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Discerning the effects of photoinhibition and photoprotection on the rate of oxygen evolution in *Arabidopsis* leaves

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ABSTRACT

Higher plants possess a set of interconnected processes to regulate light harvesting. Non-photochemical quenching of chlorophyll *a* fluorescence (NPQ) is the fastest process activated to protect the photosystem (PS) II from the absorption of excess light energy. However, damage of PSII reaction centers (RCIIs) is often inevitable, a phenomenon known as photoinhibition. Both NPQ and photoinhibition undermine PSII quantum yield (Φ_{PSII}). Recently, we devised a fluorescence-based methodology that uses the coefficient of photochemical quenching measured in the dark following illumination (qPd) to assess the intactness of RCIIs. This procedure enables to express Φ_{PSII} as a function (f) of NPQ and qPd, $\Phi_{PSII} = f(\text{NPQ,qPd})$, thus allowing to efficiently discern between the effects of protective NPQ and photoinhibition upon the efficiency of electron transport. In this study, we addressed the relationship between qPd and Φ_{PSII} measured by photosynthetic oxygen evolution in intact leaves of *Arabidopsis*. We found a linear correlation between qPd and Φ_{PSII} of oxygen evolution (as well as Fv/Fm). This relates to the fact that qPd reflects the onset of photoinhibition. These results further demonstrate the validity of the qPd parameter and underlying theory in quantitatively assessing PSII efficiency solely by using this effective and simple fluorescence technique.

1. Introduction

The quantum yield of photosynthesis can be defined by the number of moles of oxygen (O_2) evolved, or of carbon dioxide (CO_2) assimilated, per mole of photons absorbed by the photosynthetic apparatus [1]. It thus reflects the efficiency of light energy conversion into fixed carbon. The quantum yield of photosystem (PS) II (Φ_{PSII}) is affected by a multitude of environmental factors. Of these, light is one of the most variable, both, in terms of extent and frequency in intensity and quality changes [2]. Photosynthetic organisms must therefore constantly adjust their photosynthetic machinery to maintain optimal performance in limiting light conditions while minimizing photodamage in excess light [2,3]. To achieve this goal, they use a hierarchy of interconnected strategies to regulate energy transformation within PSII.

Non-photochemical quenching of chlorophyll *a* fluorescence (NPQ) is the fastest process evolved to protect against excess light [3–5]. Ubiquitous and essential among all photosynthetic lineages [6,7], NPQ results in the rapid and reversible thermal dissipation of absorbed light energy in the PSII antenna [3]. So far, the process of NPQ encompasses four components with different underlying mechanisms: the energy-dependent (qE), state-transition (qT), zeaxanthin-dependent (qZ) and photoinhibitory quenching (qI) [3,6–8]. While, in higher plants, qT mainly serves the purpose of balancing light absorption between the two photosystems under low light [9,10], the qE and qZ components are essential for photoprotection, while qI was believed to reflect the photoinhibitory damage to the PSII reaction centers (RCII) [3]. qE is the fastest (forming and recovering within minutes) and most intensely studied component. Moreover, it is the most effective in protecting RCIIs against photodamage (see [3,6,7]). When the rate of light absorption becomes excessive, a transthylakoidal proton gradient (ΔpH) is generated and activates qE [11,12]. The acidification of the thylakoid lumen protonates the PSII subunit S (PsbS) protein [13,14] and activates violaxanthin de-epoxidase, which converts the xanthophyll violaxanthin into zeaxanthin [15–17]. PsbS and zeaxanthin act as allosteric modulators and enhance the sensitivity of PSII light harvesting antenna (LHCII) to the lumen protons, hence inducing qE formation [3,7,14].

Much less is known on the molecular basis of the sustained quenching components, such as qZ and qI. However, they have also been proposed to exert protection of RCIIs [3,18–20]. qZ relaxes in tens of minutes to hours and requires the formation of zeaxanthin, but it is independent of ΔpH once this xanthophyll is synthesized [19]. Differently, qI relaxes within hours. Despite having been originally attributed to photoinhibitory damage to the RCIIs [21,22], qI itself is currently regarded as a heterogeneous process consisting of multiple mechanisms that include the photoinhibition as well as slow

Abbreviations: Φ, quantum yield; AL, actinic light; NPQ, non-photochemical quenching of chlorophyll fluorescence; pNPQ, protective non-photochemical quenching of chlorophyll fluorescence; PSII, photosystem II; qPd, photochemical quenching in the dark; RCII, PSII reaction center; SP, saturating pulse; WT, wild type.

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photoprotective components of NPQ [3,18,20]. Although photoinhibition has been studied for several decades, there is no consensus upon its molecular mechanism, functional nature and protective capacity (see [23,24] and references therein). Moreover, the role played by NPQ in preventing the occurrence of photoinhibition has not yet been clearly elucidated [3,20,24]. Often kinetic criteria have been employed to investigate the relationship underlying NPQ and photoinhibition, which is measured by fluorescence analysis. However, distinguishing NPQ components based upon the heterogeneity of their kinetics of induction and relaxation can give ambiguous and often misleading results (e.g. the recovery timescale of qZ and qI overlaps; [3,19]) and it does not provide information on the protective effectiveness of NPQ. To address this issue, a new pulse amplitude modulated (PAM) fluorescence methodology has been developed to test the protective potential of NPQ in vivo, irrespective of the recovery kinetics of the components involved [25,26]. This method essentially monitors photoinhibition as a decline in the quantum coefficient of photochemical quenching (qP) in the dark, measured immediately after illumination (qPd). qPd and NPQ values are then used to fit Φ_{PSII} data. The theory of the method is thoroughly discussed in Materials and Methods section. This procedure was proved to be successful in separating the effects of protective NPQ (pNPQ) and photoinhibition in intact leaves of *Arabidopsis* WT and mutant plants [25–27], as well as in studying the coupling of PSII antenna to RCII and protective capacity of NPQ in plants acclimated to different light intensities [28]. Moreover, we also assessed the contribution of PSI fluorescence quenching in the Fm′ state to obtain a more accurate fluorescence-retrieved Φ_{PSII} [29] and the capacity to tolerate high light during ontogenesis in *Arabidopsis* plants [30]. Overall, this method offers a rapid and non-destructive way to quantitatively measure PSII photochemistry and protective efficiency of NPQ.

Common measurements of Φ_{PSII} include gas exchange (O_2 evolution or CO_2 uptake) and chlorophyll fluorescence analysis (changes in the ratio of variable to maximal fluorescence emission, Fv/Fm). It is generally well-established that Φ_{PSII} of photosynthetic O_2 evolution and fluorescence-retrieved Fv/Fm linearly correlate [e.g. 31–35]. Comparably, CO_2 assimilation has also been shown to be linearly proportional to Φ_{PSII} [e.g. 36–38]. Both methodologies thus represent useful and quantitative indicators of PSII photosynthesis efficiency. Furthermore, by addressing the relationship between qP and non-photochemical quenching processes (NPQ) in relation to photosynthesis, it has been demonstrated that the reduction in Φ_{PSII} relates to protective NPQ (both fast and slowly relaxing components), as well as photoinhibition (damage to RCIIs) [e.g. 8,32,37,39–41].

In this study our aim was to relate the qPd parameter to PSII efficiency measured by a technique independent from fluorescence, photosynthetic O_2 evolution. Here we compared the reduction in Φ_{PSII} either measured by chlorophyll fluorescence or O_2 evolution due to photoinhibition monitored as decrease in qPd, in intact *Arabidopsis* leaves. We also discerned the impact of photoprotection and photoinhibition on the Φ_{PSII} using these two independent methods. The obtained results indicate that qPd parameter is a valid and simply retrievable criterion of photoinhibition, less ambiguous than qI.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Arabidopsis thaliana, Colombia-0, wild type (WT) seeds were sterilized with 0.1% Triton X-100 and 50% ethanol for 5 min before rinsing three times in water and being stored at 4 °C for 72 h. Treated seeds were sown onto a 6:6:1 mixture of Levington M3 potting compost, John Innes No. 3 soil, and perlite (Scotts UK, Ipswich, UK). Seeds were placed in a Sanyo growth cabinet at 100 μmol photons m^{-2} s^{-1} for one week at a 10 h light/14 h darkness photoperiod. Seedlings were then shifted to a 200 μmol photons m^{-2} s^{-1} constant moderate light shelf (same photoperiod). A constant 24 °C room temperature was maintained. Measurements were carried out on intact and fully expanded leaves of non-flowering plants in rosette stage of development (7–10 weeks old; conditions similar to [42,43]).

2.2. Theoretical Principles

Φ_{PSII} is undermined by NPQ and photoinhibition [e.g. 8,32,37,39–41]. However, while the former is a reversible and protective energy-dissipation mechanism that occurs in PSII antenna, the latter is associated with long-term damage of RCIIs. Understanding the trade-offs between these two different process is therefore crucial to quantitatively assess the effects of light stress in the photosynthetic apparatus, eventually impacting plant productivity. Ruban and Murchie [25] recently formulated an equation to discern between the two processes:

$$\Phi_{PSII} = \mathrm{qPd} \cdot \frac{\left(\frac{Fv}{Fm}\right)}{\left[1 + \left(1 - \frac{Fv}{Fm}\right) \times \mathrm{NPQ}\right]}, \quad (1)$$

where qPd is the photochemical quenching (qP) measured in the dark immediately after illumination, Fm and Fo are the maximum and minimum fluorescence levels in the dark-adapted leaf, and Fv/Fm is the maximum quantum yield of PSII in the dark, where Fv = (Fm − Fo). NPQ is defined as [(Fm/Fm′) − 1]. Thus, Φ_{PSII} is a hyperbolic function of NPQ, while linearly correlating to qPd, which is a parameter that mirrors the onset of photoinhibition [25]. Using this formula, the actual Φ_{PSII} (affected both by NPQ and qPd) and theoretical Φ_{PSII} (only affected by NPQ, i.e. qPd = 1.00) can be calculated. The absence or presence of photoinhibition (qPd = 1.00 or qPd < 1.00, respectively) determines if both yields match or diverge (closed circles and regression line plotted in Fig. 1A). It has been considered that PSII photoinhibition occurs when more than 2% of RCIIs are permanently closed (qPd < 0.98; [25]). qPd is calculated according to the following equation [25]:

$$\mathrm{qPd} = \frac{Fm' - Fo'_{act.}}{Fm' - Fo'_{calc.}}, \quad (2)$$

where Fm′ is the maximum fluorescence in the dark after actinic light illumination. $Fo'_{act.}$ and $Fo'_{calc.}$ are the measured and calculated level of minimum fluorescence in the dark after illumination, respectively. $Fo'_{calc.}$ is calculated according to Oxborough and Baker [44]:

$$Fo'_{calc.} = \frac{1}{\left(\frac{1}{Fo} - \frac{1}{Fm} + \frac{1}{Fm'}\right)}. \quad (3)$$

At low light intensities, there is usually little discrepancy between $Fo'_{act.}$ and $Fo'_{calc.}$, indicating the validity of Oxborough and Baker's equation in this light conditions [25–30]. However, under higher light exposure $Fo'_{act.}$ becomes greater than $Fo'_{calc.}$. This is due to the closure of photoinhibited RCIIs at $Fo'_{act.}$, which disguises the real fluorescence quenching effect of NPQ taken into account in Eq. (3). This deviation causes the decline of qPd below 1.00.

2.3. Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence measurements were performed using a Junior-PAM fluorometer (Walz Effeltrich, Germany). Basically, the programmed illumination procedure was: (SP)–(AL on)–(5 min)–(SP)–(AL off/FR on)–(10 s)–(SP)–(5 s)–(AL on/FR off)–repeat, where AL, SP and FR represent actinic light, saturating pulse and far red light respectively. A set of 6 *A. thaliana* WT plants was exposed to a procedure of gradually increasing blue AL intensities according to Giovagnetti et al. [29]. This illumination treatment caused a limited photoinhibition (qPd < 0.98; open circles in Fig. 1A). To obtain a much broader range of photoinhibition (i.e. qPd values) we employed constant high light