.

In all experiments, leaves were held at steady-state conditions for a minimum of 45 min before data were recorded and gas samples were collected from the outflow of the chamber. CO_2 samples (approximately 50 μmol) were purified cryogenically in a vacuum line after the air stream had passed through four dry ice-ethanol traps to remove H_2O. Pressure in the vacuum line was maintained at 5.3 kPa to prevent the condensation of O_2.

Isotopic Analysis

The 50-μmol CO_2 samples were analyzed on a gas isotope ratio mass spectrometer (Sira 12, VG Isotech, Middlewich, Cheshire, UK) at the Ottawa-Carleton Stable Isotope Facility. Isotopic compositions were expressed using the lowercase delta notation:

$$\delta = \left[\frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1\right], \quad (1)$$

where R is the molar ratio of heavy to light isotope ($^{13}C/^{12}C$ or $^{18}O/^{16}O$). The ^{18}O content of CO_2 was expressed relative to Standard Mean Ocean Water, and the ^{13}C content was expressed relative to the Pee Dee Belemnite limestone. The δ values are conveniently presented in parts per thousand.

Isotopic discrimination during photosynthetic gas-exchange (Δ) was calculated from the isotopic composition of the CO_2 leaving the chamber with (δ_o) and without (δ_e) the leaf present as in the equation:

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)}, \quad (2)$$

where $\xi = c_e/(c_e - c_o)$ and c_e and c_o are the partial pressures of CO_2 in the air, when the air is dried, entering (e) and leaving (o) the chamber while the leaf is present. The value of ξ was 5.8 ± 0.8 (mean ± SD, n = 48) for the experiments involving changing light and 11.5 ± 3.1 (mean ± SD, n = 18) for the VPD experiments. In general, the higher ξ values occurred at the high VPDs as a result of the requirement for high flow rates through the leaf chamber. The $\delta^{18}O$ value of source CO_2 entering the chamber (δ_e) was +9.97 ± 0.05‰ (n = 5), and the $\delta^{13}C$ value of the source CO_2 was −35.12 ± 0.03‰ (n = 5). As a result of using high purity gases for mixing the source air for the gas-exchange system, corrections applied to the isotope ratios for N_2O content were negligible (N_2O corrections were determined by the method of Friedli and Siegenthaler [1988]; for details, see Flanagan and Varney [1995]).

Measurements also were made of the carbon isotope ratio of leaf tissue. Foliage samples were dried at 65°C and ground to a fine powder with a mortar and pestle. The samples were prepared for measurements of carbon isotopic composition by combustion in an elemental analyzer. The CO_2 generated from the combustion was purified and passed directly to the inlet of a gas isotope ratio mass spectrometer (model 20/20, Europa Scientific, Franklin, OH). As an indication of the precision of leaf sample carbon isotope ratios measured using this technique, four replicate measurements of tissue from one plant gave a SD of 0.082‰.

Model Calculations

We used measured $\Delta^{13}CO_2$ values (Δ_{obs}) and concurrently measured gas-exchange characteristics to calculate the CO_2 partial pressure in the chloroplast using the following equations (von Caemmerer and Evans, 1991; Lloyd et al., 1992):

$$\frac{c_c}{c_a} = \frac{c_i}{c_a} - \frac{\Delta_i - \Delta_{obs} - f(\Gamma^*/c_a)}{b - a_w} \quad (3)$$

$$\Delta_i = a_b\frac{c_a - c_s}{c_a} + a\frac{c_s - c_i}{c_a} + b\frac{c_i}{c_a}, \quad (4)$$

where c is the partial pressure of CO_2, and the subscripts a, s, i, and c refer to the atmosphere, leaf surface, intercellular spaces, and chloroplast, respectively. The symbol a represents discrimination during diffusion of $^{13}CO_2$ at various steps in the atmosphere-chloroplast boundary, whereas the b subscript refers to the leaf boundary layer. The values for the diffusional fractionation factors are: a_b = 2.9‰, a = 4.4‰, and a_w = 1.8‰. The value used for b, discrimination during carboxylation, was 27.5‰ (Lloyd et al., 1992). The parameter f is the fractionation with respect to average carbon composition associated with photorespiration (7‰; Rooney, 1988). Γ^* is the CO_2 partial pressure at the compensation point in the absence of respiration during the day, calculated from an equation in Brooks and Farquhar (1985).

Discrimination against $C^{18}O^{16}O$ during photosynthetic gas-exchange ($\Delta C^{18}O^{16}O$) was calculated based on the model of Farquhar and Lloyd (1993) as described by Flanagan et al. (1994):

$$\Delta C^{18}O^{16}O = \frac{\bar{a} + \frac{c_c}{c_a - c_c}(\delta_c - \delta_a)}{1 - \frac{c_c}{c_a - c_c}(\delta_c - \delta_a)}, \quad (5)$$

where a is the net discrimination during diffusion of CO_2 from the atmosphere into the chloroplast and back out again, δ_c is the oxygen isotope ratio of CO_2 in the chloroplast, and δ_a is the oxygen isotope ratio of CO_2 in the gas-exchange chamber (equals δ_o). The oxygen isotope ratio of CO_2 in the chloroplast was estimated by assuming that isotopic equilibrium between CO_2 and water in the chloroplast is complete, and that the oxygen isotope ratio of chloroplast water is well described by an evaporative enrichment model (Craig and Gordon, 1965; Flanagan et al., 1994). Depending on the relative activities of CA and ribulose bisphosphate carboxylase, isotopic equilibrium may not be complete, and Equation 5 can be modified as follows:

$$\Delta C^{18}O^{16}O = \frac{\bar{a}(1 + 3\rho) + \frac{c_c}{c_a - c_c}([\delta_c - \delta_a] + 3\rho b)}{1 - \frac{c_c}{c_a - c_c}(\delta_c - \delta_a) + 3\rho\frac{c_c}{c_a - c_c}}, \quad (6)$$

where ρ is the ratio of the rate of carboxylation by ribulose bisphosphate carboxylase to the rate of hydration by CA, and b represents discrimination against $C^{18}O^{16}O$ during carboxylation (taken as 0‰). We used values calculated with Equation 6 to compare with values obtained during on-line discrimination measurements. Further details of the modeled discrimination calculations are presented by Flanagan et al. (1994).

RESULTS

The response of assimilation rate, stomatal conductance, transpiration rate, and C_i to VPD or light was identical in control plants (wild type) and low chloroplastic CA plants (Fig. 1). The assimilation rate did not change in response to VPD changes. In contrast, CO_2 assimilation increased in a linear manner with light from 150 to 400 μmol m^{-2} s^{-1}, at which light level the photosynthetic rate was similar to that of the VPD experiment, in which light intensity was 1000 μmol m^{-2} s^{-1} (Fig. 1, A and B). In response to an increase in VPD, the transpiration rate remained constant because of a concomitant decline in stomatal conductance (Fig. 1, C and E). With VPD held constant, transpiration increased linearly with light intensity as a result of a concomitant increase in stomatal conductance (Fig. 1, D and F). C_i remained relatively constant with increasing light, despite an increase in assimilation rate, because of the associated increase in stomatal conductance (Fig. 1H). In contrast, C_i declined with an increase in VPD at saturating light (Fig. 1G).

Although there were no measurable differences in photosynthetic gas-exchange characteristics between low-CA and wild-type plants, there were significant differences between genotypes for on-line stable isotope discrimination. When light was saturating, $\Delta^{13}CO_2$ was consistently lower in low-CA plants than in wild-type plants (Fig. 2, A and B). The $\Delta^{13}CO_2$ values, in combination with the values of C_i calculated from the gas-exchange data, can be used to calculate C_c (Caemmerer and Evans, 1991). With an increase in VPD, C_c declined in a pattern similar to that of C_i

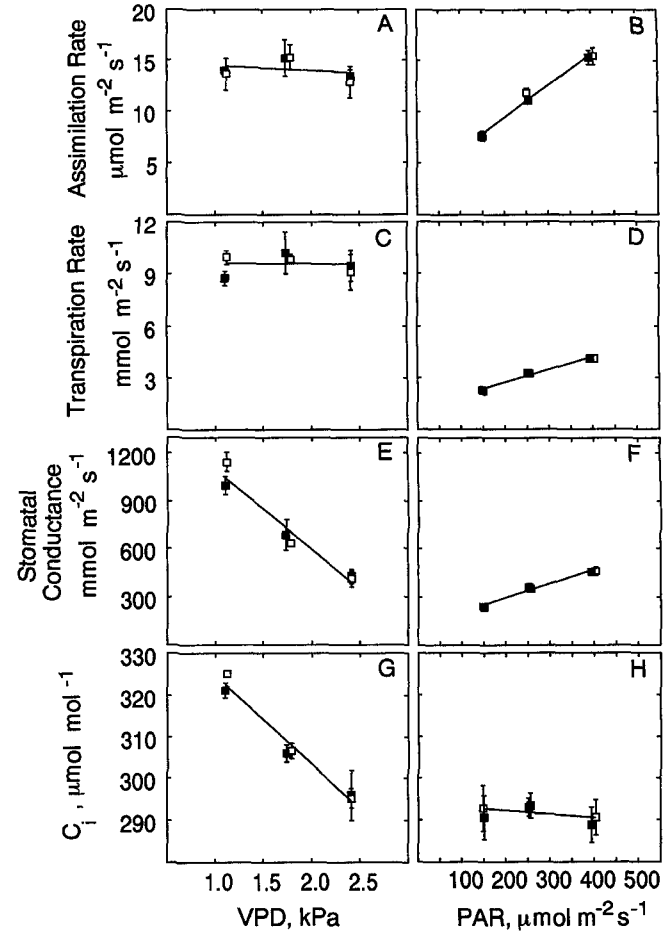

Figure 1. The effects of changes in leaf-air VPD or light intensity on steady-state values of CO_2 assimilation rate (A and B), transpiration rate (C and D), stomatal conductance (E and F), and C_i (G and H) in control tobacco plants (□) and plants transformed to have low chloroplastic CA activity (■). In experiments in which VPD was altered, the leaf temperature was 30°C and the light intensity was 1000 μmol m^{-2} s^{-1}. In experiments in which light intensity was varied, the VPD was 1.0 kPa and the leaf temperature was 25°C. Error bars represent SE (n = 3 for VPD experiments, n = 8 for light experiments).

(Fig. 2C). Whereas there was a general trend for C_c to decrease with increasing light intensity, the only statistically significant decrease occurred at saturating light (400 μmol m^{-2} s^{-1}; Fig. 2D). Values for the decrease in CO_2 concentration from the intercellular air spaces to the chloroplast ($C_i - C_c$) are shown in Figure 2, E and F. Plants with low CA had greater $C_i - C_c$ values than did control plants, reflecting the fact that, although C_i was the same for both genotypes, C_c was lower in plants with low CA. Whereas $C_i - C_c$ remained constant with changes in VPD in low-CA plants, a decrease was observed in wild-type plants (Fig. 2E). The slope of this trend, however, was not significantly different from zero. In all experiments, plants with low CA had values of $C_i - C_c$ that were between 13 and 22 μmol mol^{-1} higher than those of the control plants.

There was a significant difference between the $\delta^{13}C$ values for leaf tissue of low-CA and wild-type plants, al-

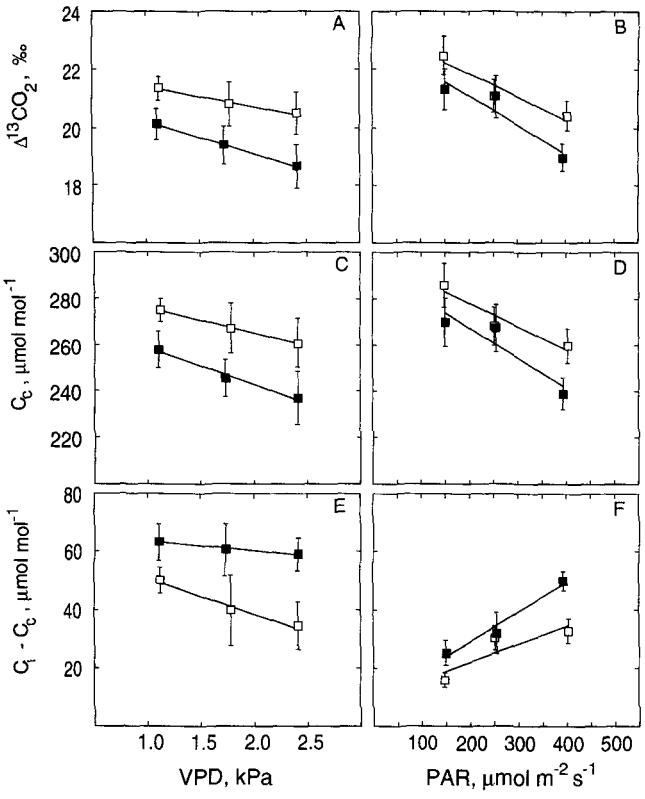

Figure 2. The effects of changes in leaf-air VPD or light intensity on discrimination against $^{13}CO_2$ (A and B), C_c (C and D), and $C_i - C_c$ in control tobacco plants (□) and plants transformed to have low chloroplastic CA activity (■) as measured on-line, concurrent with gas exchange. Environmental conditions are described in the legend to Figure 1.

though there was no significant effect of growth chamber light treatment (Table I; two-way analysis of variance; genotype effect, $F = 19.7$, $P = 0.004$; growth chamber treatment effect, $F = 4.34$, $P = 0.054$; interaction, $F = 0.011$, $P = 0.92$; $n = 5$ plants/treatment). In both light treatments, the low-CA plants had higher $\delta^{13}C$ values, which is consistent with the on-line discrimination results. The magnitude of the difference in $\delta^{13}C$ values between the low-CA and control plants was approximately 1‰, which is consistent with a difference of approximately 15 μmol mol^{-1} in C_c averaged over the life of the leaf (Farquhar et al., 1989), as was suggested by the gas-exchange results.

There were striking differences between wild-type and low-CA plants for $\Delta C^{18}O^{16}O$ values, with low-CA plants having values of approximately 20‰, and control plants having values of 100‰ (Fig. 3, A and B). Discrimination against $C^{18}O^{16}O$ did not change with VPD (Fig. 3A) but decreased as a function of increasing light (Fig. 3B). When light was held constant and VPD was changed, $\Delta C^{18}O^{16}O$ did not change in association with C_c/C_a (Fig. 3C). However, discrimination against $C^{18}O^{16}O$ increased with increasing C_c/C_a when VPD was held constant and light was changed (Fig. 3D).

The observed $\Delta C^{18}O^{16}O$ values were compared with those predicted by Equation 6, with ρ, the ratio of the rate of carboxylation by Rubisco to the rate of hydration of CO_2 by CA, set at 0.019 (the average ρ value of all experiments) (Fig. 4). Observed values for control plants were very close to those predicted by Equation 6. As expected, plants with low CA had observed $\Delta C^{18}O^{16}O$ values that were much lower than the values predicted by Equation 6.

Table I. *Carbon isotope ratio ($\delta^{13}C$, ‰) of leaf tissue from control tobacco plants (wild type) and plants transformed to have low chloroplastic CA (low CA), grown under constant light intensity or fluctuating light intensity (see text for details of growth conditions*

Values are the means ± SE, $n = 5$.

Growth Chamber Treatment	Wild-Type Plants	Low-CA Plants
Constant light	−31.11 ± 0.25	−30.09 ± 0.23
Fluctuating light	−30.64 ± 0.22	−29.57 ± 0.25

DISCUSSION

Previous studies indicated that CO_2 assimilation rates were unaffected by low chloroplastic CA in transgenic tobacco plants (Majeau et al., 1994; Price et al., 1994). However, Majeau et al. (1994) suggested that the low-CA plants compensated for the decrease in C_c that would occur as a result of low chloroplastic CA activity by increasing stomatal conductance. Price et al. (1994) could discern no difference between low-CA and control plants for any gas-exchange characteristic, including stomatal conductance. Since the two studies were conducted under different environmental conditions, an alteration in stomatal response

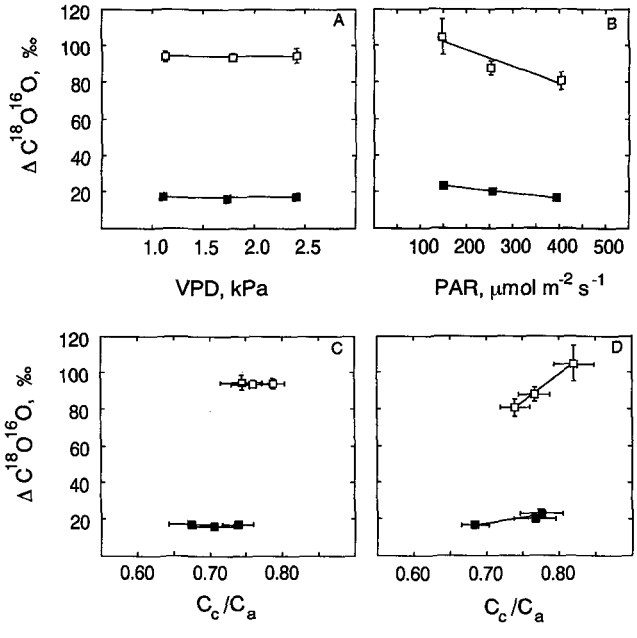

Figure 3. The effects of changes in leaf-air VPD (A and C) or light intensity (B and D) on discrimination against $C^{18}O^{16}O$ in control tobacco plants (□) and plants transformed to have low chloroplastic CA activity (■) as measured on-line, concurrent with gas exchange. In C and D, $\Delta C^{18}O^{16}O$ is plotted versus C_c/C_a for the experiments in which VPD (C) and light (D) were varied. Environmental conditions are described in the legend to Figure 1.

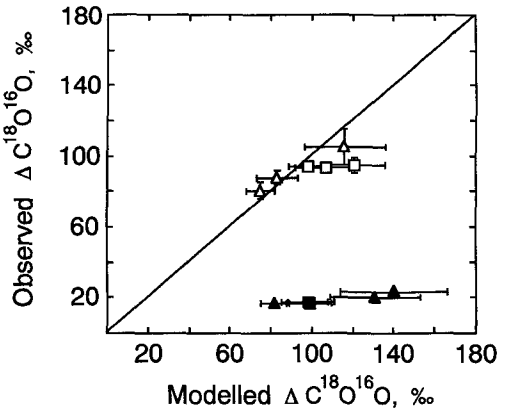

Figure 4. Comparison of modeled and observed discrimination against $C^{18}O^{16}O$ in control tobacco plants (open symbols) and plants transformed to have low chloroplastic CA activity (closed symbols). The line drawn represents a 1:1 relationship. Triangles represent results obtained from varying VPD, and squares represent results from varying light intensity as described in the text. Error bars represent SEs with $n = 3$ for VPD experiments and $n = 8$ for light experiments.

to the environment in low-CA plants could possibly explain the apparent discrepancy. Our data indicated that the steady-state gas-exchange responses to changes in VPD and light were identical in control and low chloroplastic CA plants (Fig. 1). Therefore, it is clear that under conditions of adequate water supply and normal atmospheric conditions the gas-exchange physiology of the plants was unaffected by having low chloroplastic CA activity; this confirms the results found by Price et al. (1994). The results found by Majeau et al. (1994), although inconsistent with the results found here, were obtained on primary transformants, unlike the plants in this study, which were propagated from the seeds of those transformants.

Whereas steady-state gas-exchange characteristics were indistinguishable between low-CA plants and the control plants, stable isotope discrimination was clearly different between the genotypes. Discrimination against $^{13}CO_2$ was consistently lower in plants with low chloroplastic CA activity than in control plants (Fig. 1). As VPD increased, $\Delta^{13}CO_2$ decreased in both plant types. Although this trend was not significantly different from a slope of 0, and there were no significant differences among VPD treatments for either low-CA or wild-type plants, there is a strong theoretical basis for the decrease in $\Delta^{13}CO_2$ with increasing VPD. The decline in stomatal conductance caused by an increase in VPD resulted in lower C_i values, which in turn resulted in lower $\Delta^{13}CO_2$ values (Farquhar et al., 1989). Although there is a strong relationship between C_i and $\Delta^{13}CO_2$, this is only because C_i is a reflection of C_c (Farquhar et al., 1989).

Since $\Delta^{13}CO_2$ values are clearly lower in plants with low-CA activity compared with control plants, and C_i values are the same between genotypes (Fig. 1G), it is clear that plants with low chloroplastic CA have lower C_c. We calculated that low-CA plants had C_c values lower than those of control plants (by approximately 13–22 μmol mol^{-1}). Although there is a trend toward a decline in $C_i - C_c$ with increasing VPD in wild-type plants and not in low-CA plants, there was no significant difference between the slope of the trend and a slope of 0. If the trend were real, however, it would suggest that chloroplastic CA might be of greater benefit at lower C_i. The difference in $C_i - C_c$ between low-CA plants and wild-type plants measured in this study was approximately the 15 μmol mol^{-1} differential observed by Price et al. (1994), and was consistent with the theory that chloroplastic CA facilitates supply of CO_2 to Rubisco by maintaining equilibrium between the large HCO_3^- pool and CO_2 within the chloroplast. Although the increase in supply cannot be readily observed as increases in assimilation rates, the overall fitness of the plant may be increased (Cowan, 1985; Price et al., 1994).

If CA does facilitate supply of CO_2 to Rubisco, then discrimination against $^{13}CO_2$ should become more similar between low-CA plants and controls as demand for CO_2 is reduced. As light intensity was decreased, assimilation rate decreased, thereby decreasing the demand for CO_2. The difference between $\Delta^{13}CO_2$ values for the two genotypes was only significant when light intensity saturated photosynthesis (Fig. 2B), consistent with the hypothesis that CA assists in supplying Rubisco with CO_2.

Although the steady-state response of stomata to changes in light was the same for both genotypes, it is possible that there might be differences in the transient response of stomata to fluctuating light intensity. If this were the case one might expect, given equivalent assimilation capacities, that the average C_i experienced by the genotypes might be different, a parameter that would be manifest in the $\delta^{13}C$ value of organic tissue. The $\delta^{13}C$ values of organic tissue were 1‰ higher in low-CA plants than in control plants, consistent with low-CA plants having a 15 μmol mol^{-1} lower C_c than control plants (Farquhar et al., 1989). This pattern of difference between low-CA and wild-type plants was consistent under fluctuating and constant light conditions. Although there was a trend toward plants having higher $\delta^{13}C$ values under fluctuating light, the difference between growth chamber treatments was not statistically significant.

As expected, there was a clear difference in discrimination against $C^{18}O^{16}O$ between low-CA and wild-type plants, a result similar to that seen by Price et al. (1994). A mechanistic model developed by Farquhar and Lloyd (1993) assumes that $\Delta C^{18}O^{16}O$ will be strongly influenced by the extent to which isotopic equilibrium between CO_2 and chloroplast water is achieved. In plants with low-CA activity, there will be incomplete equilibration between CO_2 and chloroplast water and, therefore, low $\Delta C^{18}O^{16}O$ values are expected. The degree to which isotopic equilibration is achieved is reflected in the ratio of the number of fixations of CO_2 to hydrations of CO_2 (ρ in Eq. 6). A value for ρ of 0.019 established a good fit between the observed and predicted values (Fig. 4) for control plants. In contrast, using a ρ of 0 in Equation 6 would result in an increase in $\Delta C^{18}O^{16}O$ values of approximately 24‰ ± 6.2 (mean ± SE, $n = 31$) above the observed values for the control plants. Therefore, chloroplast water and CO_2 are not in perfect isotopic equilibrium in the control plants, as would be

described by a ρ of 0. The average ρ value of 0.019 ± 0.003 (mean ± SE, n = 32) for tobacco is close to the value calculated for *Phaseolus* by Flanagan et al. (1994) using the same methodology.

In contrast, a ρ of approximately 0.5 was calculated for tobacco plants with low-CA activity, reflecting the lower level of hydration. The plants used by Price et al. (1994) had 2% of the activity of wild-type plants and yet had $\Delta C^{18}O^{16}O$ values of only 50% of wild type compared with 20% reported here for plants having 8% of CA activity of the wild type. Their experiments were performed at a light intensity of 1000 μmol m^{-2} s^{-1}, a VPD of 1 kPa, and a leaf temperature of 25°C, whereas the experiments performed at 25°C in this study were performed at only 400 μmol m^{-2} s^{-1}. A full comparison between studies would require a more rigorous analysis of the results of Price et al. (1994) from the perspective of the mechanistic model of Farquhar and Lloyd (1993). It must also be noted that the low-CA plants in both studies have similar absolute amounts of CA activity. The difference in percentage of CA activity relative to wild type was derived largely from the fact that the wild-type plants in this study had only 57% of the activity (on average) of the wild-type plants in the study by Price et al. (1994). Since both wild-type plants had large amounts of chloroplastic CA activity, it is unlikely that they could be distinguished using the techniques used in these two studies. Therefore, it is not surprising that similar $\Delta^{13}CO_2$ and $\Delta C^{18}O^{16}O$ values were found in the low-CA plants relative to the wild-type plants in these studies.

Flanagan et al. (1994) observed a strong relationship between changes in VPD and discrimination against $C^{18}O^{16}O$. Since chloroplast water becomes enriched in ^{18}O in direct proportion to the VPD (Craig and Gordon, 1965; Flanagan, 1993), it is also expected that $\Delta C^{18}O^{16}O$ should increase in direct proportion to the VPD. However, discrimination against $C^{18}O^{16}O$ did not change with increases in VPD in this study (Fig. 3A). This is because variation in VPD can result in two independent changes that have contrasting effects on $\Delta C^{18}O^{16}O$. This situation is shown in Fig. 5. As VPD increases, the ^{18}O content of chloroplast water increases, causing $\Delta C^{18}O^{16}O$ to increase. However, stomatal conductance may decrease in response to the VPD change, causing C_c/C_a to decrease. The decrease in C_c/C_a will result in a lower $\Delta C^{18}O^{16}O$ value (Farquhar et al., 1993). The degree to which these two effects cancel each other out will depend on how C_c/C_a is influenced by stomatal response to VPD. In this study, we observed different responses of $\Delta C^{18}O^{16}O$ to changes in C_c/C_a, depending on whether the change in C_c/C_a was caused by variations in VPD or light intensity (Fig. 3). When light intensity was increased, assimilation rate increased and C_c decreased, causing a decline in $\Delta C^{18}O^{16}O$. However, when VPD was increased, stomatal conductance declined, resulting in a reduction in C_c/C_a. The reduction in C_c/C_a compensated for the increase in the ^{18}O content of chloroplast water associated with variation in VPD, and there was no significant change in $\Delta C^{18}O^{16}O$.

In this study, chloroplast water was assumed to have the same isotopic signature as water at the sites of evaporation,

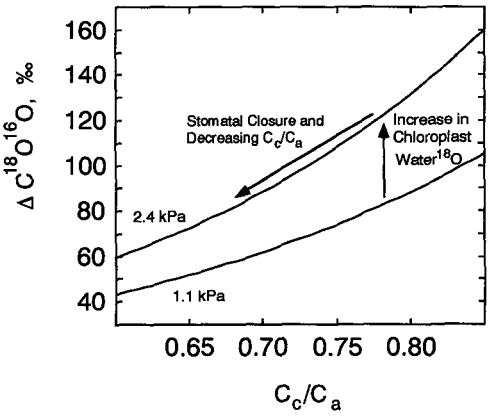

Figure 5. The combined effects of changes in C_c/C_a and VPD on discrimination against $C^{18}O^{16}O$ as described by Equation 6. As VPD increases, $\Delta C^{18}O^{16}O$ increases because of evaporative enrichment of the H_2O involved in the oxygen exchange with CO_2. As VPD increases, however, stomatal conductance decreases and C_c/C_a also declines, causing a reduction in $\Delta C^{18}O^{16}O$. The extent to which these two factors cancel each other will depend on stomatal response to VPD. The lines were calculated using Equation 6, with VPDs of 2.4 and 1.1 kPa.

as predicted by the evaporative enrichment model (Craig and Gordon, 1965; Flanagan, 1993). There is some argument as to whether this is the case. Yakir et al. (1994) suggested that the isotopic signature of water in chloroplasts is closer to that of total leaf water than to water at the sites of evaporation. The $\delta^{18}O$ of total leaf water can be up to 6‰ lower than that predicted by the evaporative enrichment model (Flanagan et al., 1991; Yakir et al., 1994). The discrepancy between predicted and measured total leaf water is a function of the transpiration rate, probably as a result of the shifting balance between the bulk flow of unfractionated source water into the leaf and the back diffusion of water enriched in ^{18}O away from the sites of evaporation (Flanagan et al., 1991). A discrepancy of 6‰ between the isotopic signature of water in the chloroplast and water at the sites of evaporation would generate a difference of approximately −20‰ in the $\Delta C^{18}O^{16}O$ values predicted in this study. Should chloroplast water actually have an isotopic signature 6‰ lower than that predicted, using a ρ of 0 would be a much better predictor of observed $\Delta C^{18}O^{16}O$ values for the control plants. If we assume that movement of water through a tobacco leaf is similar to that in *Phaseolus*, the evaporation rates in these experiments would produce only a maximum departure of 2‰ between the $\delta^{18}O$ predicted for water at the evaporative sites and that measured for total leaf water. The estimates of ρ are reasonable, but it is noted that calculations of ρ using the evaporative enrichment model may overestimate ρ by a level determined by the transpiration rate. Given this argument, one might expect that by holding the VPD constant and increasing the transpiration rate, predictions of $\Delta C^{18}O^{16}O$ using a single ρ would deviate by an increasingly large amount from the measured values. In the experiments in which light was altered and VPD was maintained at 1.0 kPa, transpiration changed more than 2-fold.

No trend in the deviation of predicted $\Delta C^{18}O^{16}O$ values from measured $\Delta C^{18}O^{16}O$ values was observed with this change in transpiration rate. This observation is consistent with the assumption that chloroplast water has an isotopic signature close to that of water at the evaporative sites within leaves.

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