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Corrections to current approaches used to calculate energy partitioning in photosystem 2

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Abstract

We analyzed several approaches dealing with the components of non-photochemical energy dissipation and introduced improved versions of the equations used to calculate this parameter. The usage of these formulae depends on the conditions of the sample (acclimation to dark or irradiation, presence or absence of the "actinic light"). The parameter known as "excess" cannot be used as a component of energy partitioning. In reality, this parameter reflects the differences between potential and actual quantum yields of photochemistry.

Additional key words: *Arabidopsis*; chlorophyll fluorescence; *Gossypium*; lincomycin; *Lycopersicon*; mutant; PsbS protein; thermal dissipation.

Introduction

Chlorophyll (Chl) fluorescence analysis is widely applied to study energy partitioning in photosystem (PS) 2 complexes. However, several different approaches using Chl fluorescence have been developed to investigate the fate of excitation energy in the leaf (reviewed by Roháček 2002 and Hendrickson *et al.* 2005, see also Kramer *et al.* 2004). The complexities of the formulae developed and the shortage of comparative studies may lead to confusion, undermining the practical application of these methodologies. However, estimations of the portions of excitation energy that enter different processes are valuable to understanding how different plant species utilize absorbed photon energy, especially when exposed to environmental stresses. Therefore, additional efforts are needed to analyze and compare the accuracy and practicality of the existing approaches.

The photon energy absorbed by PS2 can be used to drive photochemical reactions or it is dissipated as heat (non-photochemical dissipation) or fluorescence. Information about the proportion of absorbed energy entering photochemistry and non-photochemical dissipation is very important for studies aimed at better understanding the regulatory mechanisms that allow plants to deal with the photon energy they absorb. Regulatory changes in thermal dissipation are believed to be the main mechanisms protecting PS2 from photoinhibition (Müller *et al.* 2001). The extent to which energy enters each process depends on many factors. For example, the efficiency of thermal dissipation can vary depending upon the irradiance and the capacity to utilize the absorbed energy in photochemistry. Such variation is linked to the activity of the xanthophyll cycle and the phosphorylation of the light-harvesting pigment proteins of PS2, which are irradiance-dependent processes that are reversed in the dark (Horton *et al.* 1996). Therefore, as was stressed by Roháček (2002), the calculations of quantum yields of photochemistry and non-photochemical dissipation are different for leaves in the dark-acclimated and irradiance-acclimated states.

The quantum yield of photochemistry for irradiance-acclimated leaves is often measured using the parameter F_v'/F_m'. To determine F_v'/F_m', the minimal fluorescence of

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Abbreviations: Chl – chlorophyll; PS – photosystem; WT – wild type; $\Phi_{f,CON}$ – combined quantum efficiency (yield) of fluorescence and constitutive thermal dissipation; Φ_{NF} – quantum yield of thermal dissipation associated with the presence of non-functional PS2; Φ_P – actual quantum yield of photochemistry (electron transport) in PS2; Φ_{PS2} – potential quantum yield of photochemistry in PS2; Φ_{REG} – quantum yield of dark-reversible non-photochemical quenching of the excitation energy.
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irradiance-acclimated leaves, F_0', must be measured along with the maximal fluorescence level of an irradiance-acclimated sample, F_m' ($F_v' = F_m' - F_0'$). To measure F_0', researchers have routinely switched off the "actinic light" to reach the complete oxidation of Q_A pool. Thus, F_v'/F_m' really estimates the potential quantum yield of photochemistry in the irradiance-acclimated state (Φ_{PS2}) when the Q_A of all PS2 complexes is oxidized. Note that the sample is still considered as irradiance-acclimated, because it is assumed that several seconds of darkness (or far-red irradiation) used to determine F_0' will not induce any significant changes in the xanthophyll cycle or cause the transition from state 2 to state 1 (dephosphorylation of light-harvesting complex LHC2 and lateral movement of this complex from PS1 to PS2) (Schreiber *et al.* 1998).

Another well-known parameter, $1 - F_s/F_m'$ (F_s is the fluorescence signal of the sample irradiated with "actinic light") proposed by Genty *et al.* (1989) also estimates quantum yield of photochemistry in irradiance-acclimated leaves, but it is related to the situation when the Q_A pool is partially reduced (*i.e.* under irradiation). $1 - F_s/F_m'$ reflects the actual quantum yield of PS2 photochemistry denoted as Φ_P, whereas the potential quantum yield of PS2 photochemistry estimated as F_v'/F_m' is somewhat hypothetical, because there is no real photochemistry when the "actinic light" is off.

Despite the fact that most researchers agree on the interpretation of the fluorescence parameters employed to calculate potential and actual quantum yields of photochemistry, several self-excluding approaches for calculating quantum yields of non-photochemical quenching exist. The goal of this article is to carefully analyze those approaches and identify the appropriate way to estimate the quantum yields of non-photochemical quenching under various experimental conditions.

Materials and methods

Plants: Tomato plants [*Lycopersicon peruvianum* (L.) Mill., cv. Large Red Cherry] were grown in 3 000 cm^3 pots in a greenhouse at ~28/24 °C (day/night) with a natural photoperiod and were fertilized with Hoagland's solution once a week. The upper, fully expanded leaves of 4- to 5-week-old plants were used for the experiments.

Arabidopsis thaliana (L.) Heynh. (cv. Columbia and *npq4-1* mutants lacking PsbS protein) plants were grown in 500-cm^3 pots in a greenhouse under conditions similar to those for tomato plants. The youngest fully expanded leaves of 5- to 8-week-old plants were used for all analyses. In order to increase the level of PS2 photoinactivation, the leaves were treated with lincomycin, an inhibitor of PS2 repair, as described in Kornyeyev *et al.* (2004). The concentration of lincomycin in the bulk leaf tissue (C_I) was 0.8 to 1.9 mM as estimated from the formula: $C_I = C_S (M_S/M_L)$, where C_S is the inhibitor concentration in the solution, W_S is the mass of the solution taken up by a leaf, and M_L is the fresh mass of the leaf (Bilger and Björkman 1994).

Cotton plants (*Gossypium hirsutum* L. cv. Coker 312) were grown at the Texas Tech University experimental fields in Lubbock, TX (33.6°N, 101.9°W). The irrigated plot was established in mid-May of 2001. Measurements were made on the first fully expanded stem leaves (4th or 5th leaf from the top) of seedlings. The leaves were located on the south-facing side of each plant.

Fluorescence measurements: Chl *a* fluorescence emissions for *Arabidopsis* and tomato plants were measured in the laboratory with a pulse amplitude-modulated fluorometer (*PAM 101/103*, *H. Walz*, Effeltrich, Germany). The plants were kept in the greenhouse in the darkness overnight. Then the leaves were collected and placed in a temperature-controlled *Hansatech* oxygen electrode chamber (*Hansatech*, King's Lynn, Norfolk, UK) for 20 min prior to performing measurements at 25 °C. A flow of humidified air was used as the CO_2 supply. Measurements on leaf discs were conducted through a port in the chamber at various times during the treatment. The experimental protocol described by Schreiber *et al.* (1986) and nomenclature of van Kooten and Snel (1990) were employed for the fluorescence analysis. Prior to irradiation, the values of variable and maximal fluorescence were measured and denoted as F_{vM} and F_{mM}, respectively. During the irradiation, the levels of F_s, F_m', and F_0' (steady state, maximal, and minimal levels of Chl fluorescence for irradiance-acclimated samples, respectively) were recorded at different time periods after the start of irradiation. F_m' was recorded as the maximal level of fluorescence during the saturating flash and F_0' was measured as the level of fluorescence after the "actinic light" was temporarily switched off. Short-term far-red irradiation was applied to insure the oxidation of all Q_A during measurements of F_0'. Immediately after the last measurements of F_s, F_m', and F_0', leaf discs were collected using a cork borer to determine values of F_{vPI}/F_{mPI} after 3 h of dark incubation on wet *Whatman* paper in a Petri dish at room temperature. $F_{vPI} = F_{mPI} - F_{oPI}$, where F_{oPI} and F_{mPI} are minimal and maximal levels of Chl fluorescence measured for the dark-acclimated sample previously irradiated, respectively.

Field Chl fluorescence data on cotton were collected using a *FMS2* portable fluorometer (*Hansatech Instruments*, UK) following the protocol similar to the one described above. F_{vM} and F_{mM} for attached leaves were determined before sunrise (predawn). Later, F_s, F_m', and F_0' were determined at different times during the light period of the day. The leaves were kept at their natural angle during the fluorescence measurements. The magnitudes of photon flux density (PFD) and temperature

were monitored by means of the sensors located on the measuring clip of the fluorometer. The measurements of F_0' were conducted while the leaves were covered with a piece of opaque (black) cloth. In order to obtain the fluorescence parameters for dark-acclimated leaves previously irradiated, leaf discs were collected using a cork borer, placed on wet *Whatman* paper in a Petri dish and acclimated to darkness for 3 h prior to measurements of F_{oPI} and F_{mPI}.

Analysis of the equations used to calculate the contribution of photochemical and non-photochemical quenching to energy partitioning in PS2: As stated above, PS2 of irradiance-acclimated samples can be in two distinct states differing in the extent of Q_A reduction. Such a difference can be taken into account by introducing two rate constants: (*1*) the bimolecular rate constant for PS2 photochemistry (k_{PS2}) and (*2*) the (pseudo)monomolecular rate constant of photosynthesis (k_P), which reflects the actual photochemical utilization (Shinkarev and Govindjee 1993, see also Kitajima and Butler 1975). $k_P = k_{PS2}[Q_A]$, where $[Q_A]$ is the relative amount of PS2 complexes with Q_A in the oxidized state. According to the basic concept in photobiology, the quantum yield of a process is equal to the ratio of its rate constant to the sum of the rate constants of all participating processes. In terms of rate constants, the parameters F_v'/F_m' and $1 - F_s/F_m'$ can be expressed in the following ways:

$$F_v'/F_m' = \Phi_{PS2} = \frac{k_{PS2}}{k_{PS2} + k_{NP}} \quad (1)$$

$$1-F_s/F_m' = \Phi_P = \frac{k_P}{k_P + k_{NP}} \quad (2)$$

where k_{NP} is a combined rate constant of all non-photochemical processes, including fluorescence. Note that k_P was used in the equation for Φ_P, while k_{PS2} was used in the equation for Φ_{PS2}. An understanding of the distinction between the two irradiation-acclimated states of the sample (in the absence and in the presence of "actinic light") is critical for the correct interpretation of the parameters applied to calculate energy partitioning in PS2.

The very important implication of the differences between Φ_{PS2} and Φ_P is that the portion of absorbed photon energy dissipated as heat derived by Demmig-Adams *et al.* (1996), the parameter D, cannot be used to describe the quantum yield of non-photochemical dissipation under irradiation, since $D = 1 - F_v'/F_m' = 1 - \Phi_{PS2}$. Kramer *et al.* (2004) noted this problem and proposed an additional coefficient (F_s/F_0') in order to calculate non-photochemical energy dissipation (Φ_{NP}) when $[Q_A]<1$. In terms of fluorescence levels Φ_{NP} can be expressed as the following:

$$\Phi_{NP} = (1 - F_v'/F_m')\frac{F_s}{F_0'} = F_s/F_m' = \frac{k_{NP}}{k_P + k_{NP}} \quad (3)$$

Kramer *et al.* (2004) used this coefficient in a slightly different way: $\Phi_{NP} = 1 - \frac{F_v'}{F_m'}\frac{F_s}{F_0'}$ ($D = 1 - \frac{F_m' - F_0}{F_m'}\frac{F_s}{F_0'}$, in the original). Eq. 3 gives the correct ratio of the rate constants corresponding to the definition of the quantum yield of non-photochemical quenching for irradiated, irradiance-acclimated samples (with k_P in the denominator instead of k_{PS2}). Unfortunately, Kramer *et al.* (2004) did not mention that, since D could be applied only when $[Q_A] = 1$, then the usage of the parameter "excess" as a component of energy partitioning would be inappropriate. This parameter results from the use of D and Φ_P in the same balance equation describing the energy partitioning in PS2 complexes. There is no extra component, such as "excess", when Φ_{NP} from Eq. 3 is applied instead of D:

$$\Phi_P + \Phi_{NP} = 1 - \frac{F_s}{F_m'} + (1 - F_v'/F_m')\frac{F_s}{F_0'} = 1 \quad (4)$$

Because a number of researchers still use the term "excess" (reviewed in Kornyeyev *et al.* 2003), it is critical to the study of energy partitioning that this conclusion be understood and not overlooked. In our opinion, "excess" does not represent a real component of energy partitioning but rather the difference between potential and actual quantum yields of photochemistry:

$$\text{Excess} = 1 - D - \Phi_P = 1 - (1 - \Phi_{PS2}) - \Phi_P = \Phi_{PS2} - \Phi_P \quad (5)$$

The new interpretation of "excess" proposed here does not prohibit the application of the parameter as a measure of the susceptibility of PS2 to photoinhibitory irradiation as was investigated by Kato *et al.* (2003), Kornyeyev *et al.* (2003), Tsonev and Hikosaka (2003), and Hendrickson *et al.* (2005). However, it does take "excess" out of the balance equation. Also, it does mean that the approaches to studying energy partitioning that include "excess" in the balance equation should be revised. Moreover, according to Hakala *et al.* (2005) the correlation between "excess" and the level of photoinhibition is not observed under certain conditions, *i.e.* treatment with DL-glyceraldehyde and methyl viologen (see also Kornyeyev *et al.* 2004).

As mentioned above, non-photochemical quenching includes several components (Horton *et al.* 1996). The ability to distinguish between those components in physiological experiments is the key to understanding the regulatory mechanisms controlled by different processes participating in neutralizing the extra excitation energy absorbed by PS2 antennae. The traditional way to calculate these components based on the analysis of the quenching coefficients (Quick and Stitt 1989, Walters and Horton 1991, Lichtenthaler and Burkart 1999) is

greatly affected by the history of the sample (see Maxwell and Johnson 2000) and can not be easily implemented under conditions when the fibre optics can be moved in relation to the sample, for instance in field experiments. Therefore, the usage of energy partitioning, *i.e.* calculation of quantum yields for different components of non-photochemical quenching in PS2 might be an attractive alternative. Below, we analyze approaches to PS2 energy partitioning that consider the complex nature of non-photochemical quenching.

Here we divide the combined rate constant of all non-photochemical processes (k_{NP}) into several basic components, namely, fluorescence (rate constant k_F), constitutive thermal dissipation (k_{CON}), regulated, dark-reversible non-photochemical dissipation (k_{REG}), and thermal dissipation associated with PS2 photo-inactivation (k_{NF}). The mechanisms controlling those components are discussed elsewhere (Walters and Horton *et al.* 1993, Müller *et al.* 2001). The rate constant k_{NP} is equal to the sum $k_F+k_{CON}+k_{REG}+k_{NF}$. Also, it is important to remember that the rate constants are applied to the pool of PS2 complexes providing the fluorescence signal and not subpopulations of them. More detailed descriptions of this model are provided in Kornyeyev and Hendrickson (2007). Such a model originates from the matrix model by Kitajima and Butler (1975) and differs from it only in the splitting of non-photochemical dissipation into several components.

One of the common ways to check the fidelity of the equations used to estimate the contribution of different processes is to verify if such equations produce the correct ratio of the rate constants. When we analyzed the equation used by Hikosaka *et al.* (2004) and Hendrickson *et al.* (2005) for estimating the quantum yield of the photo-inactivation component of non-photochemical dissipation, Φ_{NF}, using the ratios of rate constants, it became clear that this equation reflected the value of Φ_{NF} in the dark-acclimated sample previously subjected to a photo-inactivating treatment:

$$\Phi_{NF} = 1 - \frac{F_{vPI}/F_{mPI}}{F_{vM}/F_{mM}} = \frac{k_{NF}}{k_{PS2}+k_F+k_{CON}+k_{NF}} \quad (6)$$

F_{vM}/F_{mM} is the ratio of variable to maximal fluorescence measured for a dark-acclimated sample before any photo-inactivation treatment. In terms of rate constants, F_{vM}/F_{mM} corresponds to $\frac{k_{PS2}}{k_{PS2}+k_F+k_{CON}}$, because $k_{REG} = 0$ (the regulatory component of non-photochemical quenching is relaxed in the dark) and $k_{NF} = 0$ (no photoinhibitory treatment was applied). F_{vPI}/F_{mPI} is measured for a dark-acclimated sample previously irradiated. Therefore, $k_{NF}>0$, but $k_{REG} = 0$ (the sample was acclimated to darkness after the irradiation) and F_{vPI}/F_{mPI} corresponds to the following ratio of the rate constants $\frac{k_{PS2}}{k_{PS2}+k_F+k_{CON}+k_{NF}}$. The equations contain the rate constant k_{PS2} instead of k_P, because F_{oPI}, the minimal Chl fluorescence level used to calculate variable fluorescence ($F_{vPI} = F_{mPI} - F_{oPI}$), was measured in the absence of the "actinic light" when $[Q_A] = 1$. The absence of k_{REG} in the denominator of the ratio of rate constants from Eq. 6 means that the formula describes Φ_{NF} for the dark-acclimated state of the sample, when the regulatory component is relaxed ($k_{REG} = 0$).

The introduction of the coefficient $\frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}$ to Eq. 6 meant to account for the acclimation to irradiance (see Kornyeyev *et al.* 2001) gives the following ratio of rate constants:

$$\Phi_{NF} = \left(1 - \frac{F_{vPI}/F_{mPI}}{F_{vM}/F_{mM}}\right)\left(\frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}\right) = \frac{k_{NF}}{k_{PS2}+k_F+k_{CON}+k_{NF}+k_{REG}} \quad (7)$$

The improved formula describes the situation in irradiance-acclimated samples ($k_{REG}>0$). However, there is still k_{PS2} instead of k_P in the ratio of rate constants, suggesting that the formula estimates the potential quantum yield of photo-inactivation dissipation when the "actinic light" is off and $[Q_A] = 1$. To correct this formula, we propose to add yet another coefficient, F_s/F_0', which was initially used for the parameter D (see Eq. 3):

$$\Phi_{NF} = \left(1 - \frac{F_{vPI}/F_{mPI}}{F_{vM}/F_{mM}}\right)\left(\frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}\right)\frac{F_s}{F_0'} = \frac{k_{NF}}{k_P+k_F+k_{CON}+k_{NF}+k_{REG}} \quad (8)$$

Eq. 8 reflects the value of Φ_{NF} under irradiation when the Q_A pool is partially reduced (k_P instead of k_{PS2} in the denominator). Finally, we have a set of equations describing Φ_{NF} for several experimental conditions, namely, the dark-acclimated sample in the absence of "actinic light" (Eq. 6), the irradiance-acclimated sample in the absence of "actinic light" (Eq. 7), and the irradiance-acclimated sample in the presence of actinic irradiation (Eq. 8). It is easy to see that among those equations only Eq. 8 will provide the actual value of Φ_{NF}.

One may note that if Φ_{NP} describes all non-photochemical processes combined, then the equation for Φ_{NP} can be easily obtained from Eq. 2. This would make Eq. 3 redundant. However, we included it in order to show that the coefficient F_s/F_0' works for correcting D and it might help to do an analogous job with Φ_{NF} and Φ_{REG}.

Similarly, the formula for Φ_{REG} previously proposed in Kornyeyev *et al.* (2001) (see Eq. 9) can be modified:

$$\Phi_{REG} = 1 - \frac{F_v'/F_m'}{F_{vPI}/F_{mPI}} = \frac{k_{REG}}{k_{PS2} + k_F + k_{CON} + k_{NF} + k_{REG}} \quad (9)$$

for an irradiance-acclimated sample right after the "actinic light" is switched off ($[Q_A] = 1$).

$$\Phi_{REG} = \left(1 - \frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}\right)\frac{F_s}{F_0'} = \left(1 - \frac{F_v' F_{mPI}}{F_{vPI} F_m'}\right)\frac{F_s}{F_0'} = \frac{k_{REG}}{k_P + k_F + k_{CON} + k_{NF} + k_{REG}} \quad (10)$$

for an irradiance-acclimated sample under irradiation ($[Q_A]<1$).

The combined quantum efficiency (yield) of fluorescence and constitutive thermal dissipation ($\Phi_{f,CON}$) can be estimated from the following equation:

$$\Phi_{f,CON} = 1 - \Phi_P - \Phi_{REG} - \Phi_{NF} = \frac{F_s}{F_m'} + \left(\frac{F_v'/F_m'}{F_{vM}/F_{mM}}\right)\frac{F_s}{F_0'} - \frac{F_s}{F_0'} \quad (11)$$

Results

The pie diagrams reflecting the contribution of different routes to energy partitioning in PS2 complexes of tomato leaves at the end of a 2-h irradiation (1 500 μmol m^{-2} s^{-1}) at 25 °C are shown in Fig. 1. The calculation was done for irradiance-acclimated samples in the absence (Fig. 1*A*) and in the presence (Fig. 1*B*) of irradiation. According to these data, the largest portion of the excitation energy is being dissipated through the regulatory component of non-photochemical dissipation. Despite strong irradiance (1 500 μmol m^{-2} s^{-1}), the effect of photo-inactivated PS2 complexes is minor. This can be explained by the low level of photo-inactivation due to PS2 repair occurring during the treatment (F_{vM}/F_{mM} decreased from 0.80±0.01 to F_v/F_m = 0.75±0.01 after 2 h of irradiation plus 3 h of relaxation in the darkness in room temperature) and the strong influence of other competitive processes (electron transport and zeaxanthin-dependent energy quenching).

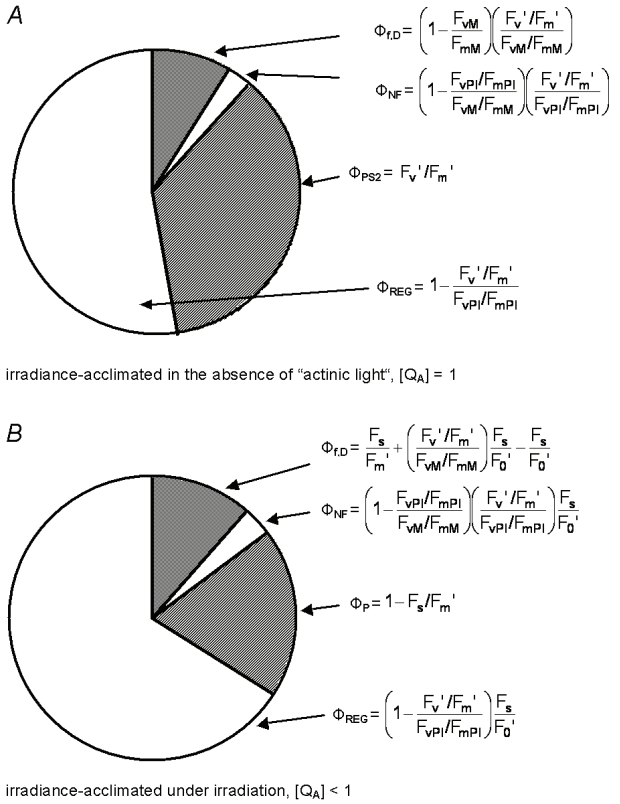

Fig. 1. The contribution of different processes (routes) to energy utilization/dissipation in PS2 complexes in tomato leaves after a 2-h exposure to irradiance (1 500 μmol m^{-2} s^{-1}) at 25 °C. The components of energy partitioning were calculated for the samples with an oxidized pool of Q_A immediately after the "actinic light" was switched off (*A*) and for irradiated samples, in which the Q_A pool was partially reduced (*B*). The data from three independent experiments were combined to calculate averaged values of the parameters.

One can notice that the parameter Φ_{REG} calculated for an irradiated sample contains a component associated with the reduction of Q_A. Therefore, in research focused on non-photochemical energy dissipation and its mechanisms, calculation of Φ_{REG} or Φ_{NF} for irradiance-acclimated samples in the absence of "actinic light" when the pool of Q_A is oxidized (see Fig. 1*A*) might be more appropriate (Kornyeyev *et al.* 2006). From this perspective, the use of F_v'/F_m' or D as indicators of changes in the regulation of non-photochemical dissipation may be valid. Numerous studies have shown that it is effective in describing total energy dissipation (Logan *et al.* 2007). However, the direct comparison of values of D with Φ_P to assess the extent to which each component contributes to energy utilization would be problematic.

We used several approaches that satisfy the rate constant criteria to calculate Φ_{NP}, Φ_{REG}, and Φ_{NF} in the experiment with tomato leaves described above. The calculations produced similar, although not identical, numbers (Table 1). Possible explanations for those minor discrepancies will be discussed below.

As an additional test of our approach to energy partitioning analysis, we calculated values of Φ_{REG} and Φ_{NF} for wild-type (WT) *Arabidopsis* plants and *npq4-1* mutants lacking psbS protein after 20 and 40 min of high irradiance exposure. These well-studied mutants exhibit impaired development of non-photochemical

Table 1. Quantum yields of non-photochemical components calculated using several approaches. The abbreviations used in the table correspond to the definitions made in the text and are different from those used in the original publications cited. NPQ = $(F_{mM} - F_m')/F_m'$, qL = $\frac{F_0'}{F_s}\frac{F_m'-F_s}{F_m'-F_0'}$ (see Kramer *et al.* 2004 for more details). *The approach proposed by Cailly *et al.* (1994) was also incorporated in more recent models (Laisk *et al.* 1997, Hendrickson *et al.* 2004, Kornyeyev and Hendrickson 2007).

Parameter	Formula	Results	Reference
Φ_N	$1-\Phi_P = F_s/F_m'$	0.808±0.043	Hendrickson *et al.* (2004)
$\Phi_{REG}+\Phi_{NF}$	$1-\Phi_P-\left(\frac{1}{NPQ+1+qL\,(\frac{F_{mM}}{F_{0M}}-1)}\right)$	0.528±0.066	Kramer *et al.* (2004)
	$\frac{F_s}{F_m'}-\frac{F_s}{F_{mM}}$	0.565±0.057	Cailly *et al.* (1996)*
Φ_{REG}	$\frac{F_s}{F_m'}-\frac{F_s}{F_{mPI}}$	0.523±0.060	Kornyeyev and Hendrickson (2007)
	$\left(1-\frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}\right)\frac{F_s}{F_0'}$	0.660±0.072	see Eq. 10
Φ_{NF}	$\frac{F_s}{F_{mPI}}-\frac{F_s}{F_{mM}}$	0.042±0.008	Kornyeyev and Hendrickson (2007)
	$\left(1-\frac{F_{vPI}/F_{mPI}}{F_{vM}/F_{mM}}\right)\left(\frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}\right)\frac{F_s}{F_0'}$	0.035±0.011	see Eq. 8
	$\left(1-\frac{F_{vPI}/F_{mPI}}{0.8}\right)\left(\frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}\right)\frac{F_s}{F_0'}$	0.035±0.011	see Eq. 14

fluorescence quenching (Li *et al.* 2000). Therefore, the comparison of the Φ_{REG} values obtained for the mutant and WT may help to confirm that the equation used to calculate this parameter is sensitive to modifications in the efficiency of the regulatory component of non-photochemical dissipation in PS2 complexes. Considering that the biggest differences between the genotypes were expected to occur at the beginning of irradiation when non-photochemical quenching was developing, we used lincomycin, an inhibitor of PS2 repair, to obtain noticeable changes in the extent of PS2 inactivation during relatively short exposure times. In accordance with published data, the mutants had lower Φ_{REG} in comparison to WT (Fig. 2*A*). At the same time, the WT plants (cv. Columbia) had less PS2 photo-inactivation and significantly lower values of Φ_{NF} than *npq4-1* mutants.

The field data obtained for cotton plants are presented in Fig. 3. The irradiance and leaf temperature (Fig. 3*A*) displayed significant diurnal variations, making obvious the need for efficient dynamic management of energy entering the photosynthetic apparatus. Using equations described above, we estimated the portions of excitation energy directed to non-photochemical quenching and its components. In accordance with abundant data in the literature, minimal levels of F_v/F_m were observed in the middle of the day (data not shown), when irradiance was maximal. During the same time period, the contribution of "down-regulation" and photo-inactivation of PS2 complexes changed in opposite directions while overall non-photochemical dissipation remained relatively stable (Fig. 3).

Discussion

A comparison of the pie diagrams (Fig. 1) suggests that the reduction state of Q_A has a noticeable effect on the distribution of the excitation energy in PS2. Also, the changes in the portion of the energy that is controlled by one component will lead inevitably to changes in the contribution of other processes. Unlike the pie diagrams published in Demmig-Adams *et al.* (1996), the diagrams in Fig. 1 not only divide total non-photochemical energy quenching into its components, but, more importantly, they distinguish between two different reduction states of Q_A in the irradiation-acclimated sample. In Demmig-Adams *et al.* (1996), the parameter $D = 1 - F_v'/F_m'$, which

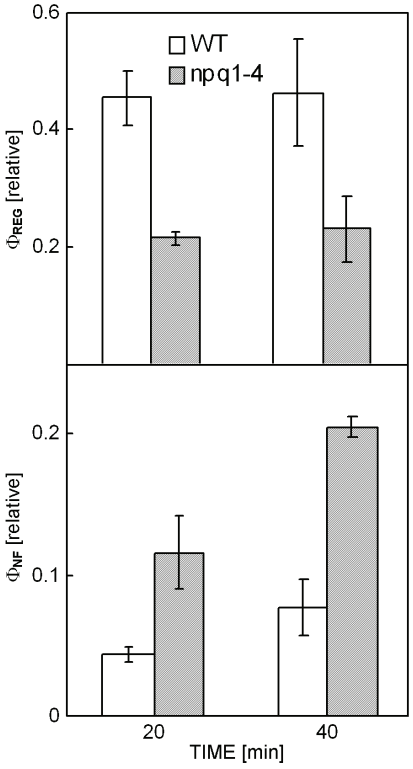

Fig. 2. The values of Φ_{REG} and Φ_{NF} calculated for *Arabidopsis* leaves of different genotypes (WT – wild type Columbia, npq1-4 – mutants lacking PsbS protein) after 20 and 40 min at a photon flux density of 1 000 μmol m^{-2} s^{-1} and 25 °C. The leaves were previously treated with lincomycin. Means±SD, n = 4–5.

estimates the quantum yield of non-photochemical quenching in PS2 in the absence of irradiation ($[Q_A] = 1$), was placed in the same diagram with the parameter $P = (F_m' - F_s)/F_m'$ describing the energy partitioning to electron transport in an irradiated sample ($[Q_A]<1$). It resulted in the generation of the parameter "excess", which, as concluded above, cannot be used in the balance equation. This is an additional reason why the usage of previously published equations for the de-convolution of the non-photochemical dissipation in PS2 complexes (Kornyeyev *et al.* 2001, Hikosaka *et al.* 2004) should be reconsidered, because they were proposed on the assumption that "excess" is a part of energy partitioning.

Recently, several new equations to estimate Φ_{REG} and Φ_{NF} were developed (Kornyeyev and Hendrikson 2007):

$$\Phi_{REG} = \frac{F_s}{F_m'} - \frac{F_s}{F_{mPI}} \quad (12)$$

$$\Phi_{NF} = \frac{F_s}{F_{mPI}} - \frac{F_s}{F_{mM}} \quad (13)$$

Different nomenclature was used in the article cited above. This is why Eqs. 12 and 13 were modified from their original forms according to the nomenclature used in the current paper. These two equations represent more detailed energy partitioning within the approach based on the calculation of ratios between the level of fluorescence under "actinic light" and the maximal levels of fluorescence reached upon application of a saturating flash under different conditions (see Laisk *et al.* 1997 for formulae estimating overall non-photochemical quenching). Eqs. 12 and 13 satisfy the rate constant criteria, as well as Eqs. 8 and 10. However, despite the simplicity of these formulae, it is difficult to implement this approach in field trials, because for correct measurements of Φ_{REG} and Φ_{NF} a stable position of the fibre optics should be maintained throughout the entire experiment. In addition, the measurement of the correct value of F_m is a challenge,

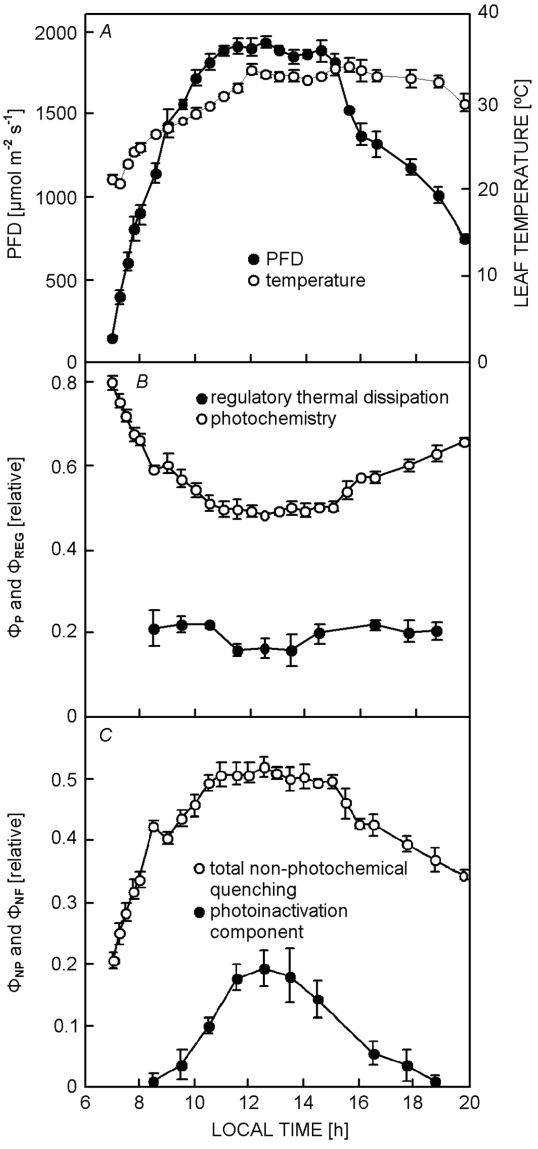

Fig. 3. Diurnal changes in leaf temperature and photon flux density (PFD) (*A*) and the partitioning of photon energy (*B* and *C*) for cotton leaves in the field. Means±SD, n = 5. See Materials and methods for details of experimental procedure.

since the sample should not have any PS2 damage or sustained down-regulation of PS2 activity from any previous environmental stress (Logan *et al.* 2007). The same problems can occur when the contributions of regulatory and photoinhibitory components are analyzed using the kinetics of dark-relaxation of non-photochemical quenching. An approach based on the comparison of F_v/F_m values (Eqs. 8 and 10) is free of the disadvantages described above. According to Eq. 10, Φ_{REG} can be estimated without knowledge of the initial state of the sample (F_{oM} and F_{mM} values before irradiation). Moreover, a maximum value of F_v/F_m known for a particular species could be used as F_{vM}/F_{mM} even without actual measurements. This makes possible the estimation of Φ_{NF} for a sample with unknown history using the following equation:

$$\Phi_{NF} = \left(1 - \frac{F_{vPI}/F_{mPI}}{0.8}\right)\left(\frac{F_v'/F_m'}{F_{vPI}/F_{mPI}}\right)\frac{F_s}{F_0'} \qquad (14)$$

The values of F_{vM}/F_{mM} between 0.80 and 0.85 are reported for most plants. Thus, Φ_{REG} may be estimated without the measurement of F_{vM}/F_{mM} prior to the photoinhibitory treatment. The advantage of this simplified version of Eq. 8 could be of substantial practical importance. The actual measurements of F_{vM}/F_{mM} can be used to generate an average level of the parameter for given plants and growing conditions. This would make such assessment more reliable. In our experiments with tomato leaves the average F_{vM}/F_{mM} was 0.8. This is why we used this value for calculating Φ_{NF} for Table 1.

Comparison of the results obtained when Φ_{REG} and Φ_{NF} were calculated using the different approaches mentioned above implies that they can produce similar results (Table 1). Some minor discrepancies may be explained by listing the sources of errors for both approaches. In the case of the approach based on the changes in the value of F_m (Eqs. 12 and 13), the potential sources of the errors are Chl bleaching during irradiation, movement of chloroplasts, incomplete relaxation of regulatory non-photochemical dissipation at the time when F_{mPI} is measured, and possible movements of the sample during the experiment. The fidelity of the results obtained using the approach based on the comparison of F_v/F_m values greatly depends on correct measurements of F_0 and F_0' and complete relaxation of regulatory non-photochemical dissipation during dark acclimation. There is a possibility to calculate the values F_0' avoiding direct measurements (Oxborough and Baker 1997). Future extensive studies may reveal the extent of the correlation between the results under various environmental conditions. The present study was mainly focused on the theory behind the equations in order to identify those formulae that would satisfy the rate constant criteria and to suggest corrections to the approaches to calculation of energy partitioning in PS2.

The data on *Arabidopsis* mutants with impaired non-photochemical quenching (Fig. 2) and the field data obtained on cotton plants (Fig. 3) serve as illustrations of how de-convolution of non-photochemical quenching in PS2 can help understand the regulation of energy partitioning under photo-inactivation conditions. The most important observation is that stable levels of Φ_P in the middle of the irradiation period in the field correspond to stable values of total (combined) irradiation-induced non-photochemical dissipation (Φ_{NP}), whereas the individual contributions of Φ_{REG} and Φ_{NF} vary noticeably during this period. Similar observations were reported earlier for other field experiments where the values of Φ_{REG} and Φ_{NF} were calculated for irradiance-acclimated samples in the absence of "actinic light" using Eqs. 7 and 9 (Kornyeyev *et al.* 2005). The implications of such results are discussed in detail in Kornyeyev *et al.* (2006) and Kornyeyev and Hendrickson (2007).

In conclusion, it is important to identify the conditions of the sample to adequately apply the various formulae for the calculation of the energy partitioning in PS2 complexes. Some formulae previously suggested to describe quantum yields of non-photochemical dissipation under irradiation should be applied to another situation, *i.e.* when "actinic light" is off and $[Q_A] = 1$ instead of $[Q_A]<1$. Corrected equations proposed in this report can simplify the procedure of the estimation of the contribution of different components of non-photochemical dissipation of absorbed photon energy in field trials.

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