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Linking light capture with CO_2 fixation: new methods enable integration from the chloroplast to the leaf.

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Introduction

There is a large amount of literature published on the photosynthetic properties of intact leaves obtained from gas exchange measurements. Often data are interpreted by applying the C_3 biochemical model of Farquhar, Von Caemmerer & Berry (1980). This model scales from the biochemistry of a chloroplast to that of a leaf by assuming that the photosynthetic capacity of any chloroplast in a leaf is directly proportional to the light absorbed by that chloroplast. It is well known that the properties of chloroplasts differ through a leaf (Terashima & Inoue, 1984, 1985a, b). It has also been shown that light is attenuated through a leaf either by direct measurement of reflected and transmitted light from paradermally sectioned leaves (Terashima & Saeki, 1983), or calculated from light measured by optical fibres inserted through leaves (Cui, Vogelmann & Smith, 1991, Vogelmann & Björn, 1984, Vogelmann, Bornman & Josserand, 1989, Vogelmann *et al.*, 1988). However, until recently, it has not been possible to rigorously examine the assumption that photosynthetic capacity per unit absorbed light is a constant.

Nishio, Sun & Vogelmann (1993) compared ^{14}C fixation profiles with the light gradient measured by optical fibres and concluded that 'the light gradient is disconnected from CO_2 fixation'. A reanalysis of the data showed that CO_2 fixation could be predicted assuming light absorption through the leaf obeyed Beer's law (Evans, 1995). More extensive ^{14}C fixation data was published by Sun, Nishio & Vogelmann (1998) and a reanalysis of the data again was able to model the experimental observations (Evans, 1999). To resolve the issue of the relationships between gradients of absorbed light and carbon fixation, we have made comprehensive measurements of the profiles of light absorption, chlorophyll, photosynthetic capacity and ^{14}C fixation in spinach leaves.

To measure the profile of light absorption through leaves, we modified the technique of Takahashi *et al.* (1994), who viewed a transversely cut leaf surface with laser light directed at the adaxial surface. Vogelmann & Han (2000) showed that fluorescence profiles peaked at progressively greater depths in spinach leaves when blue, red and green light was directed at the adaxial surface. They also showed that green light penetrated deeper into the leaf when applied to the adaxial compared to the abaxial surface, due to the presence of palisade cells. Our objectives were firstly, to quantitatively link the profiles of light absorption with that of chlorophyll. Secondly, to quantitatively link the profile of light absorption with that of CO_2 fixation.

Materials and methods

Spinacia oleracea was grown under 400 μmol quanta m^{-2} s^{-1}, with an eight hour day at 20°C and a 16 hour night at 16°C. Chlorophyll fluorescence was observed on leaf pieces cut transversely and mounted between glass slides perpendicular to the stage of an inverted microscope (Vogelmann & Han, 2000). The fluorescence images were captured with a cryogenically cooled CCD camera through a 685nm interference filter. Fluorescence was generated either by epi-illumination or by directing monochromatic light normal to the adaxial or abaxial leaf surface using a xenon lamp and interference filters (450 and 567nm). Other parts of the leaf were freeze clamped and paradermally sectioned with a freezing microtome into 40 μm layers. This was used to determine chlorophyll distribution or ^{14}C fixation. For ^{14}C fixation, leaves were exposed to $^{14}CO_2$ for 10 seconds under blue or green light incident upon the adaxial surface. Following sectioning, incorporated ^{14}C was measured by liquid scintillation counting. Details for the modelling are given by Evans (1995, 1999).

Results and discussion

Quantitative analysis of the chlorophyll fluorescence image at 685nm showed that it was directly proportional to the amount of light absorbed. The decline in fluorescence through a cuvette containing chlorophyll in 80% acetone and the relationship between fluorescence intensity and chlorophyll concentration were consistent with Beer's law. For an intact leaf, the optics are more complicated as fluorescence need not originate near the cut surface nor have the same quantum yield. It is possible for fluorescence to enter into non-absorbing tissue, such as the epidermis, and be scattered out at the cut surface. We were able to avoid this artifact by beginning our sampling from the mesophyll, by comparison with images of the leaf section. For mesophyll tissue, the strong absorption of 685nm light by chlorophyll means that fluorescence escaping from the cut surface originates near to it. Thus there should be a tight correlation between the fluorescence and absorption profiles. From other controls, we also know that absorption of light travelling parallel to the leaf surface is similar through palisade and spongy mesophyll. From these and previous measurements (Vogelmann & Han, 2000), we are confident that the fluorescence images accurately reflect the profile of light absorption. This technique enables profiles of light absorption to be obtained with relative ease and with excellent spatial resolution.

The profile of light absorption depends on the wavelength and leaf orientation (Fig. 1). Blue light is more strongly absorbed than green light, peaking near 50 μm, with 50 and 90% of blue light being captured by 110 and 310 μm depths, respectively. Green light is absorbed more evenly through the leaf, with 50 and 90% captured by 215 and 470 μm, respectively. When the leaf is inverted, both blue and green light are absorbed nearer the surface, with 50% absorbed by 60 and 130 μm for blue and green, respectively. The difference associated with leaf orientation is due to the bifacial leaf anatomy. Palisade tissue beneath the adaxial surface facilitates light penetration bypassing the chloroplasts that line the cylindrical cells. By contrast, spongy mesophyll beneath the abaxial surface efficiently scatters light, increasing the pathlength of light and the probability of absorption.

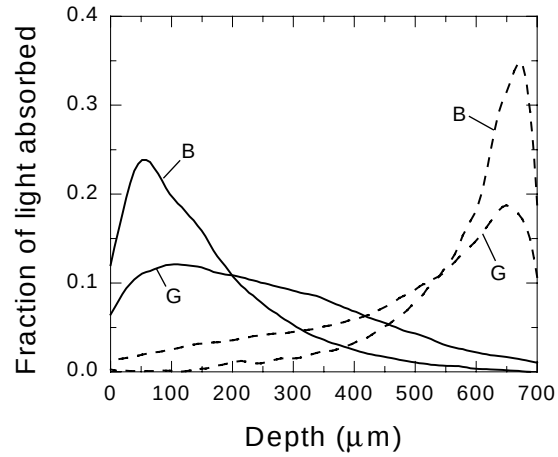


Fig. 1 Profiles of chlorophyll fluorescence through the mesophyll of spinach leaves when blue (B) or green (G) light was applied to the adaxial (solid lines) or abaxial (dashed lines) leaf surface. The area under each of the profiles is normalised to 1, with the scale giving the fraction of light absorbed per 50 microns.

The methodology used to capture fluorescence allowed us to compare fluorescence profiles when light was applied normal to the epidermis with fluorescence images under epi-illumination, which revealed the spatial distribution of chlorophyll through the leaf. We compared the profile of fluorescence under epi-illumination with the profile of chlorophyll determined from paradermal sections. The two techniques gave essentially the same profile. While epi-illumination cannot generate absolute values for the chlorophyll profile, it has much better spatial resolution than can be obtained from paradermal sectioning and is easier to obtain. By measuring the chlorophyll content per unit leaf area, the epi-fluorescence profile can be converted to an absolute basis. Epi-fluorescence is directly proportional to chlorophyll and by summing it from the adaxial surface, one can examine the relationship between light absorption and chlorophyll. The absorbance for a given wavelength of light was calculated as log(sum of fluorescence profile/(sum of fluorescence profile – cumulative fluorescence to that layer)). We were able to calculate the relationship at 3 micron intervals because of the spatial resolution of the images (Fig. 2). The data were consistent with the Beer-Bouguer law, since absorbance was nearly linear with cumulative chlorophyll. The slope of the relationships give apparent extinction coefficients of 2640 and 1200 m^2 (mol Chl)$^{-1}$ for the blue and green wavelengths, respectively. The linear relationships also show no sign of curvature around the transition from palisade to spongy mesophyll. This is probably because by that depth, light has already become quite scattered. When attenuance of light was measured with *Camellia* leaves sectioned to vary their thickness, an increase in slope was observed through spongy tissue compared to palisade tissue (Terashima & Saeki, 1983). Whether this reflects different anatomy between *Spinacia* and *Camellia* or is due to the different methodology is unclear.

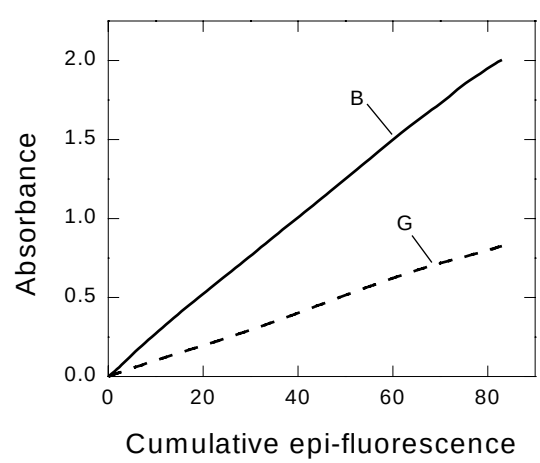
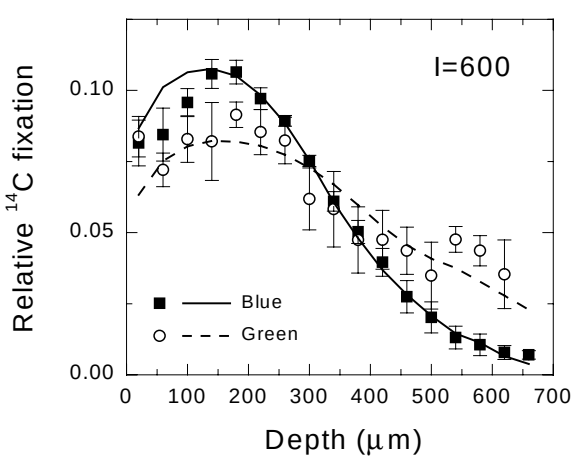


Fig. 2 Relationship between absorbance of blue (B) or green (G) light and cumulative chlorophyll from the adaxial surface of a spinach leaf. Cumulative chlorophyll is proportional to the fluorescence obtained from an image taken with epi-illumination. Absorbance was calculated from fluorescence when light was applied to the adaxial surface.

Is it possible to convert light gradients in *Spinacia* leaves calculated from inserting optical fibre probes at several angles, to profiles of light absorption? One cannot derive absorption from the change in internal fluence rate. However, if one accepts that absorption behaves according to the Beer-Bouguer law (Fig. 2), then absorbed irradiance can be approximated by $I(10^{\varepsilon C} - 1)$, where I is the internal fluence rate at a given depth, C is the chlorophyll content at that depth and ε is the apparent extinction coefficient. We applied this to profiles of internal fluence rate calculated by Cui *et al.* (1991) for 450 and 550nm light and their chlorophyll profiles, then compared them to Fig. 1 (not shown). There was remarkably good agreement for the profiles obtained by the independent methods at 550nm, but a large difference at 450nm. The profile calculated from internal fluence rate at 450nm peaked around 26 μm, compared to 50 μm shown in Fig. 1. This results in very little blue light absorption at depth, which is not consistent with the profile of ^{14}C fixation (see below). It would appear that the calculation of internal fluence rate at 450nm is in error. The internal fluence rate for *Spinacia* was calculated from profiles measured at three angles (Cui *et al.*, 1991). There was surprisingly little light scattered at 30° and the proportions were similar for 450 and 550nm light. This contrasts with the scattered light profile through *Camellia* leaves obtained by Koizumi *et al.* (1998) where considerably more blue light was scattered in the initial layers than green light.

We paradermally sectioned spinach leaves following ^{14}C labeling to determine the profiles of photosynthetic capacity and CO_2 assimilation. Photosynthetic capacity was obtained by applying light normal to the transversely cut leaf surface. The profile obtained was remarkably similar to the profile of Rubisco determined by Nishio et al. (1993) (data not shown). Profiles of CO_2 assimilation were measured with blue or green light applied to the adaxial or abaxial surface at 150 or 600 μmol quanta m^{-2} s^{-1} (Fig. 3). We modeled the profile of CO_2 assimilation according to Evans (1995) (shown as lines in Fig. 3), except that in the present case we used a direct estimate of light absorbed rather than calculating it from the Beer-Bouguer law. The agreement between the measured CO_2 assimilation and the modeled profiles was excellent. We could account for the changes in the profiles due to wavelength, leaf orientation and irradiance, which suggests that we have achieved a robust understanding of leaf photosynthesis at the cellular level.

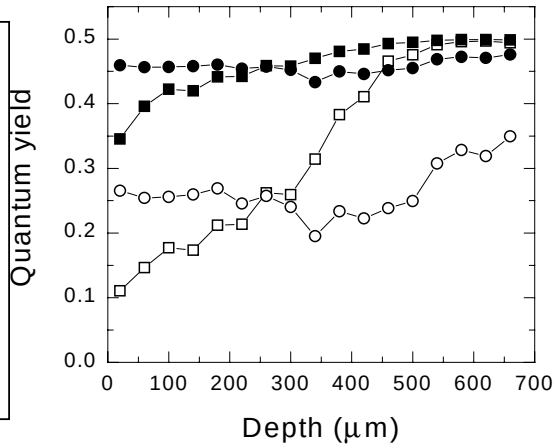
Fig. 3 Profiles of ^{14}C fixation through a spinach leaf with 600 μmol quanta m^{-2} s^{-1} of blue or green light perpendicular to the adaxial surface. Symbols represent mean values and standard errors (n = 6) from paradermal sections. The lines are calculated from profiles of light absorption and photosynthetic capacity.



Comparison of the light absorption profiles with that of CO_2 assimilation reveals that the two are not identical. CO_2 assimilation follows a broader peak that reaches its maximum deeper within the leaf than blue light absorption. This reflects the fact that the profile of photosynthetic capacity differs from the profile of blue light absorption. By contrast, photosynthetic capacity is very similar to the profile of green light absorption. Consequently CO_2 assimilation under green light peaks at the same depth as green light absorption. The relative decline with depth is, however, less for CO_2 assimilation than light absorption because of the curvilinear relationship between photosynthesis and irradiance. That is, the quantum yield of photosynthesis declines with increasing irradiance.

The wavelength dependence of the profile of light absorption means that the profile of photosynthetic capacity cannot always match that for light absorption. For spinach, a close match between the profile of photosynthetic capacity and green light absorption was observed. Consequently, the profile of quantum yield through a spinach leaf under green light is nearly constant for a given irradiance applied to the adaxial surface (Fig. 4). However, under blue light that is strongly absorbed near the surface, quantum yields are low and rise steadily with increasing depth. This has obvious implications for the interpretation of fluorescence signals obtained by applying modulated diode light as a surrogate for whole leaf electron transport. The layers of chloroplasts sampled by the modulated light used to probe fluorescence will depend on the wavelength of the excitation LED and on the fluorescence wavelength collected by the detector, which varies between instruments. The resultant photochemical efficiency calculated from the fluorescence reflects a subpopulation of chloroplasts that may not directly represent the total photosynthesis by the leaf.

Fig. 4 Profiles of quantum yield (mol e^- (mol quanta absorbed)$^{-1}$) through a spinach leaf with 600 (open symbols) or 150 µmol quanta m^{-2} s^{-1} (solid symbols) of blue (squares) or green (circles) light perpendicular to the adaxial surface. The points are calculated from profiles of light absorption and photosynthetic capacity.



Photosynthetic capacity could scale in direct proportion to the profile of absorbed white light. However, for *Spinacia*, this was not the case as it more closely reflected the profile of green light absorption. Consequently estimation of Rubisco activity by the Farquhar *et al.* (1980) model would underestimate the amount actually present in the leaf.

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