Topography of Photosynthetic Activity of Leaves Obtained from Video Images of Chlorophyll Fluorescence

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ABSTRACT

The distribution of photosynthetic activity over the area of a leaf and its change with time was determined (at low partial pressure of O₂) by recording images of chlorophyll fluorescence during saturating light flashes. Simultaneously, the gas exchange was being measured. Reductions of local fluorescence intensity quantitatively displayed the extent of nonphotochemical quenching; quench coefficients, qₑ, were computed pixel by pixel. Because rates of photosynthetic electron transport are positively correlated with (1 – qₑ), computed images of (1 – qₑ) represented topographies of photosynthetic activity. Following application of abscisic acid to the heterobaric leaves of Xanthium strumarium L., clearly delineated regions varying in nonphotochemical quenching appeared that coincided with areoles formed by minor veins and indicated stomatal closure in groups.

Until recently, photosynthesis in leaves was studied under the assumption of a uniform distribution of photosynthetic activity. However, there is evidence that this is not always the case. For instance, Farquhar et al. (8) suggested that the decline in photosynthetic capacity following an application of ABA was caused by stomata closing in groups while other stomata remained open. This would result in a nonuniform distribution of photosynthetic activity. Heterogeneity of stomatal response introduces ambiguity into the interpretation of gas analysis experiments (8); information on spatial patterns of stomatal behavior and their changes with time is therefore needed and wanting. Terashima et al. (16) and Downton et al. (5) showed the appearance of such patterns during the assimilation of CO₂ by leaves that had been given ABA by starch-printing or ¹⁴C-autoradiography. Areas differing in apparent photosynthetic activity coincided with the areoles which, in heterobaric leaves, are formed by the minor veins. However, these methods are destructive; they cannot be used routinely to assess the extent to which the distributions of photosynthetic activity over an area of leaf may change with time. We are primarily concerned with stomatal responses here, but we note that inhomogeneous patterns of photosynthetic activity might also result from exposure to pollutant gases, insect damage, infection by pathogens, water or salinity stress, or by nonhomogeneous regulation of photosynthesis.

We set out to develop a nondestructive and noninvasive method to examine the topography of the photosynthetic activity in leaves by evaluating the spatial variation of Chl fluorescence. We were encouraged to do so by the work of Björn and Forsberg (2), Ellenson and Amundson (7), and Omasa et al. (13), who showed that images of Chl fluorescence could be obtained from intact leaves. We have extended these approaches to prepare images showing the level of fluorescence quenching by nonphotochemical mechanisms.

Duyens and Sweers (6) recognized that Chl fluorescence in photosynthetic systems could be quenched by nonphotochemical dissipation, and they postulated that the level of this quenching was regulated to dissipate excess absorbed energy not needed to do photochemical work. In recent years, advances were made in the measurement and interpretation of these nonphotochemical processes. Their combined effects are represented by the quench coefficient, qₑ, (10, 11), or briefly, qₑ. This coefficient includes the 'energy-dependent' quench coefficient, qₑ, which describes the degree of quenching caused by the pH gradient across the thylakoid membrane (1, 3, 15, 17, 18) as well as transfer of quanta to PSI (10, 12) or their resonant transfer to other molecules, ultimately generating heat (4). Increases in nonphotochemical quenching can be measured as a decrease in the variable fluorescence elicited by a brief pulse of intense light applied in addition to any actinic light used to drive photosynthesis (15). It was found that these reductions in fluorescence correlated with decreases in the quantum efficiency of light-driven electron transport through PSI (17, 18). It has been proposed that these changes in nonphotochemical quenching are related to the maintenance of a stoichiometric balance between reactions that consume and those that produce NADPH during steady state photosynthesis (19). A reduction of stomatal

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conductance can restrict the rate of CO₂ fixation and consequently the rate of electron transport through PSII. If this happens and light intensity remains constant, the quantum efficiency of photosynthetic electron transport will decline and the degree of nonphotochemical quenching will rise so that stoichiometric balance is maintained. It is expected that the emission of Chl fluorescence in response to flashes of saturating light will decline in areas of the leaf where stomata are closing and, as a consequence, the rate of photosynthesis is decreasing.

In our experiments, parts of leaves were enclosed in cuvettes. Gas exchange was measured continuously while fluorescence images of the enclosed regions of the leaves were recorded during pulses of intense light, following the method of Schreiber et al. (15). We made use of digitized video which yields 8 bit (one part in 256) resolution of intensity over a 512 × 512 matrix. Images obtained after dark-adaptation ([Fₐ],[Fₒ]), when nonphotochemical quenching was at its minimum, served as references. Computer processing of reference and sample frames ([Fₐ],[Fₒ]) for the area of leaf under investigation was then used to obtain a pixel-by-pixel display of the level of qₑ. Coefficients of nonphotochemical quenching were calculated for all picture elements (pixels) of each image. Because photosynthetic electron transport is positively correlated with 1−qₑ or 1−qₑ (11, 17, 18), the quantitative evaluation of fluorescence images allowed conclusions to be drawn on the topography of photosynthetic electron transport in a leaf and, by comparing images taken at suitable partial pressures of CO₂ and O₂, also on the distribution of stomatal conductances.

**MATERIALS AND METHODS**

Plants of Xanthium strumarium L. were grown in a greenhouse under natural illumination at 25/15°C (day/night) in 15-cm pots containing a commercial peat-perlite-vermiculite mixture. The plants were fertilized with a nutrient solution every other day. Synthetic (±)-abscisic acid (Aldrich) was dissolved in 95% ethanol for application to the water supply of detached leaves to achieve a final concentration of 10⁻⁴ M. DCMU was applied correspondingly at 10⁻⁴ M.

Gas exchange and fluorescence measurements were conducted as described before (9, 17) with an open-flow steady state gas-exchange system that permitted control of the partial pressures of CO₂, O₂, and H₂O and included a temperature-regulated leaf chamber. Fluorescence was measured with a pulse-modulated fluorimeter which averaged the emission over the leaf area (PAM, Heinz Walz, Mess- und Regeltechnik, 8521 Effeltrich, FRG). High-intensity pulses of light were produced by an ILC-300 xenon arc lamp (ILC Corp., Menlo Park, CA) behind an electronic shutter (Uniblitz, A. W. Vincent Assoc., Inc., Rochester, NY).

The light was filtered through 6 mm of water and a short-pass glass filter (Corning 9782). The light flashes were guided to the leaf through the branched fiber-optic light guide of the PAM fluorimeter. Flash quantum flux was 4500 μmol m⁻²s⁻¹, and varied no more than about 5% across the field of view, when measured with a 1 mm² gallium arsenide photocell (Hamamatsu G1118), in turn calibrated against a quantum sensor (LiCor 190-S). The actinic light was supplied by a tungsten-halide lamp that was turned off during each flash.

The duration of the saturating flashes was kept to <1 s, in order to avoid perturbation of steady state photosynthesis.

Fluorescence images were obtained from the same area of leaf viewed by the PAM fluorimeter (Fig. 1). The image of an area of approximately 8 mm in diameter was projected by a Canon FD 100-mm macro lens (mounted on bellows and fitted with a Schott RG 9 long pass filter, >710 nm) onto a charge-coupled device (CCD) camera (NEC model TI-22P), with jumpers set for linear response to brightness, and for automatic gain control off. Images were recorded as analog signals on standard VHS video tape. The area-averaged determinations by the PAM instrument were recorded simultaneously with a strip chart recorder.

A Society of Motion Picture and Television Engineers (SMPTE) time encoder-comparator system was used to provide a post hoc time base on one of the audio tracks of the video tape. The system incorporated a Gray Engineering Labs (Orange, CA) SMPTE code transmitter linked to a synchronizing pulse generator, data receiver, and code comparator (DT-113, CSC-710, DR-107B, DCC-114, respectively). A Quantex DS-50 video digitizer, controlled over the IEEE-488 bus by a VAX computer (11/780), captured frames. Digitized images were transferred to a magnetic tape and further processed on a MicroVAX II computer equipped with a Traprax 5500 array processor (Recognition Concepts, Inc., Incline Village, NV). Image ratios were computed with software from Tau, Inc. (Los Gatos, CA). Distributions of qₑ within the frames were calculated with the ACUity image analysis system (20), and perspective plots were generated with a NuVision image processing system (Percepts, Inc., Knoxville, TN). The computation of the nonphotochemical quench coefficients was based on

\[
qₑ = \frac{(Fₐ)ₙ - (Fₒ)ₙ}{(Fₐ)ₙ - Fₒ}
\]

where (Fₐ)ₙ is the reference fluorescence signal during a saturating light flash on a leaf adapted to darkness, (Fₐ)ₙ is the flash-saturated variable fluorescence yield during photosynthesis induction or steady state photosynthesis, and Fₒ is the fluorescence excited by the weak measuring beam of the PAM fluorimeter (1, 15). Topographs of qₑ were computed after a modification of the equation for qₑ:

\[
qₑ = \frac{2^{14} \cdot [(Fₐ)ₙ - (Fₐ)ₙ]}{2^8 \cdot 0.8 \cdot (Fₐ)ₙ}
\]

which allowed exploitation of the eight-bit pixel density. The denominator was simplified on the assumption that Fₒ (which could not be measured with the video system) is nearly constant and approximately 0.2 · (Fₐ)ₙ (1). The resulting frame was thus scaled for an eight-bit gray scale between 0 = black and 255 = white, where black corresponds to qₑ = 0 and white to qₑ = 1.0. Subtraction of qₑ frames from a white frame (all pixels = 255) produced (1 − qₑ)-frames: their gray scales were correlated with the intensity of the rate of photosynthetic electron transport (17).

**RESULTS**

Figure 2A shows the fluorescence image of a leaf of X. strumarium during a high intensity flash given after the leaf...
TOPOGRAPHY OF PHOTOSYNTHESIS FROM FLUORESCENCE IMAGING

Figure 1. Image-recording and gas-exchange system (CCD = charge coupled device).

Figure 2. Derivation of gray scale topographies of nonphotochemical fluorescence quenching ($q_n$) and estimated photosynthetic electron transport ($1 - q_n$) from a Chl-fluorescence image. A, Fluorescence image of a detached, dark-adapted *X. strumarium* leaf ($F_a$), in an atmosphere of 350 µL·L⁻¹ CO₂, 21% O₂, and 80% RH. Saturating-flash quantum flux was 4500 µmol m⁻²s⁻¹. Nylon fibers supporting the leaf are visible at the lower left and upper right of each frame. The bar indicates 1 mm. B, Fluorescence image of the same leaf ($[F_m]$), 10 min after application of $10^{-4}$ M ABA to the solution supplying the cut petiole. Illumination with 680 µmol m⁻²s⁻¹ actinic light; 2% O₂, 80% RH; c, (computed from gas-exchange data) was 200 µL·L⁻¹. C, $q_m$ frame, formed by dividing corresponding pixel brightnesses of a difference frame ($[F_{md}] - [F_m]$) by 0.8 of the dark-adapted reference, ($[F_{md}]$). D, ($1 - q_m$)-frame derived by subtraction of the $q_m$ frame from the number 255 (corresponding to 'white').
had adapted to darkness. The reticulate pattern of the venation appears dark against the fluorescence of the mesophyll at wavelengths >720 nm. Steady state photosynthesis was established in this leaf by exposure to actinic light at a quantum flux of 680 μmol m−2s−1 (λ = 400–700 nm) in 2% O2 (v/v) and at an ambient CO2 concentration that resulted in a computed average intercellular CO2 concentration of about 200 μL·L−1. Then, (±)-ABA was added to the water supply of the detached leaf (resulting in a concentration of 10−4 M). Ten min later, clearly outlined areas of less intense fluorescence appeared during high intensity pulses of light, indicating regions of increased nonphotochemical quenching (Fig. 2B). These areas coincided with areoles formed by minor veins; often, they did not adjoin the major veins. From the images shown in Figure 2, A and B, a distribution of qN was computed (Fig. 2C). Subtraction of this distribution from a white frame resulted in an image of the distribution of (1 − qN) (Fig. 2D). In this (1 − qN)-frame, brightness is correlated with photosynthetic electron transport, and this image represents a topography of the photosynthetic activity in the area of the leaf viewed by the video camera. The (1 − qN)-frames superficially resembled their source fluorescence frames; however, subtle differences in the originating frames were accentuated. The normalization of the pixel values during the calculations of qN allowed a wider spread of the computed values across the available gray scale than with the originally recorded data.

Normalization of the difference frames against the dark-adapted frame largely eliminated the gradient of emission that resulted from the arrangement of the fiberoptic bundle. The relief effect in the derived frames was an artefact caused by the frames being slightly out of registration with one another, even though the camera was rigidly mounted. The registration difference was likely only one or two pixels across the image, and may have been the result of a slight expansion of the leaves during the experiment. Figure 2 indicates that reductions of photosynthesis caused by stomatal closure in response to ABA, in general appeared first in areas between major veins. With increasing time after application of ABA, areas of strong qN-quenching coalesced, disclosing the spread of stomatal closure (not shown). Areas adjoining veins responded last and slowly, and some areoles immediately adjacent to the minor veins were not as strongly quenched as areoles in the intervenal regions. Approximately half an hour after the application of ABA the topography of photosynthetic activity no longer changed conspicuously.

The area-averaged rate of photosynthesis remained constant at A = 15.4 μmol m−2s−1 during the first 10 min following ABA application, although the frame of Figure 2B already indicated beginning stomatal closure in some areoles. With stomatal closure spreading, the rate of photosynthesis declined within 38 min to 8.0 μmol m−2s−1. The area-averaged conductance for water vapor declined from 0.256 mol m−2s−1 at the time of ABA application to 0.242 mol m−2s−1 10 min later and to 0.125 mol m−2s−1 at 38 min. Average intercellular CO2 concentrations, as computed from the gas-exchange data, remained virtually constant at 200 μL·L−1. The development of strong nonphotochemical quenching in large portions of the viewed leaf area affirmed that constancy of intercellular CO2 concentration was not a correct conclusion. The qN values displayed the appearance of severe local CO2 deficiencies while ABA took effect.

The simultaneous area-averaging recordings with the PAM fluorimeter after Schreiber (15) were used to test the reliability of the image-analysis procedure. Despite the highly nonuniform distribution of quenching, calculations of mean pixel intensities agreed closely with the qN values obtained with the PAM instrument (Fig. 3). The images obtained were evaluated further by computing isophotes of equal quenching; one example is shown in Figure 4, for the frame illustrated in Figure 2, B to D. Repetition of this operation with increasing thresholds (20) yielded a zoning of qN values from which distributions of qN among classes

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**Figure 3.** Correlation between qN, calculated with the area-averaging modulated-light Walz fluorimeter (qN Walz), and the mean pixel intensity from corresponding video image frames (qN Video). Data from the (±) abscisic-acid application experiment with X. strumarium shown in Figure 2.

**Figure 4.** Zonation of qN as demonstrated with the frame shown in Figure 2C. The white outline is the isophote for a pixel gray value of 100 and corresponds to qN = 0.38. The isophote separates zones with qN > 0.38 from those of qN < 0.38. The frame was darkened by 15% by linear interpolation, to emphasize the contour line.
of increasing quench intensity could be derived (Fig. 5). We recognize that 80% of the viewed area belonged to the least quenched class, in agreement with the simultaneous measurements of gas exchange, which indicated that the rate of photosynthesis of the whole leaf had not yet declined during the first 10 min after ABA application (when the frame for Fig. 2 was being recorded). If the average rate of CO₂ uptake of 15.4 μmol m⁻²s⁻¹, as measured by gas exchange, applied also to the viewed area, and if a linear relationship held between assimilation rate and (1 – qₙ), the rate of CO₂ uptake was 15.8 μmol m⁻²s⁻¹ in the least quenched zone and 14.8, 13.3, and about 12.8 μmol m⁻²s⁻¹ in the three zones occupied by the other classes.

Following a return to 21% O₂, the emissions from the ABA-treated Xanthium leaf became homogenous once again, which discloses that, in this species, O₂ provided effectively alternate electron acceptors when CO₂ was no longer available.

After application of DCMU, the topography of qₙ followed a pattern different from that recorded after a supply of ABA. DCMU, an inhibitor of photosynthetic electron transport, also abolishes nonphotochemical quenching. The affected areas spread from the veins to the intervalar areas (Fig. 6A). The image obtained resembled that seen by Björn and Forsberg (2) after feeding DCMU to leaves. Topographies obtained on leaves of species other than Xanthium strumarium in response to ABA, transitions to dry air, or during the induction phase of photosynthesis reflected the anatomical differences among the examples investigated. In leaves of Zea mays L., fluorescence quenching spread in stripes parallel to the veins, in leaves of Arbutus unedo L., gradual variation of quenching across larger areoles was observed.

**DISCUSSION**

Images of the Chl fluorescence from leaves were converted into gray scale reproductions of the nonphotochemical quench coefficient, qₓ. This procedure rendered comparable information obtained among various experimental set-ups, treatments, or species. Because (1 – qₓ) was positively correlated with rates of photosynthesis (11, 17), topographies of photosynthetic activity could be obtained; extension of the method to species other than those referred to will require determinations of the correlation. These topographies of the rate of CO₂ assimilation can be used to estimate distributions of intercellular CO₂ concentrations (particularly for low partial pressures of O₂), and the computed zonations (Figs. 4 and 5), in combination with gas-exchange data, can provide a base for producing maps of stomatal conductances, as we shall show in a separate publication.

The spread of the area affected by DCMU applied through the petiole (Fig. 6, A and B) is different from the pattern of stomatal closure caused by ABA. It seems likely that both ABA and DCMU move from the veins in the transpiration stream. We speculate that the different patterns may be explained by the interaction of DCMU and ABA with the cells of the leaf mesophyll. As the transpiration front proceeds through the lamina, the DCMU (which binds tightly to sites in the chloroplasts) is scavenged by cells closely adjacent to the major veins, where it becomes bound. Because DCMU blocks electron transport at the acceptor side of PSII, this

![Figure 5. Shares of four intensity classes of qₙ in the total area of the image shown in Figure 4. Image zonation was executed with qₙ thresholds at 0.38, 0.45, and 0.50 (corresponding to pixel gray values of 100, 115, and 130). The area covered by each qₙ class was determined by forming the difference between the areas enveloped by each upper and lower bordering isophote.](image)

![Figure 6. Topography of developing intoxication of a X. strumarium leaf by 10⁻⁶ M DCMU. A, Source fluorescence frame during a saturating flash; B, perspective plot of (1 – qₙ) for (A).](image)
topography of \((1-q_N)\) is positively correlated with and inhibition of photosynthetic activity.

The development of nonphotochemical fluorescence quenching after application of ABA showed two components, (a) a general increase with time, as expressed in a gradual graying of the whole image, and (b) a localized darkening associated with particular areoles or groups of areoles. These components can be considered representing two manners of stomatal control of gas exchange, one in which the width of all stomatal apertures changes in proportion to the control requirement (proportional component), and another one, in which stomata are either closed or open and only the ratio between the numbers of closed and open stomata changes (binary component) (14).

The frame digitizing described here depended on the SMPTe system, which is both costly and not generally available. However, recently a number of ‘frame-grabbing’ circuit boards have become available for desktop computers of several makes; the boards potentially support capture of images by the same system controlling other components of a gas-exchange apparatus, and often provide software with novel display alternatives for image data (Fig. 6B) that are useful in interpretation of subtle features. Such integrated systems provide a cost-effective alternative to commercially available fluorescence analyzers, capable of resolving both temporal and spatial variation in fluorescence emission, and facilitate the use of \(q_N\) for obtaining topographies of the photosynthetic activity in leaves.

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LITERATURE CITED