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Assessing the photoprotective effectiveness of non-photochemical chlorophyll fluorescence quenching: A new approach

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ABSTRACT

The photoprotective nature of non-photochemical quenching (NPQ) has not been effectively quantified and the major reason is the inability to quantitatively separate NPQ that acts directly to prevent photoinhibition of photosystem II (PSII). Here we describe a technique in which we use the values of the PSII yield and qP measured in the dark following illumination. We expressed the quantum yield of PSII (Φ_{PSII}) via NPQ as: $\Phi_{PSII} = qP \times (Fv/Fo)/(1 + Fv/Fo + NPQ)$. We then tested this theoretical relationship using *Arabidopsis thaliana* plants that had been exposed to gradually increasing irradiance. The values of qP in the dark immediately after the illumination period (here denoted qPd) were determined using a previously described technique for Fo′ calculation: $Fo'_{calc.} = 1/(1/Fo - 1/Fm - 1/Fm')$. We found that in every case the actual Φ_{PSII} deviated from theoretical values at the same point that qPd deviated from a value of 1.0. In an increasing series of irradiance levels, WT leaves tolerated 1000 μmol m^{-2} s^{-1} of light before qP_d declined. Leaves treated with the uncoupler nigericin, leaves of the mutant lacking PsbS protein and leaves overexpressing PsbS showed a qP_d reduction at 100, 600 and 2000 μmol m^{-2} s^{-1} respectively, each at an increasing value of NPQ. Therefore we suggest that this simple and timely technique will be instrumental for identifying photoprotective NPQ (pNPQ) and that it is more appropriate than the qE component. Its applications should be broad: for example it will be useful in physiology-based studies to define the optimal level of nonphotochemical quenching for plant protection and productivity.

1. Introduction

The regulation of light harvesting in order to protect the photosystem II against photodamage, i.e. permanent /long term closure of reaction centers is a major process that manifests itself in the non-photochemical chlorophyll fluorescence quenching, NPQ. The basic properties and location are now well identified; however, the mechanism remains a subject of debate. Apart from this, another feature of NPQ has not been adequately investigated — quantification of the photoprotective effectiveness of NPQ. Some in vitro reports even proposed that NPQ plays a little or no role in photoprotection of PSII [1]. However, the common opinion on the subject is that the rapidly reversible NPQ component, qE, is necessary for photoprotection. However, the nature of remaining slowly reversible NPQ is highly heterogeneous and can be hard to resolve [2]. It is believed that zeaxanthin, trapped protons, aggregated LHCII and photodamage itself are the contributors to this component, often referred to as qI [3–6]. It seems that the temporal criterion for distinguishing photoprotective from photoinhibitory components of NPQ is often ambiguous and therefore insufficient and other independent approaches are needed to verify the amount of protective NPQ. New methods are required to define the photoprotective component(s) of NPQ. The process is clearly an effective adaptation to excessive light but the common occurrence of photoinhibition in nature shows that it may be limited in its protective power under some conditions. This has meant that its role in determining plant productivity remains theoretical and un-quantified.

Common measurements for photoinhibition include dark-adapted Fv/Fm, O_2 evolution or D1 degradation. Whilst these have been effective for assessing the threshold for damage these methods have drawbacks for physiological analyses especially where lab-based biochemical analysis is required (O_2 evolution and D1 turnover). In addition they require disruption of the light treatment, either by destructive sampling or imposition of a sustained dark period. The length of the dark period used for Fv/Fm measurements itself can lead to ambiguity.

The required approach to this problem is a simple, rapid and non-disrupting method that could test the in vivo photoprotective function of NPQ regardless how quickly or slowly it recovers. Currently this does not exist. Here we undertook an investigation on several types of plant material to develop precisely such a methodology that could radically change our understanding of the NPQ process by quantifying its

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photoprotective potential in addition to chlorophyll fluorescence induction analysis.

The technique we describe here will be essential to fully understand the trade-offs between the metabolic costs of photoinhibition and the reduction in quantum yield caused by engaging NPQ. Theoretical analyses conclude that unbalancing these trade-offs has the potential to substantially reduce plant productivity [7].

2. The approach and methods

NPQ as well as photoinhibition both diminish the quantum yield of PSII (Φ_{PSII}) [8–12]. How can one separate these effects and find out the true value of the photoprotective NPQ (here denoted pNPQ)? We can express Φ_{PSII} via NPQ using the rate constants of various dissipative processes affecting chlorophyll fluorescence. $\Phi_{PSII} = qP \times (\Phi m - \Phi o)/\Phi m$ [9], where $\Phi m = k_f/(k_f + k_d)$ and $\Phi o = k_f/(k_f + k_d + k_p)$, where k_f, k_d and k_p are the rate constants for fluorescence, internal conversion and photochemistry, respectively (for review see ref. [13]). After a simple algebraic transformation we obtain:

$$\Phi_{PSII} = qP\ x\ k_p/\left(k_f + k_d + k_p\right). \quad (1)$$

Yield at any point in the dark when NPQ is present can be expressed as:

$$\Phi_{PSII} = qP\ x\ k_p/\left(k_f + k_d + k_p + K_{NPQ}\right), \quad (2)$$

where K_{NPQ} is a nonphotochemical dissipation rate constant, that incorporates the effective quenching rate constant and concentration of the quencher.

Formula (2) can be treated as:

$$\Phi_{PSII} = qP\ x\left[k_p/\left(k_f + k_d\right)\right]/\left[\left(k_f + k_d + k_p + K_{NPQ}\right)/\left(k_f + k_d\right)\right], \quad (3)$$

resulting in

$$\Phi_{PSII} = qP\ x(Fv/Fo)/(1 + Fv/Fo + NPQ), \quad (4)$$

where $NPQ = k_{NPQ}/(k_f + k_d)$.

Taking $Fv/Fo = 1/(Fm/Fv - 1)$ the yield in the presence of NPQ in the dark will become

$$\Phi_{PSII} = qP\ x[1/(Fm/Fv{-}1)]/[1 + (1{-}1/(Fm/Fv{-}1) + NPQ]$$

or

$$\Phi_{PSII} = qP\ x(Fv/Fm)/[1 + (1{-}Fv/Fm)x\ NPQ]. \quad (5)$$

Hence, the PSII yield is a hyperbolic function of NPQ. At NPQ = 0, the yield is at maximum, $\Phi_{max} = Fv/Fm$, with all reaction centres open (preillumination condition, qP = 1). NPQ = 2 will result in a yield decrease from the average of 0.8 to ~0.57, whilst for NPQ = 4, the yield will decrease to ~0.44, etc. These considerations will hold only when qP = 1 in the dark, i.e. when photoinhibition is absent. In the case of photoinhibition taking place qP < 1. This permanent closure of photosystem II reaction centres undermines the yield and causes its deviation from the theoretical hyperbolic dependency upon NPQ.

$qP = (Fm' - F)/(Fm' - Fo')$, where Fm′, F and Fo′ are fluorescence levels at maximum, steady state illumination and dark in the presence of NPQ. In the dark, straight after turning off actinic light, F should in theory become Fo′ and qP = 1. However, since photoinhibition tends to elevate Fo′ masking the quenching effect of NPQ, F will be higher than the real Fo′. In order to estimate the true Fo′ value we can use the formula of Oxborough and Baker [14]:

$$Fo'_{calc.} = 1/(1/Fo{-}1/Fm{-}1/Fm'). \quad (6)$$

Hence, in the presence of photoinhibition the true value of qP in the dark (we will refer to it as qP_d) can be calculated using the measured dark fluorescence level (F = Fo′) and the true, calculated Fo′ magnitude ($Fo'_{calc.}$) in the following way:

$$qP_d = (Fm'{-}Fo')/(Fm'{-}Fo'_{calc.}). \quad (7)$$

The modern PAM fluorimeters, like PAM100 or Junior PAM (Walz) have this routine as standard in their software.

Arabidopsis thaliana plants were grown for 8–9 weeks in Sanyo plant growth rooms (http://sanyo-biomedical.com) with an 8-h photoperiod at a light intensity of 150 μmol photons $m^{-2}\ s^{-1}$ and a day/night temperature of 20 °C. For some experiments leaves were vacuum infiltrated with 20 mM HEPES buffer (pH 7.0) containing either 50 μM nigericin to inhibit ΔpH or 100 μM lincomycin to inhibit PSII reaction centre D1 protein synthesis while control leaves were vacuum infiltrated with only the buffer. Measurements were conducted using a Walz Junior PAM (Walz Effeltrich Germany) and monitoring leaf clip.

3. Results

Fig. 1 illustrates the method. The actinic light illumination routine was chosen as following: a leaf was illuminated with 6 phases of progressively increasing light intensities of 100, 300, 600, 1000, 2000 and 3500 μM $m^{-2}\ s^{-1}$ for 5, 5, 5, 5, 10 and 10 min respectively (for L17 plants additional light intensity of 5000 μM $m^{-2}\ s^{-1}$ was used for 20 min), to allow gradual adjustment of the photosynthetic membrane to light and formation of gradually rising levels of NPQ, enabling the maximum attainable photoprotection under these conditions. Hence, the total illumination time for most types of plant material was 40 min. This type of light treatment is more natural for the plant in comparison to a sudden illumination with one light intensity, particularly if it is at near-saturating level. The measurements for NPQ, yield and qP_d have been performed in the dark 3 s after switching of the actinic light at the end of each illumination phase using 2–3 consecutive saturated pulses. Each pulse was followed by ~7 second period of far red light illumination in the dark to aid the oxidation of Q_A via enhanced excitation of PSI leading to gradual oxidation of the whole electron transport chain (Fig. 1). This procedure also prevented significant recovery of NPQ

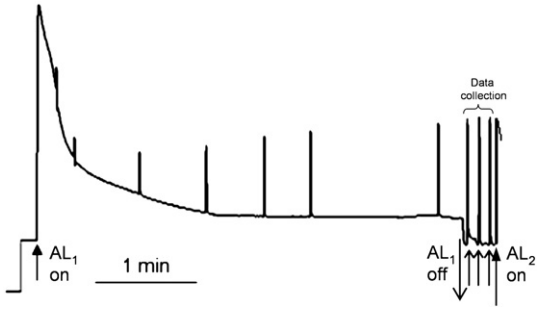

Fig. 1. Standard chlorophyll fluorescence induction trace during the first phase of actinic light illumination (the lowest light intensity AL_1 of 100 μM $m^{-2}\ s^{-1}$ for 5 min). Vertical open arrows indicate the probing saturated pulses applied in the dark on the background of far red light illumination. Fm′ and actual Fo′ levels have been registered and used for the calculations of Φ_{PSII}, qP and NPQ (see "The approach and methods" section). Promptly following the first illumination phase the second illumination phase was initiated by applying AL_2 actinic light of 300 μM $m^{-2}\ s^{-1}$ for 5 min followed by the brief period of darkness where the three probing saturation pulses were applied to gain the second set of Φ_{PSII}, qP and NPQ parameters. Total number of illumination phases was 6: AL_1 (100 μM $m^{-2}\ s^{-1}$ for 5 min), AL_2 (300 μM $m^{-2}\ s^{-1}$ for 5 min), AL_3 (600 μM $m^{-2}\ s^{-1}$ for 5 min), AL_4 (1000 μM $m^{-2}\ s^{-1}$ for 5 min), AL_5 (2000 μM $m^{-2}\ s^{-1}$ for 10 min) and AL_6, (2000 μM $m^{-2}\ s^{-1}$ for 10 min). In addition, an extra phase, AL_7 was used for PsbS overexpressor plants (L17), AL_3, (3500 μM $m^{-2}\ s^{-1}$ for 20 min).

(Fig. 1). The approach yielded satisfactory reproducible results. The statistical analysis was performed on three different leaves of the same age from different individual plants. Supplemental Figure S1 represents actinic light intensity dependency of NPQ, qP_d and Φ_{PSII} measured at the end of each illumination phase. The standard deviation did not exceed 15% of the average value of each parameter measured throughout all types of plants used for this work.

Plotting the yield, Φ_{PSII}, against the total NPQ (not qE) obtained in the course of the fluorescence induction measurement and comparing it to the theoretical relationship between the yield and NPQ (assuming qP = 1), described by formula (5), it is possible to find the point in time when photoinhibition onset takes place (Fig. 2). This point will clearly show up when the experimental data on this dependency deviates from the theoretical data. Additionally, qP estimated using the approach of Oxborough and Baker [14] can be plotted in parallel with the yield against NPQ in order to independently verify the moment of the onset of photoinhibition that was detected by deviation of the experimentally measured PSII yield from the theoretically calculated values and hence evaluate the photodamage.

Dependency of the measured yield upon NPQ is presented using round filled symbols, whilst the theoretical relationship is shown by the solid line and round open symbols. Fig. 2 shows that at the early stages of illumination, when NPQ was <2 there was very good agreement between experimental data and theoretical yield dependency upon NPQ. qP_d at these conditions was very close to 1. At the light intensity of 1000 μM m^{-2} s^{-1} the experimentally determined yield started to deviate from theoretical curve and qP_d started to decrease, both indicative of the onset of the inactivation of the PSII reaction centres resulting in their permanent closure. After division of the measured yield by qP_d all the measured yield points satisfactorily followed the theoretical relationship between yield and NPQ. Hence, the measurement routine and the presentation of the relationships between the yield, qP_d and NPQ enables establishment of the extent of photoprotective NPQ or pNPQ of the total NPQ. This method does not require measurement of qE or reversible NPQ, the criteria for which remain a subject of debate. It is assumed that the recovery time of 5–10 min is a good criterion for the photoprotective, energy dependent NPQ or qE. However, the work of recent years has explicitly demonstrated that there are much slower components in the photoprotective quenching and it is impossible to distinguish them this way from the true photoinhibitory quenching [15]. Hence, the qE term has proved to be difficult to accurately define. Therefore, our new approach seems to be more adequate for determination of the true amplitude of the photoprotective NPQ. At the same time, the approach shows how effectively NPQ protects reaction centres from the photodamage and at which maximum light intensity and plants can grow under and avoid PSII inactivation. Hence, a direct *functional*, not *temporal*, criterion is suggested here for definition of the photoprotective NPQ. It is important to note that qP_d dark recovery was found to be very slow (see Supplemental Figure S2), with the rate constant of the order of 0.008 min^{-1}, consistent with the long-term nature of the RCII damage requiring involvement of the repair cycle. Since the latter works at a much slower pace than the rate of light-triggered damage, the qP_d decline in our experiments was unaffected by the repair process when the actinic light was on as confirmed by the application of chloroplast protein biosynthesis inhibitor lincomycin (see Supplemental Figure S3).

Leaves infiltrated with the uncoupler nigericin displayed a completely different response. Fig. 3 shows the relationships between the yield, qP and NPQ. It is apparent that the photoinhibitory damage onset took place at relatively low light intensity and progressed dramatically reaching zero at NPQ levels of ~1.5. This NPQ is entirely due to photoinhibition, i.e. formation of permanently damaged PSII reaction centres that are apparently weaker quenchers than the protective NPQ. This experiment shows that the maximum contribution of qI type of quenching cannot be more than ~1.5. This level can only be reached when all PSII reaction centres are permanently closed — a rare event in quenching analysis practice. Hence, the contribution of qI quenching in NPQ seems to be relatively small in our measurements. Dividing the experimental yield points by qP resulted in generation of a dataset that began to follow closely the theoretical yield/NPQ curve, reflecting the photoinhibitory quenching (qI) effect upon the steady-state open PSII reaction centre yield, Fv/Fm′.

To explore the photoprotective potential in plants lacking one of the most discussed components of the NPQ process, PsbS protein, we applied our fluorescence analysis approach to the *npq4* mutant. Fig. 4 shows the relationship between the yield/qP and NPQ. Despite the reports that PsbS is essential to sustain qE, the quickly forming and reversible photoprotective NPQ component, the quenching generated in these plants showed photoprotective properties, that sustained a qP of 1 until the light intensity reaches ~600 μM m^{-2} s^{-1}. Unlike in

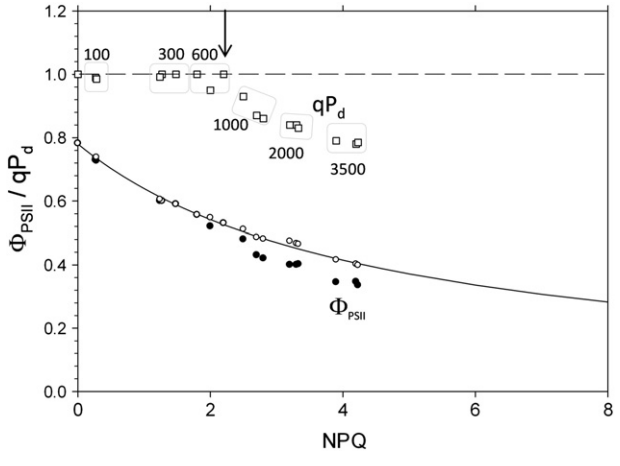

Fig. 2. Relationship between the photosystem II quantum efficiency, Φ_{PSII} and photochemical quenching measured in the dark, qP_d, and the nonphotochemical quenching parameter, NPQ, for the wild type *Arabidopsis* leaves. Vertical down arrow indicates the maximum amplitude of NPQ for the light intensity at which all reaction centres remain protected. Experimental Φ_{PSII} shown in closed circles, qP_d shown in open squares, Φ_{PSII}/ qP_d shown in open circles. The solid line is a theoretically predicted relationship between Φ_{PSII} and NPQ for conditions of open reaction centres in the dark (qP = 1, see formula (5)). Dashed line shows the level of qP = 1. Grey boxes enclose data points that correspond to the each illumination phase.

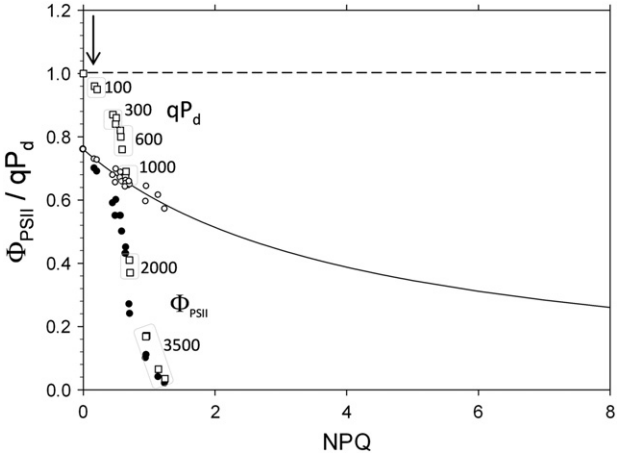

Fig. 3. Relationship between the photosystem II quantum efficiency, Φ_{PSII} and photochemical quenching measured in the dark, qP_d, and the nonphotochemical quenching parameter, NPQ, for the wild type *Arabidopsis* leaves infiltrated with nigericin. Vertical down arrow indicates the maximum amplitude of NPQ for the light intensity at which all reaction centres remain protected (close to 0 in this case). All other symbols and lines as in Fig. 2.

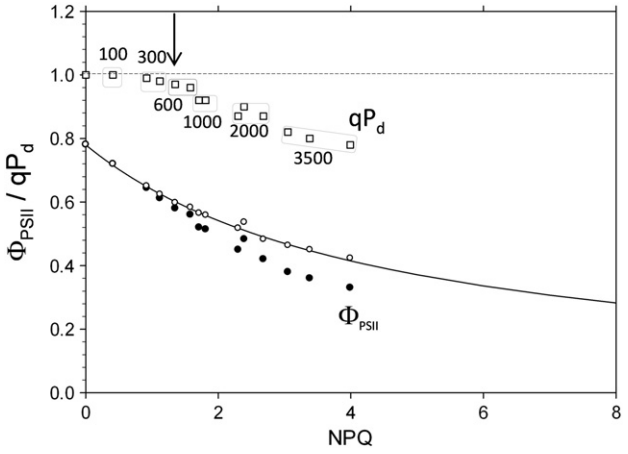

Fig. 4. Relationship between the photosystem II quantum efficiency, Φ_{PSII} and photochemical quenching measured in the dark, qP_d, and the nonphotochemical quenching parameter, NPQ, for the *Arabidopsis npq4* mutant leaves. All symbols and lines as in Fig. 2.

plants infiltrated with nigericin, NPQ reached much higher amplitude. The photodamage did occur, however it was much lower than in the nigericin-infiltrated plants and basically equal to that for the wild type (Fig. 2). The only difference between the mutant and the wild type plants was the slightly earlier onset of photodamage in the former. Therefore, we can conclude that the slowly forming NPQ in npq4 mutant is largely of a photoprotective nature and hence is similar to qE. The fact that it forms much slower than qE explains the early onset of photoinhibition in the mutant. However, once NPQ reaches the amplitude of the wild type it is protective, allowing the same extent of the photodamage as in the wild type even at the light intensity of 3500 μM m^{-2} s^{-1}. Our observations support the recent reports that NPQ in npq4 plants is of the same nature as qE and that the latter can be induced in these plants by the enhanced ΔpH [15,16]. It is likely that the light harvesting system itself can sense the lumenal protons but with a lower pK that PsbS itself [2] and PsbS plays a structural role accelerating the LHCII antenna reorganisation/aggregation in the NPQ state [17].

The role of elevated levels of PsbS in enabling the photoprotection can be learned from Fig. 5. L17 mutant plants possessing up to 5 times higher amounts of PsbS [18] have been used for the fluorescence analysis. It was possible to attain very large NPQ levels that were 100% protective even at the abnormally high light intensity of 3500 μM m^{-2} s^{-1}. No damage to the reaction centres was observed at NPQ up to 6. Such powerful quenching was previously thought to exist only in diatom algae [19]. The maximum NPQ level of 8 was observed at which only 20% of RCII's were damaged. Taking into account the data of Fig. 2 it is clear that the levels of photoinhibitory quenching qI corresponding to 20% damaged RCs were only about 0.3, leaving the 7.7 for pNPQ.

4. Discussion

The approach used here to assess the photoprotective effectiveness of NPQ enabled us to reveal that regardless of the presence of PsbS protein the relationship between the yield of open PSII reaction centres and levels of NPQ remains the same obeying formula (5). The latter involves NPQ as a process that affects the PSII yield in a manner that reveals no structural or mechanistic heterogeneity as previously proposed [20]. When the PSII yield (Φ_{PSII}) in addition to NPQ starts to be affected by the increasing permanent closure of reaction centres under certain levels of illumination, then the yield/NPQ relationship begins to deviate from formula (5). The yield simply becomes smaller than predicted. However, taking into account closed/damaged reaction centres by dividing Φ_{PSII} by qP (or dark qP) one can completely restore the relationship described by formula (5) (see Figs. 2–5). Hence, recording qP in the dark and the relationship between Φ_{PSII} vs NPQ one can obtain quantitative information about the effectiveness of NPQ under certain illumination conditions (intensity and duration). Setting up these conditions as a standard routine enables comparison of the effectiveness of photoprotection in different types of plants.

We found that the mutant devoid of PsbS protein, *npq4*, possessed significant levels of photoprotective NPQ albeit slowly forming and reversible. As a result of the slow response to the increase in the light intensity the mutant plants revealed more susceptibility to photoinhibition than the wild type. Whilst the onset of the damage to the reaction centres in *npq4* plants was observed under 300 μM m^{-2} s^{-1}, when NPQ was about 1.8 (Fig. 6). The wild type showed the first signs of damage only when the intensity reached 1000 μM m^{-2} s^{-1} and NPQ was about 2.3 (Fig. 6). However, when NPQ reached over 2 in the mutant the plants were well-protected under the highest light intensity applied as efficiently as the wild type (Fig. 4). Indeed, as in the wild type plants the qP_d reduction in the *npq4* mutant at the end of the illumination routine was around 0.8.

For plants infiltrated with the uncoupler nigericin, to inhibit qE the onset of photoinhibition had already occurred at the lowest applied

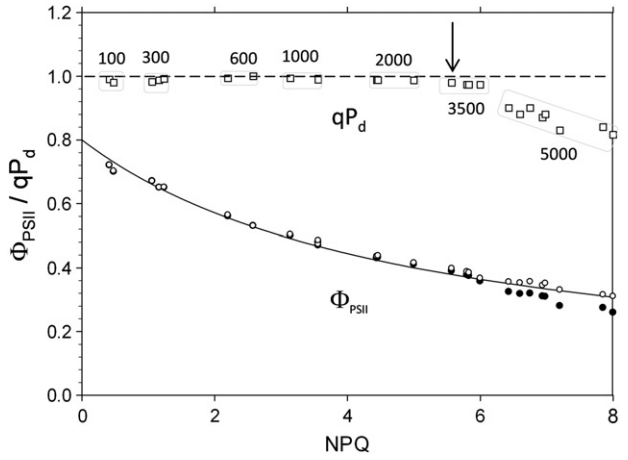

Fig. 5. Relationship between the photosystem II quantum efficiency, Φ_{PSII} and photochemical quenching measured in the dark, qP_d, and the nonphotochemical quenching parameter, NPQ, for the *Arabidopsis* PsbS overexpressor, L17, leaves. All symbols and lines as in Fig. 2.

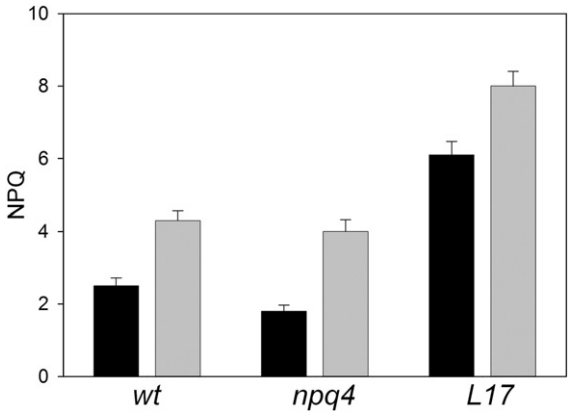

Fig. 6. Values of NPQ in Arabidopsis leaves of the wild type, lacking PsbS protein (*npq4*) and overexpressing PsbS (L17) plants. Black bars correspond to the maximum registered levels of NPQ at the conditions when $qP_d = 1$ (pNPQ). Grey bars correspond to the maximum levels of NPQ attained in the end of the actinic light illumination routine. Error bars are standard deviations from the mean of three independent measurements.

intensity of 100 μM m^{-2} s^{-1} (Fig. 3). This suggests that even small levels of pNPQ, that formed in a first 5 min of illumination (100 μM m^{-2} s^{-1}) were effective for photoprotection of reaction centres. Monitoring the relationship between Φ_{PSII}/qP and NPQ in this case enabled us to show that the quenching related to photoinhibition (qI) was of a photoprotective nature, since the yield of open reaction centres remained dependent upon the amount of quenching formed as a result of photoinhibition (qI). This observation is consistent with the recently proposed idea by Matsubara and Chow that the damaged PSII reaction centres protect the open ones from photoinhibition [21]. Other reports suggest, however, that the inhibited centres do not protect against loss of oxygen evolution [22]. The maximum level of qI when all reaction centres were damaged (Fig. 3) was found to be only ~1.5, meaning that the fraction of the quenching by the damaged reaction centres in NPQ is relatively small at these conditions. However, the level of qI quenching is likely to go up and become larger with continuing illumination. The previously published results on the amplitude of qI seem to suggest that this is the case. One can argue that nigericin in our experiment could have caused excessive reduction of PSII even at low light intensities due to the inhibition of the carbon assimilation reactions. Although, this remains a possibility, the use of far red light should have effectively caused the reoxidation as it does in the cases of a strong over-reduction in the dark at certain conditions caused by the cyclic electron transport around PSII etc. Moreover, one has to take into account that any measurements of irreversible NPQ would not enable distinction of the photoprotective from photoinhibitory parts, which was the aim of the current report. Our approach suggests that the measurements of qE underestimate the true downregulating potential of the protective component of NPQ with a part of it that becomes slowly reversible.

Plants with excess amounts of PsbS have a great potential to form NPQ that reached almost 8. All PSII reaction centres were found to be intact up to NPQ = 6 (Figs. 5 and 6). Hence this level of quenching is defined as photoprotective or pNPQ. The plants at these levels of NPQ were protected (for 10 min) against the light intensity almost twice higher than that of the brightest day in tropics (3500 μM m^{-2} s^{-1}). Naturally, the possibility exists that the longer duration of such high light could have caused some photoinhibitory damage. But the main point remains, that the proposed method has revealed that NPQ levels at which all RCII remain open in the dark are not related to photoinhibititory quenching. The Fv/Fm parameter commonly used to monitor the downregulation of PSII efficiency and related photoinhibition is rather ambiguous since the only criterion of photoinhibitory decline of the yield is time. After 15 min, when the fast NPQ component, qE, and the state transition component, qT, were recovered, the rest of NPQ and associated yield decline are regarded as monitoring photoinhibition. However, as we have demonstrated before [2,15] the presence of slowly-reversible protective NPQ components greatly undermines the use of Fv/Fm parameter in assessing the photoinhibitory damage to PSII. Both damage to the RCII and slow photoprotective components undermines the yield for a sustained period. However, whilst the photoinhibitory damage is costly to repair, the slow down regulation process is “free of charge”. Thorough field work is required to track and assess the consequences of the two processes for the plant growth, development and productivity. This assessment should include the method of monitoring of D1 protein levels as criterion of a physical photoinhibitory damage done by the onset of the photoinhibition.

The observations of large NPQ amounts in PsbS overexpressors indicate that the photoprotective quenching is much higher than previously observed on the basis of NPQ recovery, i.e. qE that revealed only a part of the protective potential in these plants. Therefore our approach is essential not only for estimation of the onset of photoinhibition and the effectiveness of pNPQ but also valuable for estimation of its true amplitude. In addition, the proposed method enables us to identify, which maximum light intensity different types of plants can tolerate (tolerated light intensity, TLI). This light intensity in our experiments was the highest actinic light intensity used at which the PSII yield still obeyed the theoretical relationship with NPQ and when qP_d was about 1, hence no damage to the reaction centres was observed. According to Figs. 2–6 TLI correlates well with amounts of pNPQ. Whilst for the wild type plants TLI was 600 μM m^{-2} s^{-1} and pNPQ of ~2.3, for the PsbS overexpressors TLI was at least 2000 μM m^{-2} s^{-1} at pNPQ of about 6.1.

Tracking the relationship between the yield and qP vs NPQ is central to the proposed methodology. Both approaches, one based on the comparison of theoretical and experimental yield/NPQ relationship and the other based on theoretical evaluation of the true Fo′ [14] yielded very consistent results. These results enabled evaluation of the true photoprotective component of NPQ, pNPQ. Naturally, this approach is based on commonly used actinic light illumination routines and a standard range of light intensities and durations. In future, it would be important to apply different illumination routines in order to find the durability of pNPQ and its dynamic range for various types of plant material. In particular, such experiments should provide invaluable insights into the NPQ dynamics and protective efficiency of plants grown in the field and allow development of standardised procedures for quantification of plant tolerance to excess light and its relation to NPQ.

Recent work has considered whether the protective role of NPQ is accurately matched to the demands placed on it by a commonly changeable light environment, and whether it may down-regulate PSII quantum yield excessively (see [23]). To understand this we must be able to empirically determine which protective components of NPQ can be correlated to other photosynthetic parameters related to electron transport and gas exchange and which are the result of photoinhibitory damage. Therefore this technique will prove extremely valuable in efforts to isolate fractions of NPQ useful for enhancing plant productivity. It is easy to envisage from this that an automated routine for measurement of qP_d could be established in PAM monitoring devices that would output both pNPQ and photoinhibition data without the need for disruption or destruction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2012.03.026.

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