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Carbon Fixation Gradients across Spinach Leaves Do Not Follow Internal Light Gradients

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In situ measurements of ^{14}C-CO_2 incorporation into 40-μm paradermal leaf sections of sun- and shade-grown spinach leaves were determined. Chlorophyll, carotenoid, and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content in similar 40-μm paradermal leaf sections was also measured. The carbon fixation gradient did not follow the leaf internal light gradient, which decreases exponentially across the leaf. Instead, the ^{14}C-CO_2 fixation was higher in the middle of the leaf. Contrary to expectations, the distribution of carbon fixation across the leaf showed that the spongy mesophyll contributes significantly to the total carbon reduced. Approximately 60% of the carboxylation occurred in the palisade mesophyll and 40% occurred in the spongy mesophyll. Carbon reduction correlated well with Rubisco content, and no correlation between chlorophyll and carotenoid content and Rubisco was observed in sun plants. The correlation among chlorophyll, carotenoids, Rubisco, and carbon fixation was higher in shade leaves than in sun leaves. The results are discussed in relation to leaf photosynthetic and biochemical measurements that generally consider the leaf as a single homogeneous unit.

INTRODUCTION

Leaves are composed of many different tissues (e.g., Fahn, 1974). The major photosynthetic tissue of C3 leaves is the mesophyll, which is separated into two types. The palisade mesophyll (PM), which may be one-to-several cell layers thick, is composed of rod-shaped cells, with the long axis perpendicular to the leaf surface. Underlying the PM is the spongy mesophyll (SM), which is composed of loosely packed, irregularly shaped cells. The SM is typified by large air cavities.

Leaf anatomy and photosynthetic characteristics depend on the species and the light conditions during growth (Wylie, 1949, 1951; Loach, 1967; Alberte et al., 1976; Boardman, 1977; Anderson, 1986; Baker and McKiernan, 1988; Cui et al., 1991). Typically, leaves grown in the shade, compared to leaves grown in full sun, have fewer layers of PM and are thinner (Wylie, 1951; Cui et al., 1991). Additionally, shade leaves have less chlorophyll on an areal basis, a lower chlorophyll *a/b* ratio, lower rates of electron transport per unit of chlorophyll, lower CO_2 fixation rates on both an areal and a chlorophyll basis, a lower light compensation point, and an increased photosystem II (PSII)/photosystem I (PSI) ratio (Boardman, 1977; Lichtenthaler et al., 1981; Barber, 1985; Anderson, 1986; Mustardy et al., 1990; McKiernan and Baker, 1991). It appears that the quality as well as the quantity of light influence leaf photosynthetic characteristics and anatomy, but the mechanisms by which the influence is exerted are not known. In general, the biochemical studies mentioned above treated the leaf as a unit and differences between the PM and SM were not emphasized.

Light impinging on the adaxial leaf surface is rapidly attenuated in the upper 20% of the leaf (Terashima and Saeki, 1983; Vogelmann et al., 1989; Cui et al., 1991). The rapid attenuation of light and change in light quality inside the leaf (Gates et al., 1965; Vogelmann et al., 1989) apparently affect the photosynthetic characteristics of chloroplasts within the leaf, and there is evidence that shade-type chloroplasts exist in the abaxial portion of sun leaves (Outlaw, 1987; Terashima, 1989). Thus, the measured photosynthetic characteristics of sun and shade leaves represent the sum of the dissimilar chloroplasts that developed in different light regimes within the leaf.

Light gradients across leaves are relatively steep, and models of carbon fixation across leaves generally predict that carbon fixation corresponds to the light gradient (Gutschik, 1984; Terashima, 1989; Fukshansky and von Remisowsky, 1992; Evans et al., 1993). In contrast to the models that imply that the PM is the major site of carbon fixation, measurements of CO_2 fixation in the SM and PM of intact leaves suggest that the SM can contribute up to 50% of the carbon fixation (Mokronosov et al., 1973; Outlaw and Fisher, 1975b). Other measurements have shown approximately three times more ^{14}C-CO_2 incorporation into the PM than into the SM (Outlaw and Fisher, 1975a).

We are interested in how component mesophyll cell layers adjust their metabolism to allow maximal performance under the saturating and nonsaturating light conditions in which plants exist. The regulation of the genes responsible for optimal performance is likely influenced by light and other signals. To properly address the problem, however, a more lucid view of

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whole-leaf photosynthesis is required. One of our present goals is to refine our knowledge of photosynthetic performance within the individual tissue layers of leaves. We report here carbon fixation rates in 40-μm paradermal sections from leaves of sun- and shade-grown spinach labeled in vivo for 10 sec at different photon flux densities. Photosynthetic pigments and Rubisco content in similar 40-μm leaf sections were also determined. The results showed that maximum rates of CO_2 fixation occur in the middle of the leaf and not at the adaxial leaf surface where light is maximal.

RESULTS

Photosynthetic Pigments

The leaf chlorophyll *a* + *b* content on an areal basis was higher (56.3 × 10^{-2} g chlorophyll m^{-2}) in sun-type leaves than in shade-type leaves (48.8 × 10^{-2} g chlorophyll m^{-2}); however, the average chlorophyll concentration per 40-μm leaf section was higher in shade leaves (3.3 × 10^{-2} g chlorophyll m^{-2} vs 3.8 × 10^{-2} g chlorophyll m^{-2}). Figure 1 shows that the greater average pigment concentration per section was due to the adaxial 280 μm of the shade leaf, which contained 30.1 × 10^{-2} g chlorophyll m^{-2} compared to only 20.8 × 10^{-2} g chlorophyll m^{-2} in the upper 280 μm of the sun leaf. The pigment concentration in the abaxial half of the shade leaf was lower than the pigment concentration in sun leaves at the same depth from the abaxial surface (Figures 1A and 1B). The carotenoid content correlated well with chlorophyll *a* + *b* (Figures 1A and 1B). Pigment concentrations were maximal at a leaf depth between ~250 and 450 μm in sun leaves, whereas the pigment concentration in shade plants exhibited a relatively steep pigment concentration curve with a maximum between ~150 and 200 μm.

The chlorophyll *a/b* ratio was quite elevated at the adaxial surface and decreased relatively linearly across the majority of the sun leaf (Figure 1C). The lowest chlorophyll *a/b* ratio of the sun leaves was lower than the lowest chlorophyll *a/b* ratio in the shade leaves. The slight increase in chlorophyll *a/b* in the abaxial section of the shade leaf was likely due to the more upright growth pattern of shade leaves compared to sun leaves, which were relatively flat rosettes.

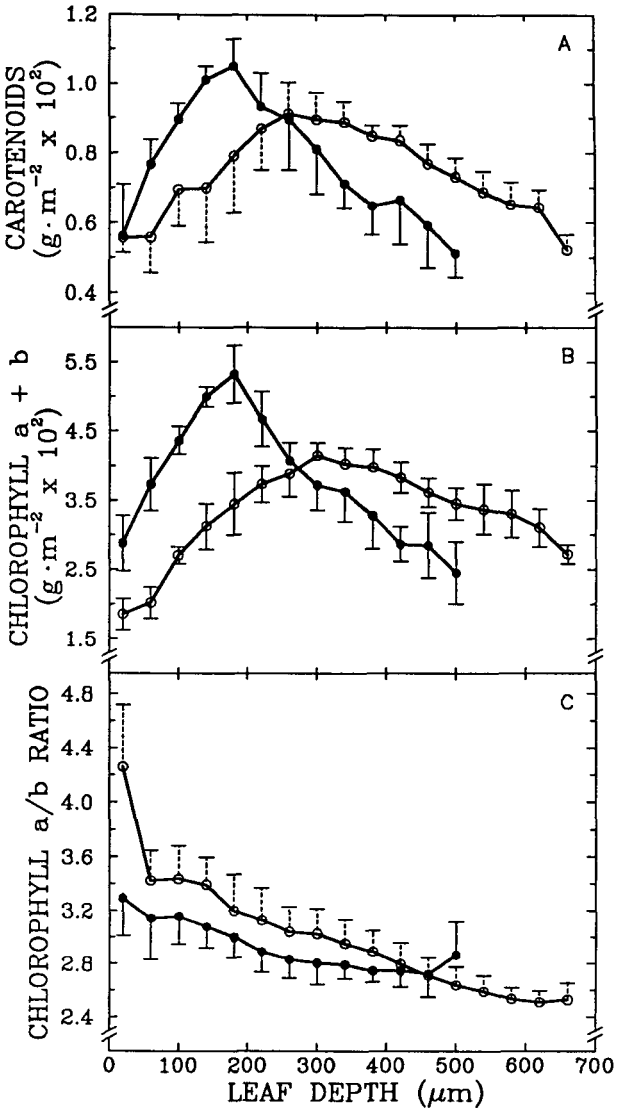

Figure 1. Pigment Composition.

(A) Carotenoids. Sun (○), *n* = 6; shade (●), *n* = 8.
(B) Chlorophyll *a+b*. Sun (○), *n* = 7; shade (●), *n* = 7.
(C) Chlorophyll *a/b*. Sun (○), *n* = 9; shade (●), *n* = 10.
Pigment composition was determined in 95% ethanol; *n* is sample size; bar represents standard deviation.

Carbon Fixation

Carbon fixation within leaves may be limited by many factors, including light and CO_2. Carbon diffusion across the leaf during a 5- to 10-sec feeding may alter patterns of carbon fixation within the leaf. Therefore, we measured carbon fixation at saturating light levels and at two different CO_2 concentrations. Carbon fixation within leaves fed 350 ppm CO_2 and 700 ppm CO_2, shown in Figure 2, exhibited identical patterns across the leaf, even though the total leaf incorporation measured as dpm was 20% higher in the leaves treated with 700 ppm CO_2 (411,810 dpm at 350 ppm vs 492,160 dpm at 700 ppm). Figures 2 and 3 show that maximum ^{14}C-CO_2 incorporation per 40-μm section occurred in the upper 20 to 40% of the leaf, a depth of 100 to 250 μm in sun leaves and 80 to 120 μm in shade leaves and not at the most adaxial surface where light was the greatest. There was a gradual decrease in CO_2 fixation toward the lower half of the leaf, but substantial fixation occurred in the bottom half. Because the increase in carbon

incorporation at 700 ppm was not accompanied by a shift in the pattern of fixation across the leaf, fixation under the steady state conditions used in our experiments does not appear to be limited by ^{14}C-CO_2 diffusion.

To test the effect of light on the CO_2 fixation gradient, ^{14}C-CO_2 incorporation at different light levels was assayed. Actual incorporation rates showed that carbon reduction in sun plants measured at 800 and 2000 μmol photons of PAR m^{-2} sec^{-1} (all further reference to photon flux is referred to as μmol PAR m^{-2} sec^{-1}) were virtually identical, whereas there was a significant increase in fixation when shade plants were assayed at 2000 μmol PAR m^{-2} sec^{-1} compared to the growth conditions of 200 μmol PAR m^{-2} sec^{-1}, as shown in Figure 3. Interestingly, the 10-fold increase in the photon flux density at which the CO_2 reduction in shade leaves was assayed increased CO_2 incorporation by only 60%, as shown in Table 1, but did not alter the general fixation pattern across the leaf (Figure 3, closed symbols). The adaxial layers increased by ~30%, whereas the abaxial layers (SM) exhibited a doubling of rates (Figure 3). In sun leaves, a 2.5-fold increase in assay light did not significantly increase the rate of fixation (Figure 3). The sun leaf is likely operating closer to saturation than a shade leaf.

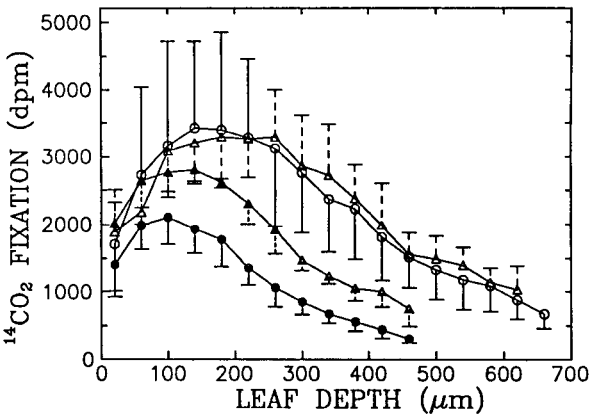

Figure 3. CO_2 Fixation across Sun and Shade Leaves of Spinach.

Sun plants were pretreated at growth conditions of 800 μmol PAR m^{-2} sec^{-1} for 1 hr. A portion of the leaf was then labeled for 10 sec with ^{14}C-CO_2 at 800 μmol PAR m^{-2} sec^{-1} (○), n = 10 and 2000 μmol PAR (△), n = 6. Shade plants were pretreated at growth conditions of 200 μmol PAR for 1 hr and then assayed at 200 μmol PAR m^{-2} sec^{-1} (●), n = 22 and 2000 μmol PAR (▲), n = 5. Each point represents average dpm ± SD; sample size given by n.

Rubisco Content

The large and small subunits (LSU and SSU) of Rubisco are easily recognizable in the polypeptide patterns from leaf sections, as shown in Figure 4. The relative amount of Rubisco determined by densitometry of the Coomassie blue–stained gels, as shown in Figure 5, was similar to the data obtained by counting ^{35}S-Met incorporated into the LSU of sun leaves, as illustrated in Figure 6. The distribution of Rubisco within the leaf was similar to the CO_2 fixation data, and the significant correlation between CO_2 fixation and Rubisco content is shown in Figure 7.

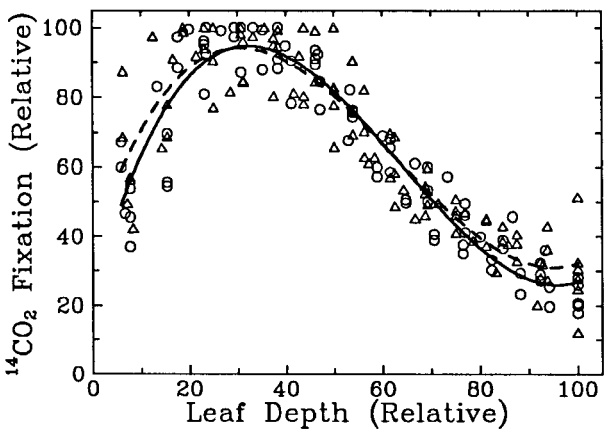

Figure 2. Effect of Increased CO_2 on Fixation Pattern across Sun Leaves of Spinach.

Sun plants were pretreated at growth conditions of 800 μmol PAR m^{-2} sec^{-1} for 1 hr. A portion of the leaf was then labeled for 10 sec with either 350 ppm (○) or 700 ppm (△) ^{14}C-CO_2 at 800 μmol PAR m^{-2} sec^{-1}.

DISCUSSION

Where Is Carbon Fixed within a Leaf?

Maximal CO_2 fixation occurred in the medial section of the leaf. In contrast, the steep light gradient across leaves (Terashima and Saeki, 1983; Vogelmann et al., 1989; Cui et al., 1991) suggests that maximal CO_2 reduction would occur near the upper leaf surface. Carbon fixation was maximal at a depth of 100 to 250 μm in sun leaves and 80 to 120 μm in shade leaves (Figure 3). Forty percent of the carbon was fixed by the SM, and only ~60% of the carbon fixation occurred in the PM, which comprises the upper 40% of the leaf. On a relative basis (percent of total carbon fixed), more fixation occurred in the PM of a shade leaf than in the PM of a sun leaf (Table 1). Studies with resolution at the SM and PM level also showed a relatively high contribution of the SM to total CO_2 fixation when plants were labeled for 10 sec; no gradient of fixation across the leaves was presented (Mokronosov et al., 1973; Outlaw and Fisher, 1975b).

The off shifting of CO_2 fixation from the light gradient is surprising. An obvious explanation is that the CO_2 fixation data are flawed, the light gradient data are flawed, or both are flawed.

Table 1. Distribution of CO_2 Fixation in Palisade Mesophyll and Spongy Mesophyll of Sun and Shade Leaves

Growth Condition	Treatment (μmols PAR m^{-2} sec^{-1}) for 1 hr	Assay Conditions (μmols PAR m^{-2} sec^{-1})	% Total CO_2 Fixation	
			PM	SM
Sun	800	800	57	43
Sun	800	2000	52	48
Shade	200	200	64	36
Shade	200	2000	57	43

It is possible that transport of products could account for the fixation in the bottom of the leaf. We attempted to minimize the possibility of transport of fixed products by keeping fixation times minimal (less than 10 sec). Conversely, we labeled long enough to overcome diffusional limitations to CO_2 and to allow significant measurable incorporation. A 5-sec pulse followed by a 25-sec chase exhibited no statistically significant differences (data not shown) between a 5-sec pulse and 5-sec chase. Labeling for 5 sec vs 10 sec did not alter the incorporation pattern, which is consistent with calculations that indicate that CO_2 diffuses across leaves within fractions of a second (Nobel, 1991). In studies done on coltsfoot labeled for 10 sec and then chased for 50 sec, the initial label in the PM decreased only ~20% (Mokronosov et al., 1973). Thus, it seems that diffusional limitations and transport artifacts are not influencing our labeling patterns.

Measurements from the upper leaf surface may be skewed by the paradermal sectioning technique and may account for the lower fixation rate at the adaxial leaf surface. The data from the first paradermal cut represent ~80% of a full section. The uppermost section also included the ~15-μm thick epidermis, which is not photosynthetically active, so the first layer would be expected to contain fewer counts. Even if we corrected for the loss of 20% of the tissue or ignored the first two points, the CO_2 fixation gradient would not be maximal at the upper leaf surface. Additionally, the pigment data collected by the same methodology exhibit a smaller slope, and the first two

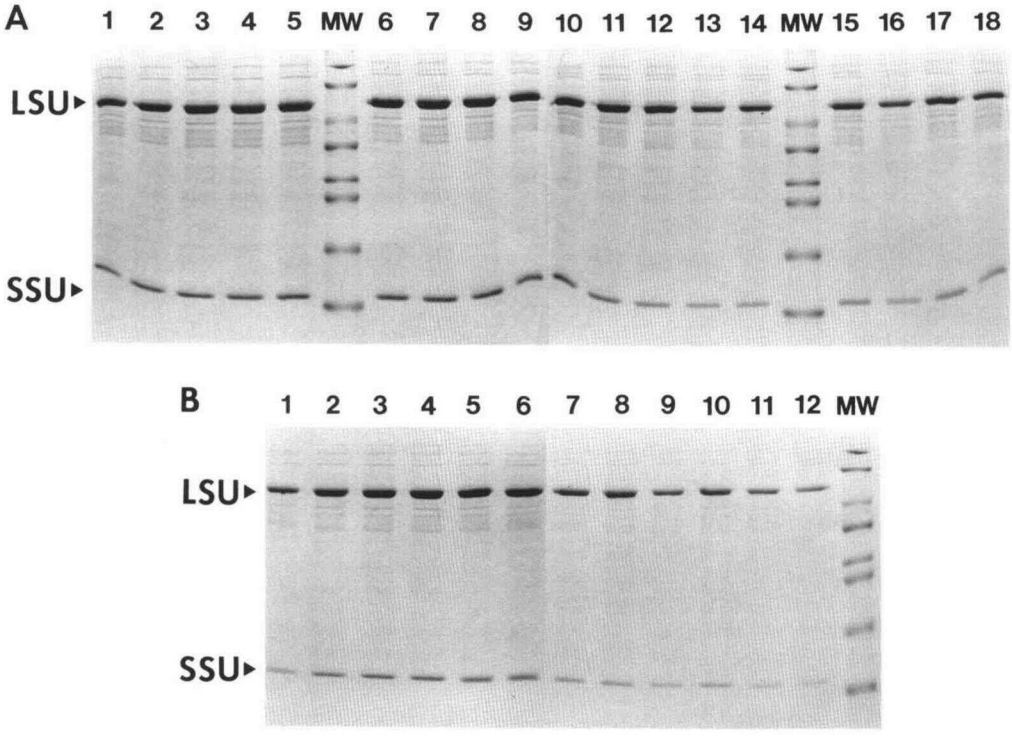

Figure 4. Rubisco Content across Sun and Shade Leaves.

(A) Sun leaf. Lanes 1 to 18 represent the most adaxial section to the most abaxial section, respectively. MW is molecular weight standards: 66, 45, 36, 29, 24, 20, 14 kD.

(B) Shade leaf. Lanes 1 to 12 represent the most adaxial section to the most abaxial section.

Polypeptides from 40-μm paradermal leaf sections were separated by SDS-PAGE and stained with Coomassie blue (see Methods). The large band represents LSU, and the distinct lower band represents SSU.

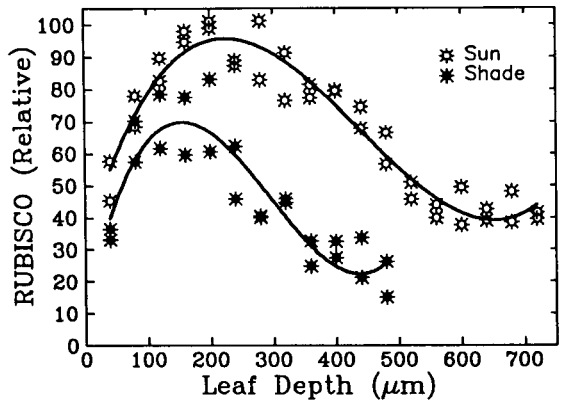

Figure 5. Large Subunit of Rubisco across Sun and Shade Leaves.

Gels similar to the ones shown in Figure 4 were scanned, and the area under the curve representing the LSU was integrated. The maximum amount of area was given a value of 100. The curves are trinomial fits (no significance to the curved tails) and represent ~93% of the data for both sun and shade leaves.

data points fall nicely within the range of what would be predicted from the slope deeper within the leaf.

Blue light (480 nm), measured with a fiber optic microprobe (Vogelmann et al., 1989; Cui et al., 1991) and by an image-analysis system (T.C. Vogelmann, J. Sun, D.A. Myers, and J.N. Nishio, unpublished data), is rapidly attenuated by the upper portion of the PM. Fiber optic measurements showed that 90% attenuation of blue and red light (680 nm) occurs within 140 μm of the adaxial leaf surface and that the SM is exposed to very low quantities of mostly green light (Cui et al., 1991). The measured exponential decrease in light across leaves agrees closely with theoretical models of light gradients across leaves (Fukshansky and von Remisowsky, 1992). Thus, it seems unlikely that the deviation between CO_2 fixation patterns and light gradients can be explained by artifacts in measuring the light gradient.

Another approach to examining the light gradient in leaves is to use the chlorophyll *a/b* as a bioassay of light quantity and quality. As expected, the chlorophyll *a/b* ratio decreased with increasing depth within the leaf. Earlier work showed that the chlorophyll *a/b* ratio is decreased in shade-grown plants (Lichtenthaler et al., 1981; Anderson, 1986; Baker and McKiernan, 1988) and that there is a decrease in the chlorophyll *a/b* ratio from the top of the leaf to the bottom, because the bottom is more shaded than the top (e.g., Outlaw, 1987; Terashima, 1989; Cui et al., 1991). If we used the chlorophyll *a/b* ratio as an indicator of the light gradient within the leaf, the light impinging on the shade leaf was similar to the light ~150 μm deep in the sun leaf. Data collected by Cui et al. (1991), using a fiber optic microprobe, showed that red and blue light are attenuated by 90% within the first 150 μm of a sun leaf. Our sun leaves were grown at 800 μmol PAR m^{-2} sec^{-1}. If the internal fluence rates (~2.3 times incident light at the leaf surface in spinach) (Cui et al., 1991) for red, blue, and green light are combined, then at a depth of 150 μm there would be light approximately equivalent to the shade-grown plant (200 μmol PAR m^{-2} sec^{-1}), with the red:far-red ratio being decreased. The relatively similar slopes in chlorophyll *a/b* vs leaf depth in sun and shade leaves suggest that after the initial 90% attenuation of the light within the upper 150 μm of the leaf, further attenuation is minimal across the rest of the leaf. Only at the most abaxial section of the sun leaf did the chlorophyll *a/b* ratio become lower than in the shade leaf, suggesting that the bottom of the sun leaf received less light than the respective portion of the shade leaf. Indeed, the shade leaves grow with a slightly erect leaf orientation compared to sun leaves, which tend to grow in flat rosettes.

There appears to be little difference in the metabolic performance and capabilities of SM and PM of C3 plants (Mokronosov, et al., 1973; Outlaw and Fisher, 1975b; Outlaw et al., 1976; Seeni et al., 1983; Shen and Outlaw, 1989). Relative fixation rates of ^{14}C-CO_2 between the PM and SM suggest that the PM fixes more CO_2 than the SM under light-limiting conditions (Outlaw and Fisher, 1975a), but the ratio approaches 1 at higher photon flux densities (Outlaw and Fisher, 1975b). Whereas Outlaw and Fisher (1975b) varied the light conditions under which the assays were made, another study varied the light conditions under which the plants were grown, and these investigators kept the measurement conditions constant (Seeni et al., 1983). Interestingly, the capacity for fixation (Seeni et al., 1983) followed the same pattern of fixation in the light saturation experiment of Outlaw and Fisher (1975b); that is, the PM:SM CO_2 fixation ratio was higher in low light–grown plants compared to high light (full sun)–grown plants (Seeni et al.,

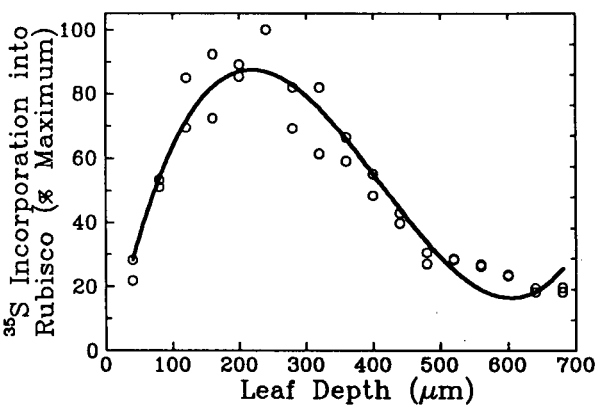

Figure 6. Large Subunit of Rubisco across Sun Leaves.

Leaf plugs were allowed to incorporate ^{35}S-Met for 1 hr, and polypeptides from 40-μm paradermal leaf sections were separated by SDS-PAGE. ^{35}S-Met incorporation into LSU was determined by cutting the LSU from gels and counting by liquid scintillation counting. The maximum amount of incorporation was given a value of 100. The synthesis of LSU corresponds well with the accumulated Rubisco shown in Figure 5.

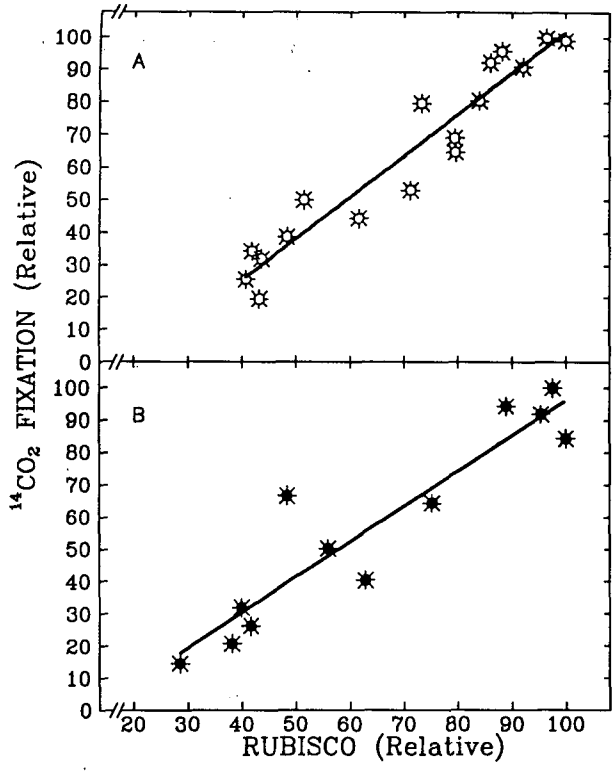

Figure 7. Relation between ^{14}C-CO_2 Incorporation and Rubisco Content across Sun and Shade Leaves of Spinach.

(A) Sun leaves. Regression is defined by the function, $y = 1.27X - 25.6245$, $r^2 = 0.923$.

(B) Shade leaves. $y = 1.10X - 13.48$, $r^2 = 0.866$.

Rubisco content determined by densitometry and ^{14}C-CO_2 incorporation (see Methods) were plotted on a relative basis with maximum amounts given a value of 100.

1983). Additionally, when ^{14}C-CO_2 incorporation was determined in isolated SM and PM under standard conditions, there was little difference in the fixation products and rates (Outlaw et al., 1976; Seeni et al., 1983). Analysis of specific enzymes and polypeptides in the SM and PM led to the conclusion that there is no significant difference in overall protein complement and photosynthetic carbon metabolism in the different mesophyll cells of *Vicia faba* (Outlaw et al., 1976; Shen and Outlaw, 1989).

In contrast to our findings, it remains a general perception that the PM is the main site of carbon fixation. The rapid attenuation of light by chlorophyll has led to statements that light limits carbon reduction in the SM (Outlaw and Fisher, 1975b; Terashima and Saeki, 1985), so changes in incident flux would have a large effect on the top of the leaf but little effect in the underlying tissue (Osborne and Raven, 1986). Additionally, models of carbon fixation across a leaf generally predict that fixation follows the light gradient (e.g., Gutschik, 1984; Evans et al., 1993). In light of the work of Mokronosov et al. (1973), Outlaw and Fisher (1975a, 1975b), and the varied approaches to measuring the light gradient, we concluded that the measured gradients of CO_2 fixation are due to interactions between light and other factors. New models of photosynthesis in leaves should incorporate the notion that the SM contributes significantly to CO_2 incorporation and that somehow the light gradient is disconnected from CO_2 fixation.

Does CO_2 Diffusion across the Leaf Limit CO_2 Fixation?

One of our experiments was aimed at determining whether the CO_2-labeling procedure was limited by CO_2 diffusion across the leaf. The light gradient across leaves (Vogelmann, 1993) is steeper than the apparent CO_2 gradient (Terashima, 1992). Typically, the PM has a greater exposed internal space than the SM, even though there are larger air spaces in the SM (Fahn, 1974; Nobel, 1991). Thus, the CO_2 gradient across the leaf under atmospheric conditions may be insignificant in terms of carbon fixation limitations. Experiments with helox suggested that a CO_2 diffusion limitation exists only at less than normal ambient CO_2 concentrations (Parkhurst and Mott, 1990). If there is a physical limitation to carbon fixation across a leaf in air, then it would likely be due to a light limitation rather than a CO_2 limitation, although the CO_2 gradient could affect enzyme distribution.

In our experiments, doubling the CO_2 concentration from 350 to 700 increased the rate of incorporation but did not change the pattern of fixation. Our data clearly showed that there is a CO_2 limitation to total leaf fixation, but the pattern of fixation across the leaf is due to an enzyme limitation, because doubling the light did not increase total carbon reduction or affect the pattern of fixation across sun leaves. We concluded that under ambient, steady state conditions, CO_2 diffusion does not limit carbon fixation in spinach. However, CO_2 gradients across leaves, if they exist, may be a factor that contributes to the Rubisco distribution within the leaf.

Is CO_2 Fixation Related to Rubisco and Pigment Content?

The Rubisco content exhibited a strong correlation between CO_2 fixation and the carboxylating enzyme ($r^2 = 0.92$ for sun and 0.87 for shade leaves). The slope of the correlations, 1.0 for shade and 1.3 for sun plants, suggested that enzyme content more than enzyme activation controls fixation across the leaf. In contrast, the correlation coefficient for CO_2 fixation and chlorophyll was low ($r^2 = 0.024$ for sun and 0.476 for shade). The correlation among chlorophyll, carotenoids, Rubisco, and carbon fixation was higher in shade leaves than in sun leaves.

Studies on C3 plants indicate that Rubisco activity in total leaf homogenates, on both an areal and a chlorophyll basis, generally decreases with decreasing light (Björkman, 1968; Bowes et al., 1972; Singh et al., 1974). In spinach, the amount

of Rubisco was greater in the PM than in the SM (Terashima and Inoue, 1985a) and greater in the upper portion of the leaf than in the lower, shaded region of the leaf (Terashima and Inoue, 1985b). In *V. faba*, however, the Rubisco activity on a chlorophyll basis was greater in the SM than in the PM (Outlaw et al., 1976). In contrast to *V. faba*, in spinach there was less Rubisco/chlorophyll in the lower part of the leaf.

What Is the Relation between Light and CO_2 Fixation within a Leaf?

The lack of carboxylation at the site of highest photon flux is surprising. Possible photoinhibitory damage or down regulation at the upper leaf surface was investigated by fluorescence measurements with a pulse amplitude modulated fluorometer (model 2000; Heinz Walz GmbH, Effeltrich, Germany). The upper part of the leaves was not damaged (F_V/F_M = 0.85), yet relatively little carboxylation occurred at the adaxial leaf surface. Photosynthetic carbon fixation rates were maximal at the point at which ~90% of the blue and red light are attenuated. Thus, green light is a particularly important light energy source deep within the leaf (T.C. Vogelmann, J. Sun, and J.N. Nishio, unpublished data). We concluded that the upper leaf surface is light saturated and enzyme limited.

Outlaw and coworkers investigated the possibility that malate may play a direct role in C3 plants similar to that in C4 plants (Outlaw et al., 1976). Using ^{14}C-CO_2 incorporation, they found that in the PM, 20% of the labeled carbon was associated with malate, whereas in the SM only 5% of the labeled carbon was incorporated into malate, but no significance was speculated. Because they used isolated cells and made measurements of CO_2 fixation under similar light conditions, they inadvertently eliminated the light gradient. The results suggest that the capacity for malate production in the light may be higher in the PM than in the SM. Actual assayed phosphoenolpyruvate carboxylase activity (on a chlorophyll basis) was lower in the PM than in the SM; possibly the SM was phosphoenolpyruvate limited in the light.

Light utilization deep within a leaf may be enhanced by altered photosynthetic systems. The increase in PSII/PSI stoichiometry associated with shade plants allows for equivalent quantum efficiency under far-red light–enhanced conditions (Chow et al., 1990) by changes in the light-harvesting complex that more efficiently funnel PSII light to PSI (McKiernan and Baker, 1991). However, by every measure, light appears to be very depleted in the lower region of the leaf.

The results raise many interesting questions about the relation between light in leaves and photosynthetic carbon fixation and electron transport. The methods for measuring CO_2 fixation and protein turnover across leaves now enable us to investigate fundamental processes within leaves. We can now test further the relationship between leaf anatomy (possibly related to leaf optics and transport and diffusion of metabolites) and metabolism (for example, enzyme distribution, gene expression, and light harvesting and electron transport considerations) that leads to optimum photosynthetic performance under saturating conditions in the sun and nonsaturating conditions in the shade or on cloudy days. Developmental studies including investigations into how enzyme distribution in cells is regulated and how internal photosynthetic performance is influenced by environmental factors such as increased CO_2 or UV light will be enlightening.

METHODS

Plant Growth Conditions

Spinach (*Spinacia oleracea*) was cultured hydroponically in controlled environment growth chambers. Lighting was provided by metal arc lamps plus incandescent lamps; PAR of sun condition was 800 μmol m^{-2} sec^{-1}; PAR of shade condition was 200 μmol m^{-2} sec^{-1} obtained with NR20SMARL film (3M; Minneapolis, MN). The film reduces the red:far-red ratio to ~0.25, which is equivalent to deep shade. Five- to 6-week-old plants were sampled.

Chlorophyll and Carotenoid Determination

Four leaf discs were paradermally sectioned into 40-μm thick layers using a cold-stage microtome (Spencer Lens Co., Buffalo, NY); corresponding layers were pooled in a small vial containing 1 mL 95% ethanol plus a little sodium ascorbate powder, which acts as an antioxidant. Samples were kept in the dark until analysis. Chlorophyll *a* and *b* and carotenoids were determined by measuring absorbency at 470, 648.6, and 664.2 nm minus absorbance at 730 nm with a spectrophotometer (Lambda 4B; Perkin Elmer, Norwalk, CT) using the extinction coefficients determined by Lichtenthaler (1987).

^{14}C-CO_2 Fixation

After 1-hr illumination, a leaf was clamped in a small leaf chamber that allowed illumination from the top and gas exchange through ports on the bottom. The leaf in the chamber was illuminated by a quartz halogen light (21 volt, 150 W, EKE) directed with a fiber optic cable. The direction of illumination was perpendicular to the leaf surface, which is similar to the direction of light under which the sun leaves developed. Because shade leaves grow upward at a slight angle, the direction of assay illumination was not exactly the same as that which the leaves experienced in the growth chamber. Such a change in angle will affect the horizontal distribution of light within a cell and can affect light penetration. On average, the change in illumination angle does not affect our interpretation of the data. ^{14}C-CO_2(g) was delivered with a syringe to the abaxial surface of the leaf for ~8 to 10 sec. The total volume injected was 2.5 mL containing 700 ppm CO_2 and ^{14}C-CO_2 with a total activity of 0.8 μCi. Immediately after labeling, a leaf disc (0.5 cm^2) was acquired with a precooled ($N_2(l)$) paper punch. Actual freezing time with the cold punch was insignificant, but manipulation of tissue and the punch resulted in approximately a 5-sec delay, so leaves were labeled for ~12 to 15 sec. The frozen leaf disc was then transferred onto the stage of a freezing microtome and cut paradermally into 40-μm thick sections. Each section was immediately put into 1 mL 95% ethanol in a liquid scintillation vial and kept at −20°C unless immediately counted. Two drops, delivered by a graduated

disposable pipette (5 mL capacity), of ~25% acetic acid were added to each sample, and the vial was then heated in a boiling water bath (95°C) for 1 min to release unincorporated ^{14}C-CO_2 and to bleach the photosynthetic pigments. The radioactivity was determined in a liquid scintillation counter (TriCarb 4430; United Technologies Packard, Downers Grove, IL).

Rubisco Determination

Polyacrylamide Gel Electrophoresis

A leaf disc was sampled and microsectioned as described in the ^{14}C-CO_2 fixation section above. Each section was put into a 1.5 mL microcentrifuge tube in a 67-μL solution containing 25 mM Hepes, pH 7.6, 0.5 mM Na_2-EDTA, 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, and 5 mM β-mercaptoethanol. Samples were then macerated with a microhomogenizer and centrifuged at 12,000*g* for 15 min at 4°C. A 30-μL aliquot of supernatant was mixed with 15 μL of sample buffer containing 100 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 6% (g/100 mL) SDS, 5% (g/100 mL) 2-mercaptoethanol (Laemmli, 1970). Samples were heated at 100°C for 2 min and centrifuged at 12,000*g* for 4 min at 4°C. The total sample was loaded on a 13% SDS–polyacrylamide gel. After electrophoresis, gels were stained with 0.25% Coomassie blue in 50% methanol and 10% acetic acid and destained with a solution containing 50% methanol and 10% acetic acid. Gels were then scanned at 565 nm on a Beckman DU1000 adapted by Gilford (Oberlin, OH). The scans were integrated on an integrator (model 3396, series II; Hewlett-Packard, Palo Alto, CA).

^{35}S-Methionine Labeling

After leaves were light treated for 1 hr at growth conditions (either 200 μmol PAR m^{-2} sec^{-1} or 800 μmol PAR m^{-2} sec^{-1}), a 0.5-cm^2 leaf disc was placed upside down in a glass vial containing 100 μL 20 mM Hepes, pH 7.7, 0.4 mM Na_2-EDTA, 0.01% (v/v) Triton X-100, 0.4 mM $MgCl_2$, 0.4 mM $MnCl_2$, 40 mM $NaHCO_3$, 0.25 mM each amino acid minus methionine and cysteine, 100 μCi ^{35}S-methionine. The ^{35}S-methionine (ICN, Irvine, CA) contained 15% labeled cysteine. A leaf plug–sized piece of white filter paper was then placed over the sample to keep the sample immersed. Samples were then vacuum infiltrated and bottom illuminated (the upper surface of the leaf faces the light) with light equivalent to the growth condition. Samples were shaken every 3 to 5 min. After 30 min, the samples were again vacuum infiltrated and placed in the light for another 30-min period. The leaf plugs were then sectioned and separated by PAGE as described above. Protein was blotted to PVDF membrane at 1 A for 2 hr at 4°C. The filters were dried and exposed to Kodak XAR film. Film was developed with GBX (Eastman-Kodak, Rochester, NY). The piece of blot containing the large subunit of Rubisco was cut from the gel and incorporation was determined by liquid scintillation counting (TriCarb 4430; United Technologies Packard).

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