

Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo

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Key Words

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Abstract

The use of chlorophyll fluorescence to monitor photosynthetic performance in algae and plants is now widespread. This review examines how fluorescence parameters can be used to evaluate changes in photosystem II (PSII) photochemistry, linear electron flux, and CO₂ assimilation in vivo, and outlines the theoretical bases for the use of specific fluorescence parameters. Although fluorescence parameters can be measured easily, many potential problems may arise when they are applied to predict changes in photosynthetic performance. In particular, consideration is given to problems associated with accurate estimation of the PSII operating efficiency measured by fluorescence and its relationship with the rates of linear electron flux and CO₂ assimilation. The roles of photochemical and non-photochemical quenching in the determination of changes in PSII operating efficiency are examined. Finally, applications of fluorescence imaging to studies of photosynthetic heterogeneity and the rapid screening of large numbers of plants for perturbations in photosynthesis and associated metabolism are considered.

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Heat loss: occurs when excitation energy within pigments is lost as heat; often termed nonradiative decay or thermal deactivation

Excitation energy: energy within a pigment molecule after a photon is absorbed and generates an excited state of the molecule

Q_A: primary quinone electron acceptor of PSII

Photochemical quenching: results from using excitation energy within photosystem II (PSII) to drive electron transport from P680 to Q_A

INTRODUCTION

The use of chlorophyll *a* fluorescence measurements to examine photosynthetic performance and stress in algae and plants is now widespread in physiological and ecophysiological studies. This has come about owing to the development of a sound understanding of the relationships between fluorescence parameters and photosynthetic electron transport in vivo and the commercial availability of a range of affordable, easy to use portable fluorimeters. Fluorescence can be a very powerful tool to study photosynthetic performance, especially when coupled with other noninvasive measurements such as absorption spectroscopy, gas analyses, and infrared thermometry. This review examines how some key fluorescence parameters can be used to assess photosynthetic performance in vivo and to identify possible causes of changes in photosynthesis and plant performance; it is aimed at plant biologists who seek to use fluorescence as a tool in their research. However, the underlying theoretical bases of fluores-

cence changes in vivo are complex and correct interpretation of changes in fluorescence parameters can often be difficult. Consideration is given to some problems associated with the measurement of these parameters and the assumptions made when using these parameters to evaluate changes in photosynthetic performance.

BACKGROUND

Following the observation by Kautsky & Hirsch (55) that changes in fluorescence induced by illumination of dark-adapted leaves are qualitatively correlated with changes in CO₂ assimilation, it became evident that under some circumstances fluorescence emissions in photosynthetic organisms could be correlated to their photosynthetic rates (54, 56, 77). Butler (21) developed a simple model for photosystem II (PSII) photochemistry in which photochemistry competes with the processes of fluorescence and heat loss for excitation energy in the pigment antenna of PSII (**Figure 1**). This model followed from the proposal that electron transfer from the reaction center chlorophyll of PSII (P680) to the primary quinone acceptor of PSII (Q_A) quenches fluorescence (28), a process termed photochemical quenching. Increases in the rate of heat loss result in nonphotochemical quenching of fluorescence. The model predicts that PSII fluorescence emission could be used to monitor changes in photochemistry, provided that the rate constants for fluorescence and heat loss do not change (21). However, it is now well established that large changes can occur in the rate constant for heat loss from the PSII antenna (61, 65). Consequently, to estimate PSII photochemistry from fluorescence, it is essential to determine the fluorescence quenching that results from both photochemical and nonphotochemical processes.

Separation of fluorescence quenching into photochemical and nonphotochemical components was first achieved by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea

(DCMU) to intact chloroplasts and *Chlorella* cells at points throughout the fluorescence induction curve (64, 66). DCMU inhibits electron transfer from Q_A to the secondary quinone acceptor of PSII (Q_B), which results in a rapid reduction of Q_A and an increase in fluorescence as photochemical quenching is prevented. A slower increase in fluorescence follows, which is associated with the decay of nonphotochemical quenching. Unfortunately, this DCMU technique is not suitable for analyzing fluorescence quenching in leaves owing to the slow and uneven penetration of DCMU into leaf tissues. Also, the irreversibility of the DCMU inhibition of electron transport makes the technique unsuitable for continuous measurements on individual leaves. However, maximal Q_A reduction in leaves in the light can be achieved by rapidly exposing leaves to a very large increase in light (17). This light-addition technique is used to quantitatively determine the fraction of fluorescence quenching that is attributable to photochemical and nonphotochemical quenching processes (18). The development of fluorimeters that use weak modulated measuring beams in which phase and frequency decoding are used to detect fluorescence yield changes enabled the routine, nondestructive, quantitative determination of photochemical and nonphotochemical processes in leaves by the application of a brief (less than 1 s) saturating flash of light sufficiently intense as to maximally reduce the Q_A pool in the sample (26, 102). The value of the modulated technique is that it provides a continuous measure of the relative quantum yield of fluorescence (101). This technique was used to demonstrate that the quantum yield of PSII photochemistry of a leaf at a given actinic light intensity can be estimated from the modulated fluorescence yield prior to the application of the saturating flash and the maximum modulated fluorescence yield during the flash (37). In the absence of photorespiration, which competes with CO_2 assimilation for the products of electron transport, the quantum yield of PSII photochemistry is directly re-

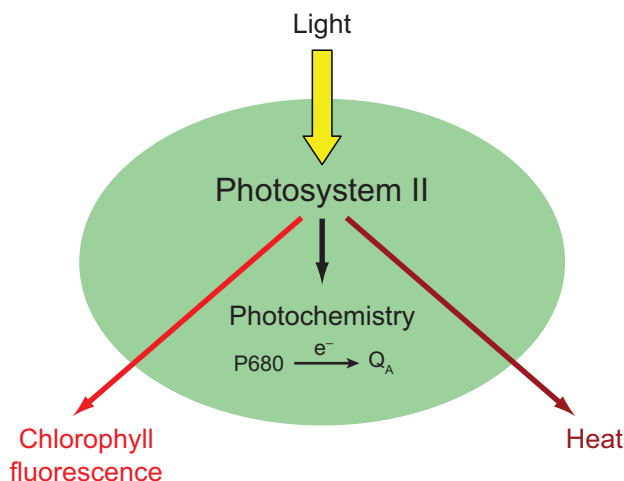


Figure 1

Simple model of the possible fate of light energy absorbed by photosystem II (PSII). Light energy absorbed by chlorophylls associated with PSII can be used to drive photochemistry in which an electron (e^-) is transferred from the reaction center chlorophyll, P680, to the primary quinone acceptor of PSII, Q_A . Alternatively, absorbed light energy can be lost from PSII as chlorophyll fluorescence or heat. The processes of photochemistry, chlorophyll fluorescence, and heat loss are in direct competition for excitation energy. If the rate of one process increases the rates of the other two will decrease.

lated to the quantum yield of CO_2 assimilation by the leaf, ϕ_{CO_2} (37), thus allowing, under certain conditions, the application of fluorescence measurements to provide a rapid, nondestructive probe of CO_2 assimilation. A list of the fluorescence parameters used in this review, their definitions, and comments on their physiological relevance are given in **Table 1**.

PHOTOSYSTEM II PHOTOCHEMISTRY

Dark-Adapted State

When a leaf is kept in the dark, Q_A becomes maximally oxidized and the PSII reaction centers are referred to as being 'open', i.e., capable of performing photochemical reduction of Q_A . Exposure of a dark-adapted leaf to a weak modulated measuring beam [photosynthetically active photon flux density (PPFD) of ca. $0.1 \mu mol m^{-2} s^{-1}$] results in the minimal level of fluorescence, F_o (**Figure 2**). The intensity of the measuring beam must be nonactinic

Nonphotochemical quenching: occurs when there is an increase in the rate at which excitation energy within photosystem II is lost as heat

Quantum yield (quantum efficiency) of a process: number of molecules undergoing the process divided by the number of photons absorbed by the system

Actinic light: light that is absorbed by the photosynthetic apparatus and will drive electron transport

Table 1 Chlorophyll fluorescence parameters frequently used in studies of photosystem II photochemistry

Parameter	Definition	Physiological relevance
F, F'	Fluorescence emission from dark- or light-adapted leaf, respectively.	Provides little information on photosynthetic performance because these parameters are influenced by many factors. F' is sometimes referred to as F_s' when at steady state
F_o, F_o'	Minimal fluorescence from dark- and light-adapted leaf, respectively	Level of fluorescence when Q_A is maximally oxidized (PSII centers open)
F_m, F_m'	Maximal fluorescence from dark- and light-adapted leaf, respectively	Level of fluorescence when Q_A is maximally reduced (PSII centers closed)
F_v, F_v'	Variable fluorescence from dark- and light-adapted leaves, respectively	Demonstrates the ability of PSII to perform photochemistry (Q_A reduction)
F_q'	Difference in fluorescence between F_m' and F'	Photochemical quenching of fluorescence by open PSII centers.
F_v/F_m	Maximum quantum efficiency of PSII photochemistry	Maximum efficiency at which light absorbed by PSII is used for reduction of Q_A .
F_q'/F_m'	PSII operating efficiency	Estimates the efficiency at which light absorbed by PSII is used for Q_A reduction. At a given photosynthetically active photon flux density (PPFD) this parameter provides an estimate of the quantum yield of linear electron flux through PSII. This parameter has previously been termed $\Delta F/F_m'$ and ϕ_{PSII} in the literature.
F_v'/F_m'	PSII maximum efficiency	Provides an estimate of the maximum efficiency of PSII photochemistry at a given PPFD, which is the PSII operating efficiency if all the PSII centers were 'open' (Q_A oxidized).
F_q'/F_v'	PSII efficiency factor	Relates the PSII maximum efficiency to the PSII operating efficiency. Nonlinearly related to the proportion of PSII centers that are 'open' (Q_A oxidized). Mathematically identical to the coefficient of photochemical quenching, qp .
NPQ	Nonphotochemical quenching	Estimates the nonphotochemical quenching from F_m to F_m' . Monitors the apparent rate constant for heat loss from PSII. Calculated from $(F_m/F_m') - 1$.
q_E	Energy-dependent quenching	Associated with light-induced proton transport into the thylakoid lumen. Regulates the rate of excitation of PSII reaction centers.
q_I	Photoinhibitory quenching	Results from photoinhibition of PSII photochemistry.
q_L	Fraction of PSII centers that are 'open'	Estimates the fraction of 'open' PSII centers (with Q_A oxidized) on the basis of a lake model for the PSII photosynthetic apparatus. Given by $(F_q'/F_v')(F_o'/F')$
q_T	Quenching associated with a state transition	Results from phosphorylation of light-harvesting complexes associated with PSII
ϕ_F	Quantum yield of fluorescence	Number of fluorescent events for each photon absorbed

PPFD:
photosynthetically
active photon flux
density

to ensure that Q_A remains maximally oxidized. If the period used for dark adaptation is not long enough Q_A may not become maximally oxidized. Then a pulse of weak far-red light, which preferentially excites photosystem I (PSI) and removes electrons from Q_A , should be applied prior to the measurements

of F_o . In some leaves (32) and algae (10) significant accumulation of reduced Q_A can occur in the dark owing to nonphotochemical reduction of plastoquinone by chlororespiration; the reduced plastoquinone must be re-oxidized by a pulse of weak red light before measurement of F_o . If after reaching F_o the

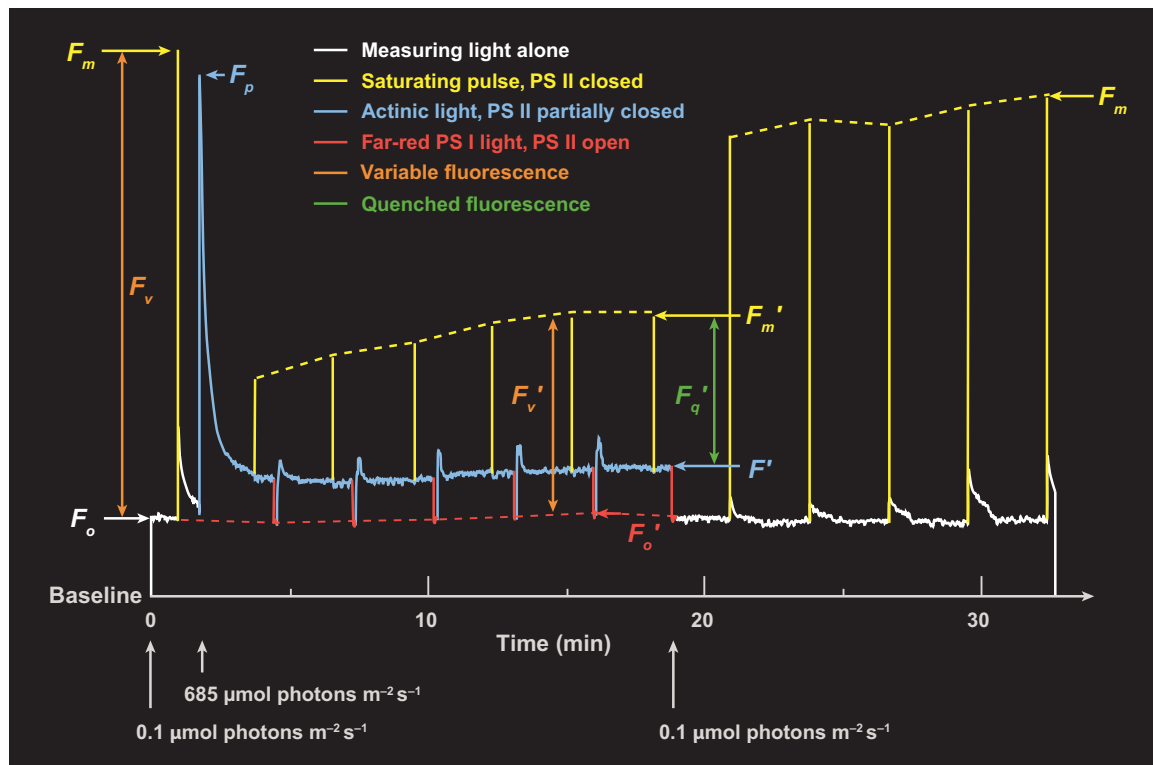


Figure 2

Fluorescence quenching analysis using modulated fluorescence. A dark-adapted leaf is exposed to various light treatments. The parameters denoted with a prime (') are from the leaf exposed to actinic light. The parameters without a prime are obtained from the leaf in the dark-adapted state. The different colors of the trace denote different light treatments. White: weak measuring light alone ($0.1 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) that gives F_0 . An important feature of this measuring beam is that its intensity must be low enough so it does not drive significant PSII photochemistry. Yellow: saturating light pulse (≤ 1 s duration, $> 6000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) that gives F_m in darkness and F_m' in light. Blue: actinic light ($685 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) that drives photosynthesis and gives F' . Red: far-red light ($30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at $720\text{--}730$ nm for 4 s) that excites photosystem I (PSI) preferentially, and thus oxidizes the plastoquinone and Q_A pools associated with PSII and gives F_o' . Orange: variable fluorescence calculated as $F_v = F_m - F_0$ from the dark-adapted leaf and $F_v' = F_m' - F_o'$ from the illuminated leaf. Green: fluorescence that is quenched from F_m' to F' by PSII photochemistry in the illuminated leaf, calculated as $F_q' = F_m' - F'$. All parameters, except F_q' , F_v' , and F_v , are measured from the baseline. Figure reproduced from Reference 8, with permission.

leaf is now exposed to a short actinic pulse of high PPFD (typically less than 1 s at several thousand $\mu\text{mol m}^{-2} \text{s}^{-1}$), Q_A will be maximally reduced and the maximal fluorescence level, F_m , is observed (**Figure 1**). PSII reaction centers with reduced Q_A are referred to as being 'closed'. The difference between F_m and F_0 is defined as the variable fluorescence, F_v . The ratio of F_v/F_m can be used

to estimate the maximum quantum yield of Q_A reduction, i.e., PSII photochemistry, from the simple model of Butler (21). The fluorescence emission from a leaf, F , is defined by $I A_{\text{leaf}} \cdot \text{fraction}_{\text{PSII}} \cdot \phi_F$, where I is the incident PPFD on the leaf, A_{leaf} is the proportion of incident PPFD that is absorbed by the leaf, $\text{fraction}_{\text{PSII}}$ is the fraction of absorbed PPFD that is received by PSII and ϕ_F is the quantum

Open center: photosystem II (PSII) reaction center in which the primary quinone acceptor of PSII, Q_A , is oxidized and capable of photoreduction

Closed center:

PSII reaction center in which Q_A is reduced and unable to perform photochemistry

I: PPFD incident on the leaf

A_{leaf} : proportion of incident PPFD on the leaf that is absorbed by the leaf

$fraction_{PSII}$: fraction of absorbed PPFD that is received by PSII

yield of fluorescence. ϕ_F is defined by $k_F/(k_F + k_H + k_P P)$, where k_F , k_H , and k_P are the rate constants for the decay of excitation energy in PSII by fluorescence, heat loss, and photochemistry, respectively, and P is the fraction of PSII reaction centers that are open. At F_o , PSII reaction centers are maximally open, $P = 1$, and the fluorescence quantum yield, ϕ_{F_o} , is given by $k_F/(k_F + k_H + k_P)$. At F_m , the PSII reaction centers are maximally closed, $P = 0$, and photochemistry cannot occur, thus $k_P P = 0$ and the fluorescence quantum yield, ϕ_{F_m} , is given by $k_F/(k_F + k_H)$. Thus, ϕ_{F_o}/ϕ_{F_m} is given by $(\phi_{F_m} - \phi_{F_o})/\phi_{F_m} = k_P/(k_F + k_H + k_P)$, which shows that this ratio estimates the maximum quantum yield of PSII photochemistry. Assuming that I , A_{leaf} , and $fraction_{PSII}$ are constant for measurements of F_o and F_m , then F_v/F_m can be used to estimate the maximum quantum yield of PSII photochemistry. This simple model requires a number of other assumptions that are not necessarily correct for all situations (15). For example, fluorescence at both F_o and F_m is assumed to be emitted from a homogeneous system where all the excited states of the chlorophylls are the same. Clearly this is generally not the case; consequently, F_v/F_m should not be considered to provide a rigorous quantitative value of the quantum yield of PSII photochemistry (15). However, F_v/F_m does provide a very useful relative measure of the maximum quantum yield of PSII primary photochemistry; F_v/F_m values for nonstressed leaves are remarkably consistent at *ca.* 0.83 (14).

When plants are exposed to abiotic and biotic stresses in the light, decreases in F_v/F_m are frequently observed. This is such a widespread phenomenon that F_v/F_m measurements provide a simple and rapid way of monitoring stress. Unfortunately, the reasons for stress-induced decreases in F_v/F_m are often complex. Stressing photosynthetic tissues in the light can result in increases in nonphotochemical quenching processes, which decrease F_m . Such quenching may not recover during a short period of dark adaptation, or even overnight, and results

in decreases in F_v/F_m (1, 2). However, identification of the intrinsic causes of such decreases can often be difficult. In many stress situations increases in nonphotochemical quenching can often be accompanied by photoinactivation of PSII reaction centers, which then dissipate excitation energy as heat rather than as photochemistry (79). Photoinactivation can lead to oxidative damage and loss of PSII reaction centers (4), both of which are associated with an increase in F_o (19, 90). However, caution must be exercised when attempting to interpret the significance of decreases in F_m or increases in F_o that occur as a result of a stress treatments. These fluorescence levels are determined both by the physicochemical properties of PSII and the optical properties of the leaf. Unfortunately, during many stress treatments, especially when changes in leaf water status occur, the optical properties of the leaf can change markedly and modify A_{leaf} . Changes in $fraction_{PSII}$ can occur owing to changes in thylakoid membrane structure and organization. Such modifications will result in changes in F_o and F_m that are independent of changes in ϕ_{F_o} and ϕ_{F_m} . In such situations, absolute changes in F_o and F_m cannot be used with confidence to indicate loss of PSII reaction centers or increases in nonphotochemical quenching. However, when ratios of fluorescence parameters, such as F_v/F_m , are considered, the influence of changes in A_{leaf} and $fraction_{PSII}$ are canceled out and changes in the ratio are indicative of changes in the ratio of quantum yields of the two parameters; for example F_v/F_m is defined by $(I \cdot A_{leaf} \cdot fraction_{PSII} \cdot \phi_{F_v}) / (I \cdot A_{leaf} \cdot fraction_{PSII} \cdot \phi_{F_m}) = (\phi_{F_v} / \phi_{F_m})$.

In many ecophysiological studies it is suggested that stress-induced decreases in F_v/F_m imply that the photosynthetic efficiency of the leaves under ambient light conditions is compromised. This is not necessarily the case, because the quantum yield of PSII photochemistry under ambient light may be considerably below the observed F_v/F_m value, which estimates the maximum quantum yield of PSII

photochemistry, not the yield at which PSII is operating under the ambient light (see below). The maximum quantum yield of PSII photochemistry is only achieved at very low ambient light levels.

Light-Adapted State

A leaf in continuous actinic light has a fluorescence level termed F' , which rises to the maximal fluorescence level, F_m' , when the leaf is exposed to a brief saturating light pulse that maximally reduces Q_A (**Figure 2**). A prime notation (') used after a fluorescence parameter indicates that the sample is exposed to light that will drive photosynthesis, i.e., actinic light. The difference between F_m' and F' is designated F_q' and results from quenching of F_m' by PSII photochemistry. The ratio F_q'/F_m' is theoretically proportional to the quantum yield of PSII photochemistry prior to application of the saturating light pulse (37). Genty and coworkers empirically confirmed this theory from mass spectrometric measurements of oxygen evolution (38). For leaves exposed to actinic light the quantum yield of PSII photochemistry is equivalent to the quantum yield of linear electron flux (LEF) through PSII reaction centers, and hereafter is referred to as the PSII operating efficiency. Measurements of F_q'/F_m' provide a rapid method to determine the PSII operating efficiency under different light and other environmental conditions; F_q'/F_m' has previously been termed $\Delta F/F_m'$ and ϕ_{PSII} in the literature.

There are a number of potential sources of error associated with measurements of F_q'/F_m' , which can be important when evaluating changes in PSII operating efficiency. These sources of error can also be a problem when measuring dark-adapted F_v/F_m . The relationship between F_q'/F_m' and the true quantum yield of PSII photochemistry can be affected if PSI contributes significantly to the measurements of the fluorescence parameters (41, 57, 97). When using F_q'/F_m' to determine the quantum yield of PSII photochem-

istry all the measured fluorescence is assumed to originate from PSII. Although this is true for variable fluorescence, it is not the case for F_o if fluorescence is monitored at wavelengths above 700 nm (70, 85). PSI is generally assumed to make a negligible contribution to fluorescence at wavelengths below 700 nm. Unfortunately, most commercial instruments measure a significant amount of fluorescence at wavelengths above 700 nm. The PSI contribution to F_o at wavelengths above 700 nm has been estimated at *ca.* 30% and 50% for C_3 and C_4 leaves, respectively (41, 97). Consequently, decreases in F_q'/F_m' will occur and therefore give estimates of PSII operating efficiency that are lower than the true values. As PPFD increases F_q'/F_m' decreases (**Figure 3**), but PSI fluorescence yield remains reasonably constant (25), thus the PSI contributions that result in depression of F_q'/F_m' and the consequent errors that lead to underestimation of PSII operating efficiency become proportionally greater. Measurement of fluorescence at wavelengths below 700 nm minimizes such errors by markedly reducing the PSI contribution to the signals (41, 97). However, measurements at these shorter wavelengths result in an increase in the contribution of fluorescence from the upper regions of the leaf because the probability of reabsorption of emissions at the shorter wavelengths is greater than for emissions above 700 nm (71).

Another error can arise in estimations of F_q'/F_m' via the use of saturating light pulses that induce multiple turnovers of PSII reaction centers, as is the case with most commercial instruments. Such saturating pulses can result not only in the reduction of Q_A , but also in the reduction of plastoquinone to plastoquinol. Plastoquinone, but not plastoquinol, is a quencher of chlorophyll fluorescence. A decrease in plastoquinone during the application of the saturating light pulse will result in a decrease in quenching and an overestimation of F_m' that can be as large as 20% (62, 105). Such errors are significant only in leaves with high plastoquinone/plastoquinol ratios prior to application of the saturating light pulse,

LEF: linear electron flux

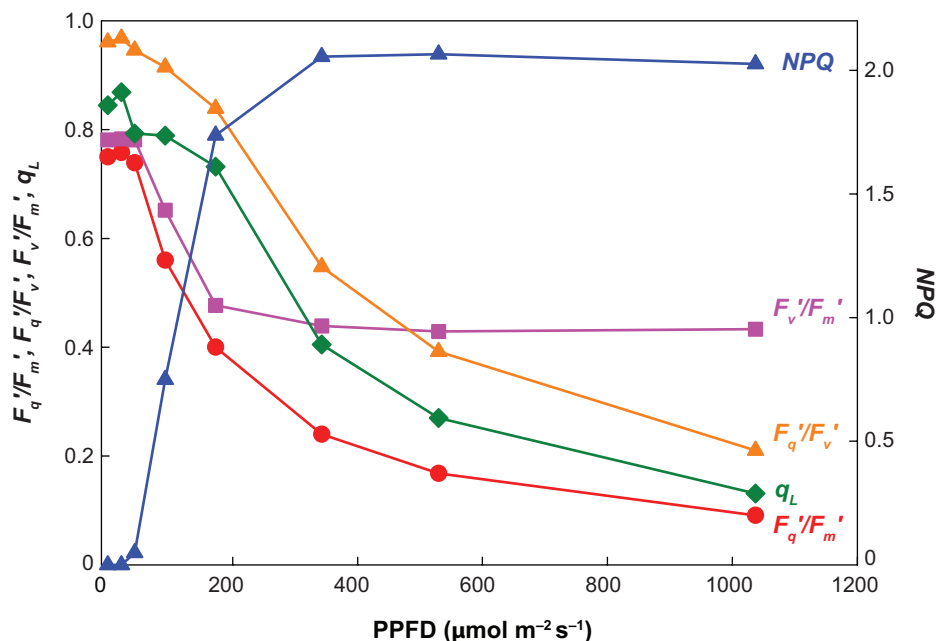


Figure 3

The responses of photosystem II (PSII) operating efficiency (F_q'/F_m'), maximum PSII quantum efficiency (F_v'/F_m'), the fraction of the maximum PSII efficiency that is realized in the light (F_q'/F_v'), the fraction of PSII reaction centers that are open (q_L), and nonphotochemical quenching (NPQ) in a tobacco leaf to increasing photosynthetically active photon flux density (PPFD). The leaf was kept in an atmosphere containing $100 \mu\text{mol mol}^{-1} \text{CO}_2$ and 2% O_2 to reduce CO_2 assimilation and eliminate photorespiration, respectively. Data taken from Reference 63 with permission.

which is the case at very low light levels, and even then overestimates of F_q'/F_m' will be less than 10% (6).

Fortunately, errors in the measurement of F_q'/F_m' due to PSI fluorescence and plastoquinone quenching are small in many cases. The frequently observed linear relationship between F_q'/F_m' and the quantum yield of CO_2 assimilation with increasing light in leaves from a wide range of species in which photorespiration was absent or suppressed (e.g., 23, 24, 27, 29, 37, 39, 40, 51, 58, 59, 60) matches what is theoretically predicted (see below). Also, the yield of oxygen evolution from PSII determined by mass spectrometry is linearly related to F_q'/F_m' (38). If large errors in the measurement of F_q'/F_m' existed, then such linear relationships would not be observed. However, it is possible that errors could be more significant in leaves with un-

usual pigment or plastoquinone contents and caution should be exercised in such situations.

RELATIONSHIP BETWEEN PHOTOSYSTEM II OPERATING EFFICIENCY, LINEAR ELECTRON FLUX, AND CO_2 ASSIMILATION

The operation of linear electron flux (LEF) from water through PSII and PSI to electron acceptors requires similar electron fluxes through the reaction centers of both PSII and PSI. When the quantum yield of PSI photochemistry and PSII operating efficiency are measured simultaneously over a range of light intensities, linear relationships between the two parameters are observed frequently (34, 35, 39, 43, 44, 45, 57). In mature C_4 leaves, where CO_2 assimilation is the main sink for

the products of LEF (i.e., ATP and NADPH) (29), the PSII operating efficiency should be directly related to the quantum yield of CO₂ assimilation, ϕ_{CO_2} (37). Such linear relationships between the PSII operating efficiency and ϕ_{CO_2} have been observed over a range of light intensities (37, 58, 59, 60, 103), over a range of atmospheric CO₂ concentrations (37, 103), and during induction of photosynthesis when dark-adapted leaves are exposed to actinic light (37). When photorespiration is inhibited in mature C₃ leaves by reduction of the atmospheric oxygen from 21% to 2% and CO₂ assimilation is the only major sink for ATP and NADPH, a linear relationship is also observed between PSII operating efficiency and ϕ_{CO_2} (23, 24, 27, 39, 40, 51). These observations demonstrate that PSII operating efficiency is a very good monitor of LEF.

In principle, the linear relationship between PSII operating efficiency and LEF allows the use of F_q'/F_m' to estimate the noncyclic electron transport rate through PSII (ETR), where $ETR = I \cdot A_{leaf} \cdot fraction_{PSII} \cdot (F_q'/F_m')$. As discussed above, care should be taken when determining and interpreting F_q'/F_m' , but often difficulties arise in the accurate determination of the other parameters involved in the estimation of ETR. A_{leaf} is frequently assumed to be 0.84, i.e., 84% of incident PPFD is assumed to be absorbed by leaves. This assumption may be reasonable for many mature green leaves, but is not always the case and large deviations from this value can frequently occur (30, 47, 53). A_{leaf} should be measured using an integrating sphere with a light source similar to that used to drive photosynthesis and a spectroradiometer or quantum sensor. Similarly, $fraction_{PSII}$ for leaves is frequently assumed to be 0.5, which is unlikely to be the case in many situations. Although $fraction_{PSII}$ has been estimated for leaves, the procedure is not straightforward and involves numerous assumptions (67, 68, 83). Another problem is that leaves of many species accumulate nonphotosynthetic pigments, such as anthocyanins, which can markedly mod-

ify not only A_{leaf} but also $fraction_{PSII}$; this is often the case when leaves experience environmental stresses during development. Unfortunately, commercial modulated fluorometers automatically calculate values of ETR by assuming that leaves have values of A_{leaf} and $fraction_{PSII}$ of 0.84 and 0.5, respectively, often leading to substantial errors in calculations of ETR. ETR values calculated by such instruments should not be used unless the assumed values of A_{leaf} and $fraction_{PSII}$ have been validated for the leaves being measured. In cases where such validations have not been made, changes in F_q'/F_m' should be used only to determine changes in the relative quantum yield of LEF and not used to estimate differences in ETR.

If the allocation of the ATP and electrons that result from LEF to sinks other than CO₂ assimilation is negligible or constant, then PSII operating efficiency also provides a good relative measure of the quantum yield of CO₂ assimilation. The relationship between the PSII operating efficiency and the quantum yield of CO₂ assimilation (ϕ_{CO_2}) is defined by $\phi_{CO_2} = (F_q'/F_m') \cdot fraction_{PSII} \cdot (1/k)$, where k is the number of electron equivalents produced by LEF required to reduce one molecule of CO₂. For C₃ leaves in which photorespiration is inhibited and other electron sinks are negligible k is assumed to be 4. If k and $fraction_{PSII}$ are constant then F_q'/F_m' is a good indicator of changes in ϕ_{CO_2} . In many cases k and $fraction_{PSII}$ will not remain constant between treatments and F_q'/F_m' should not be used to monitor changes in ϕ_{CO_2} . The value of k is dependent upon the proportion of reductants produced by LEF used for CO₂ assimilation. k will change when other sinks for these reductants change relative to CO₂ assimilation. Differences in k occur in leaves at different stages of growth and in response to environmental stresses. In C₃ leaves large changes in k occur with changes in intracellular CO₂ and O₂ concentrations, which modify the relative rates of CO₂ assimilation and photorespiration. The difficulties in the accurate determination of k and $fraction_{PSII}$ preclude the use of

ETR: electron transport rate through photosystem II

estimations of ϕ_{CO_2} from F_q'/F_m' to calculate actual rates of CO_2 assimilation from $(I \cdot A_{leaf} \cdot \phi_{CO_2})$. However, relative changes in rates of CO_2 assimilation can be evaluated from estimations of ϕ_{CO_2} provided that k and $fraction_{PSII}$ are constant.

A linear relationship between PSII operating efficiency and ϕ_{CO_2} is not found in many situations. Linearity is lost if the proportion of electrons consumed by CO_2 assimilation relative to other metabolic processes changes. In such cases F_q'/F_m' should not be used to estimate changes in ϕ_{CO_2} unless the relationship between F_q'/F_m' and ϕ_{CO_2} has been determined for the particular system under investigation. This is the case for C_3 leaves when photorespiration is operating; the ratio of PSII operating efficiency to ϕ_{CO_2} increases with increasing photorespiratory activity relative to carbon assimilation (39, 42, 45). Environmental stresses can induce large increases in the PSII operating efficiency: ϕ_{CO_2} ratio. For example, when the leaves of some C_4 species develop at suboptimal growth temperatures, PSII operating efficiency: ϕ_{CO_2} is increased significantly (31, 36). These increases are accompanied by increases in the levels of antioxidants and activities of enzymes involved in scavenging reactive oxygen species, which suggests that an increased electron flux to oxygen, relative to CO_2 assimilation, is occurring via the Mehler reaction (31, 36). Similar increases in PSII operating efficiency: ϕ_{CO_2} were observed in leaves of mangrove, a C_3 species, growing at high temperatures in tropical Australia (22).

FACTORS THAT DETERMINE PHOTOSYSTEM II OPERATING EFFICIENCY

PSII operating efficiency, F_q'/F_m' , is given by the product of two important fluorescence parameters, F_v'/F_m' and F_q'/F_v' (37), where F_v' is equal to $F_m' - F_o'$ and is the variable fluorescence of the light-adapted leaf and F_o' is the minimal fluorescence level in the light when Q_A is maximally oxidized (Figure 2). F_v'/F_m'

estimates the maximum quantum yield of PSII photochemistry (hereafter termed maximum PSII efficiency) that can be achieved in the light-adapted leaf when Q_A is maximally oxidized. Consequently, this parameter can be used to assess the contributions of nonphotochemical quenching to changes in the PSII operating efficiency of leaves in the light. F_q'/F_v' provides an estimate of the fraction of the maximum PSII efficiency that is actually realized in the leaf under the environmental conditions during the measurement, and is hereafter termed the PSII efficiency factor. The PSII efficiency factor is nonlinearly related to the fraction of PSII reaction centers with Q_A oxidized, i.e., the fraction of PSII centers that are open, and is mathematically identical to the frequently used coefficient of photochemical quenching, q_P . F_q'/F_v' is determined by the ability of the photosynthetic apparatus to maintain Q_A in the oxidized state, which is a function of the relative rates of Q_A reduction and oxidation. Determination of F_v'/F_m' and F_q'/F_v' makes it possible to assess whether changes in PSII operating efficiency are attributable to changes in nonphotochemical quenching or the ability of an excited PSII reaction center to drive electron transport.

Calculation of F_v'/F_m' and F_q'/F_v' requires determination of F_o' , which can often be difficult. F_o' is usually measured by exposing the leaf to a pulse of weak far-red light, after removing the actinic light, to maximally oxidize Q_A (101). However, in many situations maximal oxidation of Q_A may not be achieved during the far-red pulse and also nonphotochemical quenching can partially relax, thus resulting in an overestimation of F_o' (6). This problem can be overcome by calculating F_o' from values of F_m' at the point of measurement and dark-adapted values of F_o and F_m using $F_o' = F_o / [(F_v'/F_m) + (F_o/F_m)]$ (92). Maxwell & Johnson (78) suggested that this calculation should not be used if leaves are stressed and significant photoinhibition has occurred. However, a problem exists only if F_m is measured after F_m' and recovery

from photoinhibition occurs during the dark adaptation period prior to measurement of F_m (6).

The PSII operating efficiency of a leaf decreases as PPFD increases owing to decreases in both F_v'/F_m' and F_q'/F_v' (Figure 3). However, the relative contributions of these two parameters can change markedly with increasing PPFD. Generally, increases in nonphotochemical quenching, indicated by decreases in F_v'/F_m' , saturate at much lower light levels than decreases in F_q'/F_v' , which demonstrates that a decrease in the ability to oxidize Q_A , not an increase in nonphotochemical quenching, is the major factor that determines the large changes in PSII operating efficiency at high light intensities. Also, increases in the PSII operating efficiency during induction of photosynthesis when a dark-adapted maize leaf is exposed to actinic light are primarily associated with increases in F_q'/F_v' and not changes in F_v'/F_m' (92). This finding demonstrates that the ability of processes downstream of PSII to utilize the products of LEF, rather than nonphotochemical quenching, is most important in the regulation of the induction of photosynthesis in this leaf.

The rate of consumption of NADPH and ATP are major factors that determine PSII operating efficiency in many situations. Changes in carboxylation efficiency, the rate of regeneration of ribulose 1,5-bisphosphate, the supply of CO_2 from the atmosphere to the sites of carboxylation via the stomata, photorespiration, and the rate of transport of carbohydrates out of the cell can all influence the rate of NADPH and ATP utilization (Figure 4), and consequently the PSII operating efficiency. Many environmental stresses impact on CO_2 assimilation, although the sites of photosynthesis limitation during these stresses can be quite varied. Stress-induced decreases in stomatal conductance, carbon metabolism, and transport processes can all decrease PSII efficiency. The specific mechanisms by which a restriction in metabolic turnover can result in decreases in PSII operating efficiency are not fully understood. In-

creases in NADPH and ATP decrease LEF and the rate of Q_A oxidation, which can be monitored by decreases in F_q'/F_v' . However, acidification of the thylakoid lumen as ATP levels increase also results in an increase in nonphotochemical quenching and a decrease in F_v'/F_m' (see section on Nonphotochemical Quenching, below).

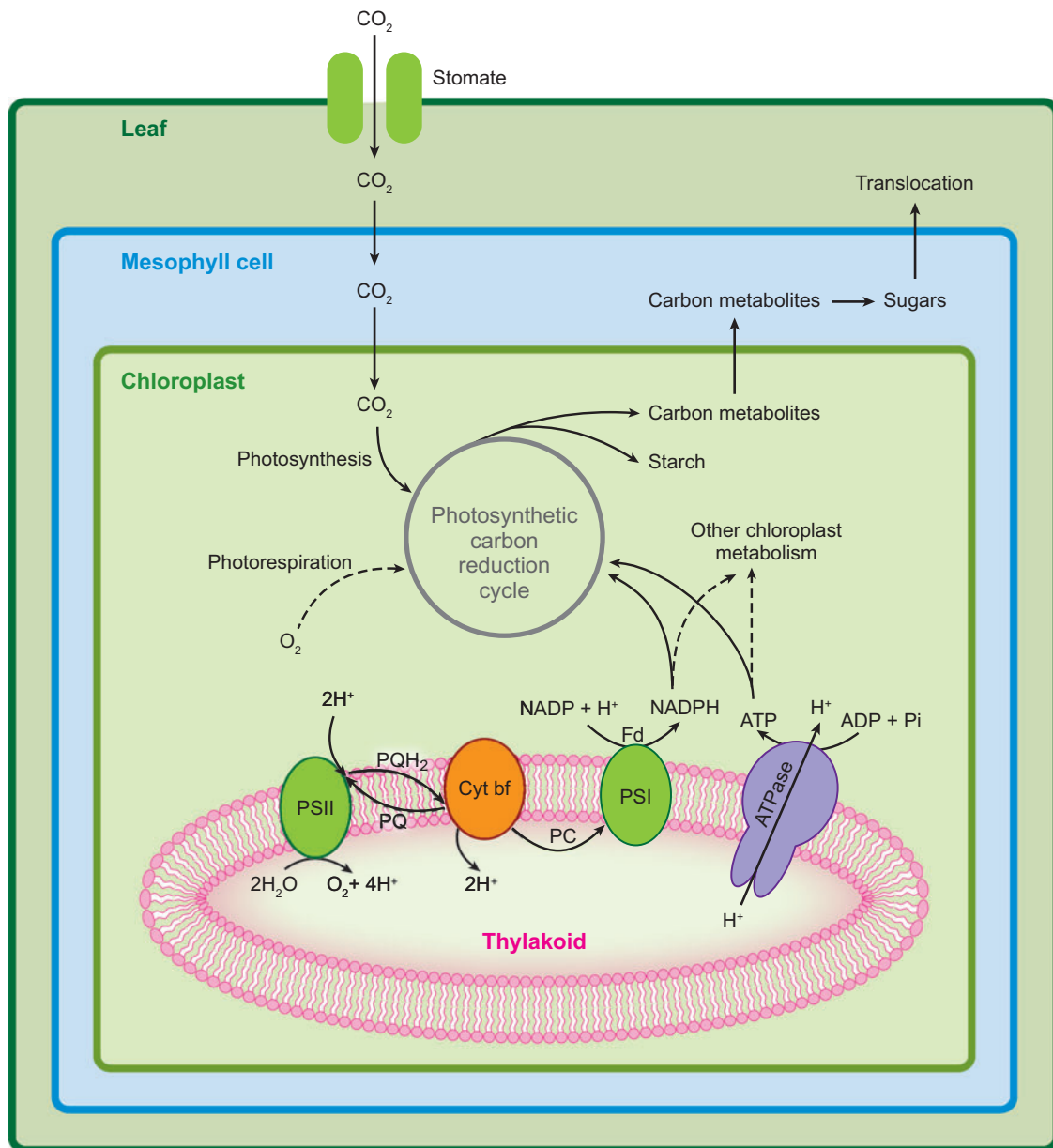
Photochemical Quenching

An important factor in determining the probability of PSII photochemistry is the redox state of Q_A , i.e., the fraction of PSII reaction centers that are open and capable of photochemistry. Frequently, the PSII efficiency factor (or the mathematically equivalent q_P) is used to estimate the redox state of Q_A . Unfortunately, in most situations the relationship between the PSII efficiency factor and the fraction of PSII centers in the open state is not linear and consequently changes in F_q'/F_v' (or q_P) cannot simply be used to estimate the redox state of Q_A . The relationship between the PSII efficiency factor and the fraction of open PSII centers is only linear if there is negligible excitation energy transfer among individual PSII complexes and associated antennae. This is the 'puddle model' in which each PSII reaction center and its associated antenna cannot transfer excitation energy to the antennae of other PSII reaction centers. It is widely accepted that this is not the case and excitation in PSII antennae can be competed for by a number of reaction centers (21, 69, 72). If all the PSII reaction centers are considered to be embedded within a single antennae matrix and are capable of receiving excitation energy from antenna pigments throughout the matrix ('lake model'), then the relationship between F_q'/F_v' and the redox state of Q_A is curvilinear (7, 52). However, the degree of curvilinearity is dependent not only upon the fraction of PSII centers that are open but also on the amount of light-induced nonphotochemical quenching that is occurring; for a fixed oxidation state of Q_A increases

in nonphotochemical quenching decrease the curvilinearity (7).

Assuming a lake model for PSII, the redox state of Q_A is linearly related to the fluorescence parameter $(F_q'/F_v')(F_o'/F')$, which has been termed q_L (63). Consequently, if an accurate assessment of the redox state of the Q_A pool is required then q_L , and not F_q'/F_v' (or

q_P), should be used. When the PSII operating efficiency is modified by exposing leaves to a range of PPFDs, although the patterns of change of F_q'/F_v' and q_L with increasing PPFD are similar, values of q_L are always lower than for F_q'/F_v' (or q_P), and at high PPFDs q_L values can be almost half of F_q'/F_v' (63) (Figure 3). Consequently, large errors can occur when



estimating changes in the redox state of QA using F_q'/F_v' , rather than q_L , especially at high light intensities.

Nonphotochemical Quenching

Although F_v'/F_m' can be used to evaluate the contribution of changes in nonphotochemical quenching to changes in PSII operating efficiency, levels of nonphotochemical quenching are often assessed by the parameter *NPQ*. *NPQ* is calculated from $(F_m/F_m') - 1$ (13) and estimates changes in the apparent rate constant for excitation decay by heat loss induced by light relative to this rate constant in the dark (65). Because *NPQ* compares nonphotochemical quenching from a dark-adapted leaf at F_m to that at F_m' for the leaf exposed to actinic light, *NPQ* values can only be compared for leaves that have similar nonphotochemical quenching characteristics in the dark-adapted state, e.g., leaves with similar F_v/F_m values. Changes in *NPQ* are nonlinearly related to and rise to higher values than F_v'/F_m' for a given change in nonphotochemical quenching (Figure 3). Consequently, changes in *NPQ* do not allow evaluation of the proportion of changes in PSII operating efficiency that are attributable to changes in nonphotochemical quenching.

Nonphotochemical quenching in leaves can consist of three components: energy-dependent quenching, q_E , photoinhibitory

quenching, q_I , and state transition quenching, q_T (65). Researchers have resolved nonphotochemical quenching into q_E , q_I , and q_T from analyses of the relaxation kinetics of these quenching components in the dark (49, 98, 106). However, care must be taken when attempting to quantify the contributions of these components because the characteristics of their relaxation kinetics can vary as a result of changing environmental conditions imposed on leaves. Generally, in nonstressed leaves under moderate to saturating light q_E is the major component, and q_I becomes prominent at light levels well in excess of that required to saturate photosynthesis or when stresses severely restrict the consumption of reductants produced by photosynthetic electron transport. Quenching associated with state transitions, q_T , is important only at low light levels, but can be very significant in algae (3, 33). Development of q_E is associated with quenching in the PSII antennae owing to the acidification of the thylakoid lumen resulting from electron transport (66). This acidification results in activation of violaxanthin de-epoxidase (109) and protonation of some carboxylic acid residues of the PsbS, a protein associated with the PSII antennae (74, 75) (Figure 5). Protonation of PsbS and binding of zeaxanthin to PSII produces conformational changes in the antennae that result in increases in the quantum yield of thermal dissipation of excitation

Figure 4

Relationships between photosynthetic electron transport, carbon metabolism and transport, and CO₂ supply. Electron transport, driven by the excitation of photosystem I (PSI) and photosystem II (PSII), results in the reduction of NADP to NADPH and the accumulation of protons in the thylakoid lumen. The resulting proton motive force is used to make ATP by driving protons back across the membrane through ATP synthase (ATPase). Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the assimilation of CO₂ with ribulose 1,5-bisphosphate (RuBP) in the carboxylation reaction of the photosynthetic carbon reduction cycle in the chloroplast stroma. Stomata regulate the diffusion of CO₂ from the atmosphere to the sites of carboxylation. Other reactions of the photosynthetic carbon reduction cycle utilize NADPH and ATP to produce triose phosphates, which are required for the synthesis of carbohydrates. NADPH and ATP are also used in a range of other chloroplast metabolic activities, e.g., nitrogen and sulfur metabolism and lipid and pigment synthesis. Rubisco can also catalyze the oxygenation of RuBP in the process of photorespiration, which also involves consumption of NADPH and ATP by the photosynthetic carbon reduction cycle. Abbreviations: Cyt bf, cytochrome b₆f complex; Fd, ferredoxin; PC, plastocyanin; PQ, plastoquinone; PQH₂, plastoquinol.

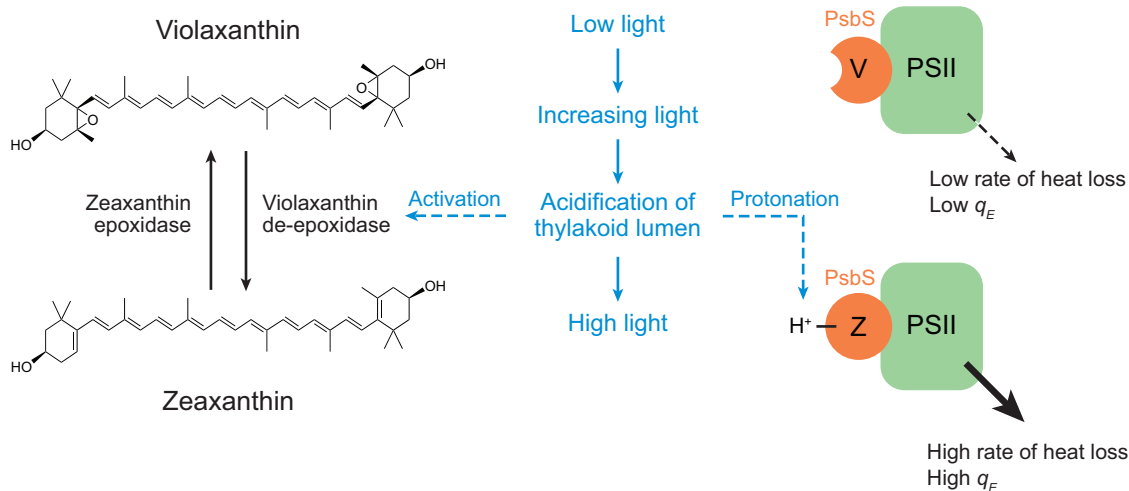


Figure 5

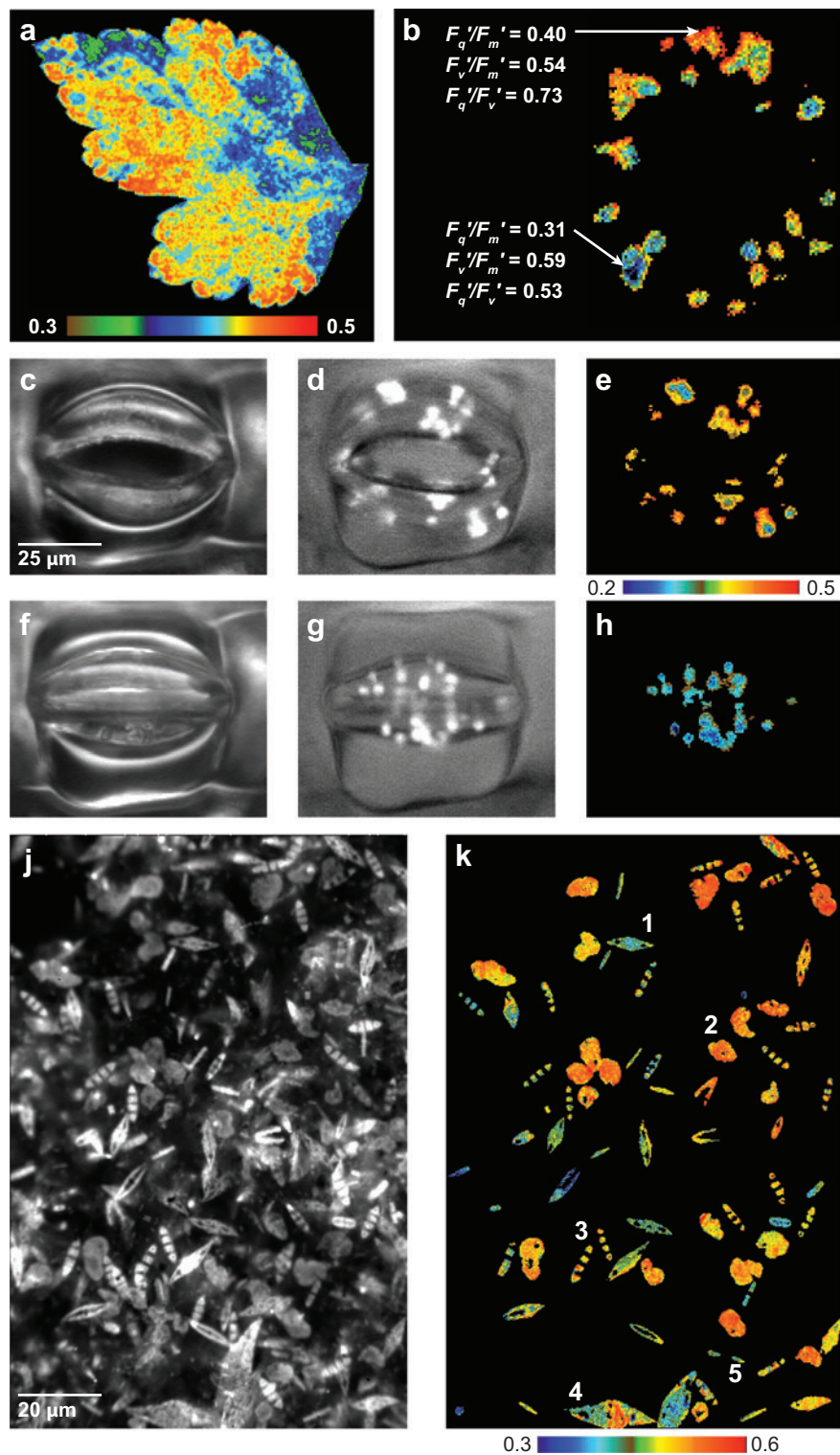
Mechanism of light-induced energy-dependent quenching of excitation energy in photosystem II (PSII). At low light that is limiting for photosynthesis a xanthophyll pigment, violaxanthin (V), is associated with the PSII antenna and PSII has a low rate of heat loss and consequently a low level of energy-dependent quenching, q_E , which is an important component of nonphotochemical quenching (NPQ). At higher light intensities increased electron transport results in acidification of the thylakoid lumen. When the lumen pH drops below *ca.* 6 violaxanthin de-epoxidase is activated and converts violaxanthin to zeaxanthin (Z) and PsbS becomes protonated. The zeaxanthin associated with PSII is an efficient quencher of excitation energy in the PSII antenna and the rate of heat loss from PSII increases, which increases q_E . When light intensity decreases deprotonation of PsbS occurs and zeaxanthin epoxidase converts zeaxanthin back to violaxanthin, which decreases q_E .

energy (50, 65, 95). Photoinactivation of PSII and zeaxanthin-related quenching can be involved in the development of q_I (65).

More detailed analyses of nonphotochemical quenching can resolve the excitation energy fluxes into light-induced quenching processes and non-light-induced quenching

Figure 6

Imaging the heterogeneity of photosynthetic activities of leaves, individual cells, and chloroplasts. (a) Image of F_q'/F_m' for a mildly water-stressed leaf of a Japanese anemone (*Anemone × hybrida*) collected from a local park on a warm and windy day and exposed to an actinic photosynthetically active photon flux density (PPFD) of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. This image demonstrates the large heterogeneity in photosynthetic activity across the leaf. The colored bar indicates the range of F_q'/F_m' values. (b) Image of F_q'/F_m' of chloroplasts in pair of stomatal guard cells of an attached leaf of *Tradescantia albiflora* exposed to a PPFD of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$. Values of F_q'/F_m' , F_v'/F_m' , and F_q'/F_v' shown for two individual chloroplasts demonstrate the heterogeneity of photosynthetic activity between chloroplasts in similar cells; this is primarily attributable to differences in F_q'/F_v' . (c–h) Images taken from a pair of guard cells of an attached leaf of *Commelina communis* with the stomate open (c–e) and after closure by decreasing the relative humidity (f–h). (c, f) are reflected light images; (d, g) are images of F_m' . (e, h) Images of F_q'/F_m' at a PPFD of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ showing the large decrease in PSII operating efficiency that occurs on closure of the stomata. (j) Reflected light image from an intertidal benthic biofilm collected from a salt marsh mud flat at Colne Point, Essex, UK and (k) image of F_q'/F_m' from these cells demonstrating the very large differences in the PSII operating efficiency between species. A number of different species can be identified in the biofilm: *Gyrosigma limosum* (1); *Euglena* sp. (2); *Plagiotropis vitrea* (3); *Pleurosigma angulatum* (4); and *Navicula* sp. (5). Images in (b–h) are taken from Reference 7, with permission; images in (j) and (k) are taken from Reference 94 with permission of copyright holder, American Society of Limnology and Oceanography.



processes (46, 63). The quantum yield of quenching due to light-induced processes, ϕ_{NPQ} , can be calculated (63):

$$\phi_{NPQ} = 1 - \frac{F'_q}{F'_m} - \frac{1}{\frac{F_m - F'_m}{F'_m} + 1 + \frac{F'_q}{F'_v} \cdot \frac{F'_v}{F'_o} \cdot \left(\frac{F_m}{F_o} - 1\right)} \quad (1)$$

Because the sum of the quantum yields of PSII photochemistry (ϕ_{PSII}), light-induced quenching processes, and non-light-induced quenching processes is assumed to equal 1, the quantum yield of non-light-induced quenching processes, ϕ_{NO} , can be estimated from $\phi_{NO} = 1 - (F'_q/F'_m) - \phi_{NPQ}$ (63).

IMAGING OF FLUORESCENCE

The development of instruments capable of imaging chlorophyll fluorescence has provided a powerful tool to resolve spatial heterogeneity of leaf photosynthetic performance (86, 91). Photosynthetic heterogeneity has been identified in many situations, e.g., during induction of photosynthesis (20, 92), with changes in carbohydrate translocation (80), at the onset of senescence (108), in response to changes in leaf water status (82, 87, 107) (**Figure 6a**), chilling (48) and ozone (73) stresses, wounding (99), and infection with bacteria (12, 16) and fungi (100, 104). Non-imaging fluorescence measurements would often not detect such heterogeneity. Imaging of appropriate fluorescence parameters can provide information about the causes of the heterogeneity. During induction of photosynthesis in a maize leaf, large changes in the degree of heterogeneity of the PSII operating efficiency occur (7). Similar patterns of heterogeneity are found in the images of F'_q/F'_m and F'_q/F'_v , which are not seen in the F'_v/F'_m images. Consequently, the heterogeneity is attributable to differences in the ability of cells to oxidize Q_A , which results from an inability to consume NADPH and ATP in CO_2 assimilation.

For C_3 leaves in which photorespiration is inhibited, the mean PSII operating efficiency (determined from images of F'_q/F'_m) is linearly related to ϕ_{CO_2} (determined from gas exchange), which allows quantitative visualization of the spatial distribution of photosynthesis (40). From gas exchange measurements made in conjunction with fluorescence imaging, Meyer & Genty (81) determined the relationship between PSII operating efficiency and intercellular CO_2 concentration (C_i) and constructed images of C_i from images of F'_q/F'_m . This approach has made it possible to map the two-dimensional distribution of C_i across leaves to study the lateral diffusion of CO_2 in leaf tissues (84). However, this procedure requires the assumption of spatially homogenous light absorption across the leaf area under study, which may not be the case in many leaves, such as when leaves have developed under stress or have been infected with pathogens.

High-resolution imaging has been used to examine the photosynthetic activities of single cells and even individual chloroplasts (93). The responses of electron transport in individual stomatal guard cells and adjacent mesophyll cells in intact leaves to changes in light, atmospheric CO_2 concentration, and humidity have been studied by imaging F'_q/F'_m (71) (**Figure 6c–b**). The isolation of individual chloroplasts from images of the guard cells of *Tradescantia albiflora* exposed to a PPFD of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ indicates that they show a wide range of mean F'_q/F'_m values, ranging from 0.27 to 0.43 (7). Such differences in the PSII operating efficiencies of individual chloroplasts are primarily attributed to differences in the ability to utilize ATP and reductants, not to differences in nonphotochemical quenching, because differences in F'_q/F'_v are considerably greater than those for F'_v/F'_m (7) (**Figure 6b**). Imaging has also resolved large differences in photosynthetic performance among benthic diatom species in biofilms (94) (**Figure 6j,k**). One problem in the production of images of F'_q/F'_m of such biofilms is that some of the cells can move

between the time that the images of F' and F_m' are captured, and therefore these cells must be moved within one image to allow them to be superimposed before calculation of the F_q'/F_m' image (91, 94).

Fluorescence imaging can be used in screening procedures to identify organisms with modified photosynthetic performance, as has been done for algae (11, 88) and *Arabidopsis* (89) mutants. Perturbations of metabolic processes not directly involved in photosynthetic metabolism often induce changes in fluorescence parameters (9, 96), which can be used to screen for such perturbations. The development of commercial fluorescence imaging

instruments that can image areas greater than 100 cm² allows the screening of large numbers of plants simultaneously. High-throughput screening of metabolic perturbations in *Arabidopsis* seedlings can be achieved by growing plants in the wells of 96-well microtiter plates (9) (**Figure 7**). Fluorescence imaging can also be used to estimate leaf area and consequently estimate growth; one application is the early growth of seedlings that have planophile, nonoverlapping leaves, such as *Arabidopsis*, from images of F_m . The total area from which the fluorescence is emitted is directly related to the leaf area that contains chlorophyll (9). However, for plants in

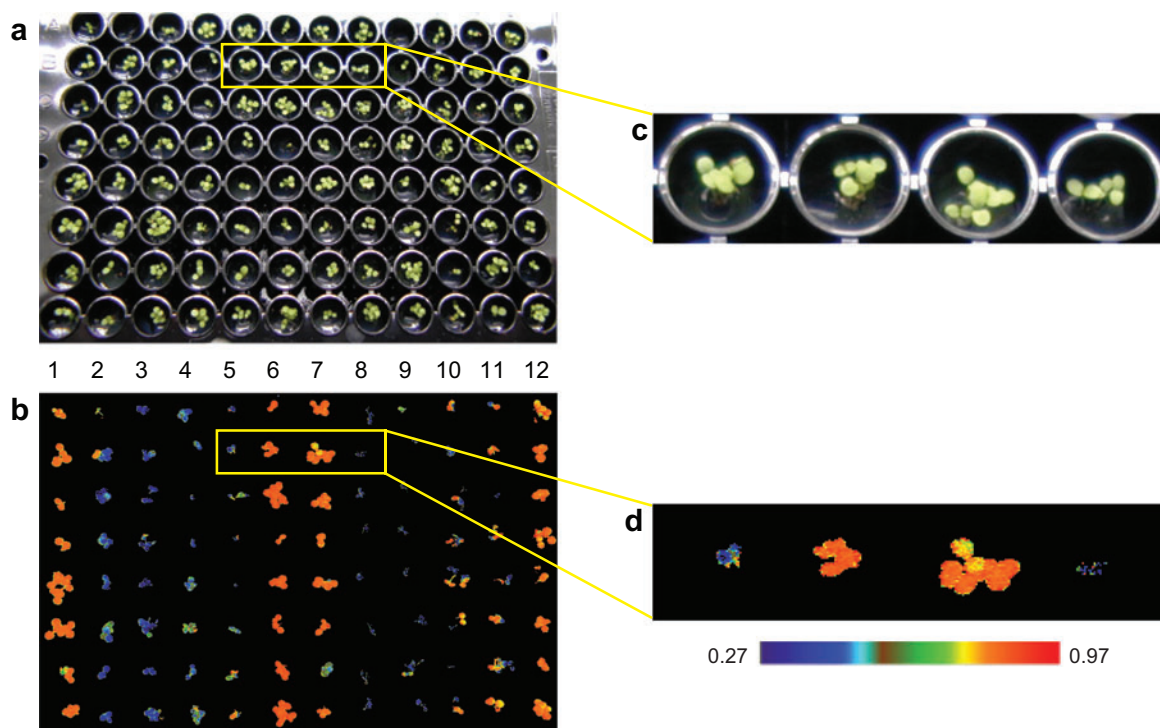


Figure 7

High-throughput screening for metabolic perturbations in *Arabidopsis*. (a) Five-day-old *Arabidopsis* plants in a 96-well plate 24 h after being treated with 0.4 (rows 5 and 11), 0.8 (rows 4 and 10), 4 (rows 3 and 9), and 8 (rows 2 and 8) mM Imazapyr, a herbicide that inhibits acetolactase synthase and consequently the synthesis of branched chain amino acids. Untreated controls are in rows 1, 6, 7, and 12. (b) Images of F_v/F_m for these plants. (c,d) Enlargements of the plants and images outlined by the yellow boxes in (a) and (b) respectively. Although differences in growth cannot be detected by visual observation, there are very large differences in the images of F_v/F_m between the control and herbicide-treated plants. Taken from Reference 9 with permission of copyright holder, American Society of Plant Biologists.

which the leaves overlap significantly or the leaves cannot be positioned normal to the camera this relationship does not necessarily hold. In such cases examination of the re-

lationship between the area of fluorescence and leaf growth is required before the use of fluorescence to screen for differences in growth.

SUMMARY POINTS

1. Dark-adapted F_v/F_m is a useful relative measure of the maximum quantum yield of photosystem II (PSII) photochemistry, but does not provide an accurate quantitative value of this quantum yield.
2. F_q'/F_m' is a useful relative measure of the quantum yields of PSII photochemistry and linear electron flux through PSII.
3. F_q'/F_m' can be used to estimate the rate of linear electron transport. This requires accurate determination of the photosynthetically active photon flux density (PPFD) incident on the leaf, the proportion of incident PPFD that is absorbed by the leaf, and the fraction of absorbed PPFD that is received by PSII.
4. In certain circumstances F_q'/F_m' is a good indicator of changes in the quantum yield of CO₂ assimilation by the leaf, ϕ_{CO_2} , but it should not be used to estimate absolute rates of CO₂ assimilation.
5. Many metabolic and physiological factors influence F_q'/F_m' by determining the rate of consumption of ATP and NADPH.
6. Fluorescence imaging can identify spatial heterogeneity of photosynthetic performance and offers new possibilities for understanding the operation and regulation of photosynthesis. Fluorescence imaging can also be used to image other physiological phenomena indirectly if they interfere with the operation of photosynthesis and its associated metabolism, e.g., herbicide effects and stomatal heterogeneity.

FUTURE DIRECTIONS

1. Chlorophyll fluorescence parameters can now be easily measured and provide useful probes of photosynthetic performance in vivo and the extent to which performance is limited by photochemical and nonphotochemical processes.
2. Coupling of appropriate fluorescence measurements with other noninvasive techniques, such as absorption spectroscopy (5), gas exchange (76), and thermal imaging (107), can provide insights into the limitations to photosynthesis under given conditions.
3. Fluorescence imaging has great potential in future plant screening programs and other areas of applied plant physiology. The selection of appropriate fluorescence parameters and careful calibration of their changes with key plant performance indicators is important. Once a satisfactory calibration has been achieved, fluorescence can offer rapid, high-throughput screening. The use of automated sampling devices in conjunction with increases in the areas than can be imaged will enhance the rates of screening procedures even further.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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