Investigating energy partitioning during photosynthesis using an expanded quantum yield convention

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Abstract

In higher plants, regulation of excess absorbed light is essential for their survival and fitness, as it enables avoidance of a build up of singlet oxygen and other reactive oxygen species. Regulation processes (known as non-photochemical quenching; NPQ) can be monitored by steady-state fluorescence on intact plant leaves. Pulse amplitude modulated (PAM) measurements of chlorophyll a fluorescence have been used for over 20 years to evaluate the amount of NPQ and photochemistry (PC). Recently, a quantum yield representation of NPQ (ΦNPQ), which incorporates a variable fraction of open reaction centers, was proposed by Hendrickson et al. [L. Hendrickson, R.T. Forbush, W.S. Chow, Photosynth. Res. 82 (2004) 73]. In this work we extend the quantum yield approach to describe the yields of reversible energy-dependent quenching (ΦqE), state transitions to balance PC between photosystems I and II (ΦqT), and photoinhibition quenching associated with damaged reaction centers (ΦqI). We showed the additivity of the various quantum yield components of NPQ through experiments on wild-type and npq1 strains of Arabidopsis thaliana. The quantum yield approach enables comparison of ΦqE with data from a variety of techniques used to investigate the mechanism of qE. We showed that ΦqE for a series of A. thaliana genotypes scales linearly with the magnitude of zeaxanthin cation formation, suggesting that charge-transfer quenching is largely responsible for qE in plants.

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1. Introduction

Photosynthesis begins with the absorption of sunlight in antenna proteins that bind chlorophyll and carotenoid pigments followed by the subsequent transfer of the excitation energy to reaction centers where electron transfer occurs [1]. The rate of absorption of sunlight routinely exceeds the down-stream capacity of CO2 assimilation [2–4]. The excessive absorbed energy can lead to a variety of harmful side reactions, some of which produce reactive oxygen species (ROS) [5], and some to acid-induced damage to electron transfer carriers exposed to the thylakoid lumen, thereby threatening the survival of the organism [6]. In addition to possessing a robust anti-oxidant system [3], higher plants are capable of reducing the negative effects of excess light absorption by a composite of processes collectively termed non-photochemical quenching, or NPQ [7].

To explore and quantify the amount of NPQ, the pulse amplitude modulated (PAM) chlorophyll a fluorescence yield (ΦF) has been the primary observable [8]. The information from PAM studies has broad implications in photosynthesis research [9]. The PAM fluorescence technique uses three excitation sources: the fluorescence monitoring light, the actinic light, and a saturation pulse to drive the chlorophyll a fluorescence yield (ΦF) either in leaves or in extracted thylakoid membranes. The parameters extracted from such measurements provide information about how energy is partitioned during photosynthesis. Interpretation relies on the fact that singlet excited chlorophyll a can decay by intrinsic photo-physical relaxation pathways and, when integrated into the photosynthetic system (i.e. bound by proteins that are organized into photosystems, etc.), the chlorophyll a ΦF can also report on auxiliary pathways capable of quenching chlorophyll excited states [10–12]. The photosynthetic apparatus (i.e. interconnected photosystems, etc.) is comprised of various ‘traps’ for excitation energy, some of which are chemically productive and lead to electron transfer (i.e. reaction centers), whereas others non-productively...
dissipate excitation energy. PAM chlorophyll a fluorescence analyses can provide information regarding how energy is partitioned to these pathways during photosynthesis. This technique was recently reviewed by Kramer et al. [12].

Quantitative and qualitative comparisons of various parameters derived from the PAM technique have proved invaluable for understanding the regulatory aspects of photosynthesis. For example, qualitative estimates of the magnitude of NPQ in various mutant species of Arabidopsis thaliana have confirmed essential roles for the xanthophyll cycle [13] and the photosystem (PS) II-associated protein PsbS in the regulation of excitation flow to the reaction center of PSI [14,15]. Quantitative comparison, obtained from PAM analyses, of NPQ with estimates of steady-state electron flow (i.e. linear electron flow, or LEF), also during photosynthesis has led to novel insights regarding how both light capture and the output ratio of the light reactions, e.g. the ATP:NADPH ratio, are regulated [16–21]. Despite these successes, it is becoming clear that a more thorough, quantitative description of the PAM parameters is essential for comparison to and interpretation of data derived from other approaches [22].

An important step in this direction was taken by Hendrickson et al. [10,11] who introduced a quantum yield description, including the quantum yield of NPQ ($\Phi_{npq}$, which they called the yield of “non-functional” reaction centers), in order to provide a more quantitative analysis of energy partitioning. Hendrickson et al. extended this quantum yield analysis to the study of Capsicum annum leaves and demonstrated contributions of the quantum yield of NPQ to dissipation of chlorophyll excited states [10]. In these studies, $\Phi_{npq}$ was assumed to represent the pH- and zeaxanthin (Z)-dependent component of NPQ that is generally referred to as energy-dependent quenching, or qE [2,7]. Although qE is the major component of NPQ under most conditions, state transitions (qT) and a quenching related to photoinhibition (qL) contribute to total NPQ [2,23]; the former to balance the PC between PSI and PSII and the latter reflecting the pH independent quenching induced by binding of Z to lhc proteins and the rates of photodamage to and repair of PSI [24]. Photoinhibition, which remains a rather mysterious phenomenon, has been reviewed recently [25,26]. Under certain conditions and in various mutant species, qT and qL may be larger than qE, information that could be hidden in the total $\Phi_{npq}$ parameter. Therefore, it should be useful to decompose $\Phi_{npq}$ into these three components.

An important aspect of the quantum yield convention is that it reflects the contributions of the rate constants for all of the processes that are capable of quenching chlorophyll excited states. Therefore, values of $\Phi_{npq}$ obtained for wild-type and mutant plants can report on the set of processes in the leaf under natural light conditions [10,11]. For example, estimates of NPQ and qE, as they are typically reported in the literature, are based solely on PAM chlorophyll a fluorescence parameters that are obtained under conditions (i.e. supersaturating pulses of actinic light) in which the reaction centers of PSI are completely ‘closed’ [2,7,13,17], and so the rate constant for photochemistry is assumed to approach zero. This is not representative of what occurs in a leaf in nature. In contrast, the quantum yield convention takes into account the fraction of reaction centers which remain open and thereby quantitatively reflects the fraction of absorbed quanta that are dissipated by the various processes. Advances in our understanding of photosynthesis are becoming increasingly dependent upon approaches involving comparison of parameters derived from multiple techniques [16,27]. In some cases, data from these approaches are obtained under conditions in which a fraction of the reaction centers remain open and therefore require explicit comparisons with the quantum yield parameters of PAM analyses.

In this paper we compare and contrast estimates of the various photosynthetic parameters that can be derived from PAM chlorophyll a fluorescence analyses based on the quantum yield approach under the assumptions that the reaction centers are either closed or open. In addition, we extend the previous approach of Hendrickson et al. [10,11] by decomposing $\Phi_{npq}$ into $\Phi_{qE}$, $\Phi_{qT}$, and $\Phi_{qL}$ and demonstrate the importance of this decomposition by comparing these estimates in A. thaliana wild-type and npq1, a mutant that lacks a functional violaxanthin de-epoxidase (VDE) and cannot accumulate Z in plants [13]. Furthermore we demonstrate the linear correlation between $\Phi_{qE}$ and the transient charge-transfer absorption signal previously assigned to Z cation radicals [22,27] in various A. thaliana mutants and wild-type. Therefore we suggest the quantum yield of qE is the appropriate way to quantify the influence of specific factors on energy-dependent quenching and to establish causal relationships between specific spectroscopic signals and qE.

2. Materials and methods

2.1. Plant material

A. thaliana (ecotype Col-0) wild-type and npq1-2 mutant were grown in commercial potting mix. The plants were grown in a controlled growth chamber with a short-day photoperiod of 10 h light (22–23 °C) and 14 h dark (19–20 °C), under a light intensity of 150 µmol photons m⁻² s⁻¹ (150 µE). Plants between the ages of 5 and 6 weeks, prior to bolting, were used for all experiments.

2.2. Pulse amplitude modulated chlorophyll a fluorescence analyses

Chlorophyll a fluorescence traces were measured using a FMS2 fluorimeter (Hansatech instruments, Norfolk, England). $F_{m}$ and $F_{s}$ are maximum levels of fluorescence before and after the steady-state during the actinic illumination, respectively. $F_{s}$ is the steady-state fluorescence level. $F_{m}^\prime$ is the maximum level of fluorescence measured at 10 min after turning off the actinic light. The saturating pulse intensity was more than 5000 µE.

2.3. The photophysics of the regulation of light harvesting

When a molecule is excited by absorption of light, it can decay to the ground state directly via fluorescence and/or internal conversion (IC) or indirectly through intersystem crossing (ISC) into the triplet state, which then decays to the ground state directly via fluorescence and/or internal conversion. The quantum yield of fluorescence, $\Phi_{F}$, is the sum of these rate constants, respectively [28]. For chlorophyll a, the major chromophore in the chloroplast of higher plants, $\Phi_{F}$ and the triplet yield ($\Phi_{T}$) are reported to be 0.32 and 0.64 in ether solvent, implying that the predominant relaxation pathway for chlorophyll a in solution is ISC to the triplet state and that the yield of IC is negligible [29]. In the intact photosynthetic system, the triplet state of chlorophyll can generate highly toxic singlet oxygen [2] with almost unity efficiency [30,31], eventually threatening plant survival [6]. However, when large networks of chlorophyll molecules are bound within light-harvesting complexes, singlet excited states of chlorophyll can be efficiently quenched by energy transfer to reaction centers, and by NPQ, effectively attenuating $\Phi_{T}$.
2.4. Parameters in PAM fluorescence measurements

The PAM chlorophyll α fluorescence technique detects relative changes in chlorophyll α φr and, by employing a variety of experimental manipulations, can provide accurate analyses of the various pathways that can quench chlorophyll excited states and thereby alter φr. Several useful quantities are defined as follows: $F_m = G_{\text{ISC}} = G_{\text{F}}$, $F_s = G_{\text{F}} - k_p/F_m$, and $F_a = G_{\text{F}} - k_p/F_m$, where $k_p$ and $k_{NPQ}$ are the rate constants for photochemistry leading to down-stream electron transfer and NPQ, respectively, and $G$ is the instrumental gain coefficient [1]. $k_p$ is more accurately described as an apparent rate constant and equal to $k_p/[Q_A]$, where $[Q_A]$ is the concentration of non-reduced (oxidized) quinones in the reaction center and $k_p$ is an elementary rate constant [29,32,33]. Similarly, $k_{NPQ}$ may be regarded as $k_{NPQ}/[\text{Quencher}]$, where $[\text{Quencher}]$ is the concentration of putative quencher (see below) and $k_{NPQ}$ is the simple rate constant [1]. Therefore, $k_p$ and $k_{NPQ}$ are expected to be sensitive to environmental factors such as actinic light intensity and the capacity of down-stream metabolism to consume the output of the light reactions, both of which regulate $[Q_A]$ and $[\text{Quencher}]$. Moreover, $k_{NPQ}$ can be further dissected into the sum of the apparent rate constants for $q_E$, $q_T$, and $q_l$ ($k_{NPQ} = k_{q_E} + k_{q_T} + k_{q_l}$). In this regard, another useful parameter that can be obtained from PAM analysis is referred to as $F_m'$ and can be defined as

$$ F_m' = \frac{G_{\text{F}} - k_p}{k_{NPQ} + k_{q_E} + k_{q_T}} $$

and which is typically obtained ~10–20 min post actinic illumination, e.g. after the $q_E$ component of NPQ has relaxed, leaving $q_T$ and $q_l$ as the only active components of NPQ [2,12,17,23,34,35–37]. Finally, since the actual fluorescence decay of intact thylakoid membranes requires more than three exponential functions to fit the above mentioned rate constants are assumed to be weighted averaged rate constants.

2.5. Interpretation of rate constants

NPQ has been expressed according to the Stern–Volmer quenching mechanism as $F_m - F_m = k_{NPQ}$, where $k$ is a proportionality coefficient and $[\text{Quencher}]$ is the concentration of putative quencher in photosystem (PS) II [1]. The left side of the equation, which can be measured by fluorescence, is defined as the magnitude of NPQ, i.e. $NPQ = \frac{F_m - F_a}{F_m}$ [2,23,38]. From the above, this relation corresponds to a ratio of rate constants for the NPQ processes relative to the sum of the rate constants ($k_C$) for the intrinsic relaxation pathways of singlet excited chlorophyll α:

$$ \frac{F_m - F_m}{k_{NPQ}} = \frac{F_m - F_a}{F_m} = \frac{F_m}{k_C}. $$

Similarly, the ratio of the rate constant for PC ($k_P$) can be derived as:

$$ \frac{k_P}{k_C} = \frac{F_m - F_s}{F_m} = \frac{F_m}{k_C}. $$

The sum of these rate constants divided by $k_C$ equals $F_m/F_C$, i.e. $\sum_{NPQ,PC} k_i/k_c = F_m/F_C$.

2.6. Preparation and PAM fluorescence measurement of thylakoid membranes

One gram of leaves were homogenized in 200 ml of grinding buffer (0.3 M sorbitol, 2 mM EDTA, 5 mM EGTA, 5 mM MgCl2, 10 mM NaHCO3, 20 mM Tricine, pH 8.2, 0.5% BSA, and protease inhibitors). The solution was centrifuged 2600g for 4 min at 4 °C. The chloroplast pellets were re-suspended in resuspending buffer (0.3 M sorbitol, 2.5 mM EDTA, 5 mM MgCl2, 10 mM NaHCO3, 20 mM Hepes, pH 7.8, 0.5% BSA), and then centrifuged 2600g for 4 min at 4 °C. The chloroplast pellets were re-suspended in a small volume of resuspending buffer to match the same chlorophyll concentration (20 μM) to measure PAM fluorescence. During the PAM fluorescence measurement, the actinic light and saturating flash intensities were set to be 500 μE and 2500 μE (0.8 s), respectively. The measuring buffer (0.3 M sorbitol, 5 mM MgCl2, 10 mM NaCl, 20 mM KCl, 30 mM Tricine, pH 7.8, 20 μM methylviologen, 0.3 mM ATP, 30 mM ascorbate, and 0.2% BSA) was used also.

3. Results

Fig. 1 shows $k_{NPQ}$ and $k_P$ divided by $k_C$, respectively, versus elapsed time after the actinic light is turned on using three different actinic light intensities (150, 350, and 1250 μE). Within 5 min PC evolves rapidly and reaches saturation. The saturated PC rate decreases with an increase in actinic light intensity, whereas the NPQ rate increases according to the light intensity. At 350 μE, NPQ quickly reaches a maximum which is followed by a slow decrease as the PC rate reaches a constant value. This means that NPQ, most likely $q_E$, abruptly increases to compensate while the rate of photochemistry builds up [39]. Interestingly the saturated $k_{NPQ}/k_C$ value at 150 μE (2.38) is the same as $k_{NPQ}/k_C$ at 1250 μE, indicating that NPQ compensates PC perfectly under high actinic light.

To explore the light-dependent dynamics of these rate constants under steady-state conditions, we plotted the ratios of $k_{NPQ}$, $k_P$, and $k_C$ relative to $k_C$ (Eqs. (3) and (4)) as estimated in leaves of Arabidopsis thaliana exposed to actinic light intensities ranging from 25 to 2500 μE (Fig. 2). While $k_C$ component is constant (~1.0) over the entire range of light intensities, as expected (see above) both $k_P/k_C$ and $k_{NPQ}/k_C$ exhibit dynamic behavior in response to this variable light regime, the former decreasing sharply from 25 to ~500 μE, followed by a gradual decrease out to 2500 μE, while
the latter increases steadily from 25 to 2500 μE. The sum of all rate constants ( ∑ k_i, i = C, NPQ, and P) over the range of actinic light intensities utilized is not constant, because while k_p/k_c decreases sharply, k_{NPQ}/k_c increases more gradually. Given this result, a more comprehensive and quantitative description of energy partitioning to the respective pathways can be obtained using the quantum yield convention which appropriately accommodates for changes in the relevant rate constants.

3.1. Assessment of quantum yields

In contrast to describing NPQ, PC, and intrinsic decays, in terms of ratios of rate constants, simple equations can be obtained using the abovementioned PAM fluorescence parameters (F_m, F_s, etc.) that represent the quantum yields of the respective pathways. For example, the quantum yield of PSII-mediated photochemistry (Φ_II), a very robust and extensively utilized parameter [10–12,33], has been previously defined as

\[
\Phi_{II} = \frac{k_0}{k_c + k_{NPQ} + k_p} = \frac{F_m - F_s}{F_m}.
\]

Note that the rate constants for all of the processes capable of dissipating chlorophyll singlet excited states in the intact system appear in the denominator. The quantum yield of NPQ, or Φ_{NPQ}, can be similarly estimated from [10,11]

\[
\Phi_{NPQ} = \frac{k_{NPQ}}{k_c + k_{NPQ} + k_p} = \frac{F_m - F_{m-1}}{F_m} \times \frac{F_s}{F_m}.
\]

The quantum yield of the chlorophyll photophysical decay pathways (Φ_C) can be obtained as

\[
\Phi_C = \frac{k_c}{k_c + k_{NPQ} + k_p} = \frac{F_s}{F_m}.
\]

Interestingly Φ_C equals the steady-state PAM fluorescence trace itself without saturation pulse when it is normalized by the maximum fluorescence, F_m. Further equations that express the quantum yields of the various processes that comprise Φ_{NPQ}, namely Φ_{ql}, Φ_{qT}, and Φ_{qE}, can be written. For example, Φ_{ql} defined as

\[
\Phi_{ql} = \frac{k_q}{k_c + k_{NPQ} + k_p} = \frac{F_m - F'_m}{F_m} \times \frac{F_s}{F_m}.
\]

while the sum of Φ_{qT} and Φ_{ql} can be obtained from

\[
\Phi_{qT+ql} = \frac{k_{qT} + k_q}{k_c + k_{NPQ} + k_p} = \frac{F_m - F'_m}{F_m} \times \frac{F_s}{F_m}.
\]

It should be emphasized that estimates of Φ_{II}, Φ_{NPQ}, Φ_C, Φ_{qE}, and Φ_{qT+ql} according to Eqs. (5)–(9) correspond to energy partitioning via the respective processes not only under steady-state illumination, but more importantly with the additional assumption that a certain fraction of the reaction centers remain in the open state. Thus these equations reflect the actual quantum yields of the respective processes in a leaf under naturally fluctuating light conditions that result in variability in the extent to which the reaction centers remain open.

Fig. 3A shows values of Φ_{II}, Φ_{NPQ}, Φ_C, Φ_{qE}, and Φ_{qT+ql} for wild-type A. thaliana leaves that were exposed to actinic light intensities ranging from 25 to 2500 μE. Φ_C reaches a maximum of ca. 0.3 at ~400 μE, an increase that can be attributed to the fact that while k_c remains constant, the sum of k_{NPQ} and k_p is decreased at ~400 μE (Fig. 2). These data are consistent with an increase in ISC at intermediate light intensities, possibly enhancing the production of toxic singlet oxygen. However, both Φ_T and the triplet quantum yield (Φ_T = k_{ISC}/k_{NPQ}) have been reported to be significantly attenuated (0.06 and 0.1, respectively at room temperature) in the protein environment (i.e. CP47) [40], implying that the quantum yield of IC of excited chlorophyll is more likely significantly enhanced. X-ray crystallography shows that various carotenoid species, all of which are capable of quenching triplet chlorophyll
and singlet oxygen, are positioned in the protein in close association with the chlorophylls [41–43].

The total area between the dashed lines corresponds to \( \Phi_{\text{NPQ}} \) and the decomposition of \( \Phi_{\text{NPQ}} \) into \( \Phi_{\text{qE}} \) and \( \Phi_{\text{qT+qI}} \) is shown by the hatched and open areas, respectively. These data demonstrate that the major portion of \( \Phi_{\text{NPQ}} \) over a wide range of light intensities is attributable to \( \Phi_{\text{qE}} \). Furthermore, the remaining upper portion of the quantum yield plot corresponds to \( \Phi_{\text{II}} \) since \( \Phi_{\text{C}} + \Phi_{\text{NPQ}} + \Phi_{\text{II}} = \frac{1}{\Phi_{\text{C}} + \Phi_{\text{NPQ}} + \Phi_{\text{II}}} = 1 \). These quantum yield plots provide quantitative information on the fate of absorbed quanta demonstrating the capacity of \( \Phi_{\text{NPQ}} \) and distinguishing the relative contributions of \( \Phi_{\text{qE}} \) and \( \Phi_{\text{qT+qI}} \). A reader new to this field might find it surprising that, over a wide range of actinic light intensities, these data indicate that the major fraction of absorbed quanta are dissipated by processes that do not contribute to the production of organic matter (i.e. plant biomass).

Fig. 3B shows the same quantum yield parameters for the A. thaliana mutant npq1 which lacks the capacity to generate 2 [13]. The \( \Phi_{\text{C}} \) for npq1 exhibits an overall shape similar to that of the wild-type, but reaches a maximum of -0.46 at -400 \( \mu \)E, implying that the summation of \( k_{\text{NPQ}} \) and \( k_{\text{II}} \) in npq1 is likely lower than that of the same summation in the wild-type (Fig. 1). Considering that \( \Phi_{\text{II}} \) of npq1 is same as that of the wild-type within the error range, the main difference between the two genotypes is that \( \Phi_{\text{NPQ}} \) under the equivalent of full sunlight (2500 \( \mu \)E), is attenuated by ~18% in the npq1 strain. Interestingly, while \( \Phi_{\text{qE}} \) in npq1 is only ~36% relative to that of the wild-type, \( \Phi_{\text{qT+qI}} \) of npq1 is almost 70% larger than that of the wild-type at 2500 \( \mu \)E. Therefore, a significant decrease of \( \Phi_{\text{qE}} \) and an increase of \( \Phi_{\text{qT+qI}} \) (mainly \( \Phi_{\text{II}} \)) are the major changes in the npq1 mutant. In addition, \( \Phi_{\text{C}} \) of npq1 mutant is much higher than that of wild-type throughout this range of actinic light intensities. Therefore, in the absence of Z formation, the intrinsic relaxation of excited chlorophyll a likely generates more triplet states in npq1, thereby increasing the probability of singlet oxygen generation through triplet–triplet quenching [44], effectively sensitizing the npq1 strain to light, cold temperatures, etc. [41,45]. Additionally, in both cases, \( \Phi_{\text{II}} \) cannot be ignored, which is estimated to be approximately 0.1 or less under full sunlight (2500 \( \mu \)E), indicating that roughly 10% of the excited chlorophyll states can still be transferred to the reaction center (Fig. 2).

### 4. Discussion

#### 4.1. Photophysical meaning of \( \Phi_{\text{qE}} \) and \( \Phi_{\text{NPQ}} \)

As mentioned above, NPQ as typically defined (Eq. (3)) corresponds to a rate constant (\( k_{\text{NPQ}} \)) relative to the intrinsic rate constants (\( k_{\text{c}} \)) of the natural relaxation pathways of excited chlorophyll a. As such, NPQ may have a value over 1 at a light intensity higher than 500 \( \mu \)E in wide-type A. thaliana leaves (Fig. 4). However, it does not delineate the contribution of different processes to the fate of the chlorophyll excited states [10,11]. This information is inherent in the quantum yield convention discussed above. This approach also allows for quantitative comparisons of the specific parameters (\( \Phi_{\text{NPQ}} \), etc.) at different light intensities, conditions over which, as was demonstrated in Fig. 1, the sum of \( k_{\text{C}}, k_{\text{C}}, \) and \( k_{\text{NPQ}} \) is not constant. Fig. 4 illustrates the discrepancy between \( \Phi_{\text{NPQ}} \) and NPQ in wide-type A. thaliana leaves.

It should also be emphasized that the quantum yield parameters accurately reflect energy partitioning allowing that a variable fraction of the reaction centers remain open. This contrasts with typical estimates of NPQ, which are obtained from PAM fluorescence parameters that are derived with completely closed reaction centers. Similarly, in the literature, estimates of qE are measured from PAM fluorescence parameters (\( F_{\text{m}}^{\text{qE}} \), etc.) that are obtained using super saturating pulses of light to effectively close the reaction centers. It is possible to obtain estimates of the quantum yield of qE, when the reaction centers are completely closed, (i.e. \( k_{\text{C}} = 0 \), \( \Phi_{\text{qE,RCC}} \) from the following expression:

\[
\Phi_{\text{qE,RCC}} = \frac{k_{\text{qE}}}{k_{\text{C}} + k_{\text{NPQ}}} = \frac{F_{\text{m}} - F_{\text{m}}^{\text{qE}}}{F_{\text{m}}^{\text{qE}}},
\]

where RCC indicates that the Reaction Centers are Closed. Likewise the sum of such quantum yield representations for qT and qI can be similarly estimated as

\[
\Phi_{\text{qT+qI,RCC}} = \frac{k_{\text{qT+qI}} + k_{\text{qI}}}{k_{\text{C}} + k_{\text{NPQ}}} = \frac{F_{\text{m}} - F_{\text{m}}^{\text{qT+qI}}}{F_{\text{m}}^{\text{qT+qI}}},
\]

This manner of expressing qE and qT+qI is not simply related to values of NPQ obtained in the standard way (Eq. (3)). For example, a plot of the sum of qE, qT, and qI, estimated according to Eqs. (10) and (11), versus NPQ determined by Eq. (3) is not linear (Fig. 5A), indicating that these parameters are not directly comparable. However, NPQ can be derived in a way that directly correlates with representations of qE, qT, and qI according to Eqs. (10) and (11), indicating that the two representations are directly related. For example, when it is necessary to decompose NPQ into its constituent components, we suggest the use of the following equation:

\[
\Phi_{\text{NPQ,RCC}} = \frac{k_{\text{NPQ}} + k_{\text{II}}}{k_{\text{C}} + k_{\text{NPQ}}} = \frac{F_{\text{m}} - F_{\text{m}}^{\text{NPQ}}}{F_{\text{m}}^{\text{NPQ}}},
\]

As shown in Fig. 5B, a plot of \( \Phi_{\text{NPQ,RCC}} \) estimated according to Eq. (12) versus the sum of qE, qT, and qI estimated according to Eqs. (10) and (11) is linear with a slope of one, showing these parameters are equivalent. This particular representation of NPQ can be regarded as a special case of \( \Phi_{\text{NPQ}} \) when the reaction centers are closed (\( k_{\text{C}} = k_{\text{NPQ}} \) can therefore be ignored). In this case, the following relation is valid:

\[
\Phi_{\text{NPQ,RCC}} = \Phi_{\text{qE,RCC}} + \Phi_{\text{qT+qI,RCC}}.
\]

It should also be noted that a plot of \( \Phi_{\text{NPQ}} \) and the sum of \( \Phi_{\text{qE}} \) plus \( \Phi_{\text{qT+qI}} \), which are based on a variable fraction of open reaction centers and defined according to Eqs. (6), (8) and (9), is linear with a slope of one (Fig. 5C). The additivity of the components of \( \Phi_{\text{NPQ}} \) is the key benefit of this approach.

#### 4.2. Mechanism of the various components of NPQ

As demonstrated above, \( \Phi_{\text{NPQ}} \) in npq1 was ~82% that of the wild-type level at 2500 \( \mu \)E (Fig. 3). Given that this mutant completely lacks detectable levels of Z, this might suggest a minor role for Z in photoprotection. However, while \( \Phi_{\text{NPQ}} \) was only modestly
lower, $\Phi_{qE}$ in npq1 was less than $\sim$50% that in the wild-type, indicating a significant role for Z in qE. It may be necessary to separate the contributions of $\Phi_{qT}$ and $\Phi_{qI}$ to understand mutants like npq1 in detail. At present we cannot specify if the twofold increase in $\Phi_{qT}$ originates with qT, qI or both components. $\Phi_{qT}$ quenching has been suggested to involve damage to the reaction centers [23]. If so, it might be expected that electron transfer yield would be lower in npq1. However, estimates of $\Phi_{II}$ were identical in the wild-type and npq1 within our error range (Fig. 3). Since, npq1 has extremely low levels of Z [13], other pigments such as antheraxanthin or lutein [46] could account for this sustained quenching. Alternatively, the increase in the sum of $\Phi_{qI}$ and $\Phi_{qI}$ in npq1 may reflect an increase in $\Phi_{qT}$. A complete description of a mutant such as npq1 would clearly be facilitated by extending the quantum yield approach to include separate estimates of $\Phi_{qT}$ and $\Phi_{qI}$.

4.3. Correlation of $\Phi_{qE}$ with charge-transfer quenching

Recently there has been a surge in interest in exploring the molecular mechanism(s) for qE [22,47,48]. Our group has identified charge-transfer (CT) quenching in thylakoids, through a combination of quantum chemical calculations [49–51], ultra-fast transient absorbance (TA) spectroscopy in the near infrared region (NIR) and genetic modifications [22,27]. Holt et al. demonstrated transient Z$^+$ formation in thylakoids from wild-type A. thaliana, whereas almost negligible transient signal was observed in thylakoids from npq1 and npq4, mutant strains of A. thaliana that lack the ability to generate Z and a functional psbS, respectively [27]. Both factors are necessary for fully functional qE [13–15].

Recently, we demonstrated the CT quenching occurs in isolated minor complexes of the PSII antenna, but not in LHCl trimers or monomers [22,47]. All three minor complexes (CP24, CP26, and CP29) that show the Z$^+$ signal have been proposed as sites of CT quenching in PSII during qE [22]. We explored the molecular details of CP29 and proposed that an excitonically coupled Chl dimer interacting with Z are the key components for CT quenching [47]. We suggested that the low pH in the lumen combined with binding of PsbS to the CP complexes triggers a protein conformational change in the excitonic dimer which activates the CT quenching, thereby modulating the energy transfer to RC [47].

We introduced the following quantitative CT quenching yield to compare with $\Phi_{qE}$:

$$\Phi_{CT} = \frac{[Z^+]_0}{[Chl]\_0\_M}$$

where $[Z^+]_0$ was derived from the maximum difference of the NIR TA kinetic probed at 1000 nm and $[Chl]\_0\_M$ corresponds to the estimated concentration of minor complexes that were excited within the probe volume [22]. These NIR TA experiments were performed under steady-state actinic illumination of the thylakoids (i.e. with a variable fraction of the reaction centers open), requiring explicit comparison of $\Phi_{CT}$ with $\Phi_{qE}$. We estimated quite similar value of $\Phi_{CT}$ compared with $\Phi_{qE}$ [22], suggesting that a significant fraction of qE likely occurs though CT quenching [22].

We can extend the correlation shown by Holt et al. [27] of Z$^+$ absorption with magnitude of qE via the quantum yield approach. A series of A. thaliana phenotypes consisting of npq4 (no PsbS), npq1, wild-type, and PsbS overexpressors in order of increasing NPQ were used. To construct Fig. 6, we compared TA traces at 1000 nm, where Z$^+$ absorbance dominates the signal, with $\Phi_{qE}$ values obtained under the same photon flux (500 μE). The [Z$^+$] values were estimated by subtracting the dark-adapted TA intensity from the light-adapted value in thylakoid membranes using data from Holt et al. [27]. $\Phi_{qE}$ was estimated from Eq. (8) based on fluorescence measurements on leaves of A. thaliana. Error bars along x and y axes are standard errors using five points near maximum values at TA traces and more than three fluorescence trials, respectively. The dash-dot line is the best least-square fit. Fig. 6A shows an excellent linear relationship between the amount of CT signal and $\Phi_{qE}$. Assuming that the annihilation processes are the same for each A. thaliana phenotype, the normalized [Z$^+$] amplitudes on the x axis can be regarded as the quantum yield of CT quenching ($\Phi_{CT}$) in Eq. (14). Fig. 6A suggests a form of $\Phi_{qE} = m[Z^+]_0 + C$, where C is a Z independent correlation to qE. The fit in Fig. 6 gives $m = 0.81 \pm 0.04$ and $C = 0.07 \pm 0.013$. The small value of C suggests that Z$^+$ formation accounts for about 90% of qE. We would expect a slope of unity and the fact that we see 0.81 may simply arise because the TA signals are so small in the npq4 and npq1 phenotypes. Fig. 6B shows a similar plot for thylakoid membranes which exhibit a resonably linear relationship. The point for the npq1 mutant is significantly off the straight line. The npq1 mutant is reported to have a non-detectable amount of Z but still contains a similar amount of lutein to the wild-type. The enhanced qE of the npq1 mutant in thylakoid membranes is still under investigation, and most likely results from lutein-related quenching. The dash-dot line Fig. 6B is the least-square fit excluding npq1, which can be expressed as from of $\Phi_{qE} = m[Z^+]_0 + C$ with $m = 0.656 \pm 0.089$ and $C = 0.008 \pm 0.038$.

A linear relationship between NPQ and a relatively slow absorbance change in leaves (AΔA535) has been reported previously [15,38,52]. The AΔA535 has been attributed to a red-shift in the absorbance of 1 or 2 zeaxanthin molecules per PSII [53], possibly
reflecting the formation of the CT quenching species. Hence, the $\Delta A_{355}$, which is proportional to the concentration of quenchers, would scale linearly with NPQ but would not exhibit a linear relationship with $\Phi_{qE}$, as defined above. This result is entirely consistent with the analysis presented here.

Overall the successful combination of the yield of qE with TA data, suggests that use of quantum yields of quenching will provide important insights into the regulation of light harvesting and should become a standard approach.

5. Conclusions

We propose that partitioning of NPQ using quantum yield parameters (Eqs. (5)–(9)) will enable improved understanding of the natural photoprotection and regulation in intact leaves and thylakoid membranes where a variable fraction of the reaction centers remain open. In addition, the components of the quantum yield of NPQ are additive, which is the key benefit of this approach. The successful correlation of yield of qE with time-resolved spectroscopic data, suggested CT quenching involving Z$^+$ is the major component of qE.

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References


Fig. 6. (A) Plot of $\Phi_{qE}$ (leaves) versus $[Z^-]$ (each were calculated quantum yield of qE from Eq. (8) using PAM fluorescence of intact leaves and estimated $Z^-$ signal magnitude by subtracting dark-adapted TA intensity from light-adapted one in thylakoid membranes from Ref. [27], respectively). The dash-dot line is the best least-square fit giving $\Phi_{qE} = (0.805 \pm 0.035) \times [Z^-] - (0.067 \pm 0.013)$. (B) Plot of $\Phi_{qE}$ (thylakoids) versus $[Z^-]$ (each were estimated yield from Eq. (8) using PAM fluorescence of thylakoid membranes and the same $Z^-$ signal magnitude in Panel A, respectively). The dash-dot line is the least-square fit giving $\Phi_{qE} = (0.656 \pm 0.089) \times [Z^-] - (0.083 \pm 0.038)$ which excluded the point of npq1 phenotype (see the text).