# Quantifying and monitoring functional photosystem II and the stoichiometry of the two photosystems in leaf segments: Approaches and approximations

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REVIEW

# Quantifying and monitoring functional photosystem II and the stoichiometry of the two photosystems in leaf segments: approaches and approximations

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**Abstract** Given its unique function in light-induced water oxidation and its susceptibility to photoinactivation during photosynthesis, photosystem II (PS II) is often the focus of studies of photosynthetic structure and function, particularly in environmental stress conditions. Here we review four approaches for quantifying or monitoring PS II functionality or the stoichiometry of the two photosystems in leaf segments, scrutinizing the approximations in each approach. (1) Chlorophyll fluorescence parameters are convenient to derive, but the information-rich signal suffers from the localized nature of its detection in leaf tissue. (2) The gross $O_2$ yield per single-turnover flash in $CO_2$-enriched air is a more direct measurement of the functional content, assuming that each functional PS II evolves one $O_2$ molecule after four flashes. However, the gross $O_2$ yield per single-turnover flash (multiplied by four) could overestimate the content of functional PS II if mitochondrial respiration is lower in flash illumination than in darkness. (3) The cumulative delivery of electrons from PS II to $P700^+$ (oxidized primary donor in PS I) after a flash is added to steady background far-red light is a whole-tissue measurement, such that a single linear correlation with functional PS II applies to leaves of all plant species investigated so far. However, the magnitude obtained in a simple analysis (with the signal normalized to the maximum photo-oxidizable P700 signal), which should equal the ratio of PS II to PS I centers, was too small to match the independently-obtained photosystem stoichiometry. Further, an under-estimation of functional PS II content could occur if some electrons were intercepted before reaching PS I. (4) The electrochromic signal from leaf segments appears to reliably quantify the photosystem stoichiometry, either by progressively photoinactivating PS II or suppressing PS I via photo-oxidation of a known fraction of the P700 with steady far-red light. Together, these approaches have the potential for quantitatively probing PS II in vivo in leaf segments, with prospects for application of the latter two approaches in the field.

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**Abbreviations**

| | |
|---|---|
| Chl | Chlorophyll |
| Cyt | Cytochrome |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| ECS | Electrochromic signal |
| $F_o$, $F_m$ | Chl fluorescence corresponding to open and closed PS II traps in the dark-adapted state, respectively |
| $F_v/F_m$ | $(F_m - F_o)/F_m$ |
| P700 | Special Chl pair in the PS I reaction center |
| PQ | Plastoquinone |
| PS I, II | Photosystem I, II, respectively |
| $\phi_{PS\ II}$ | Effective photochemical yield of PS II |
| $Q_A$, $Q_B$ | Primary, secondary quinone acceptor in PS II, respectively |
| $qP$ | Indicator of the oxidation state of $Q_A$ |
| $\Sigma$ | Integrated flash-induced transient flow of electrons from PS II to $P700^+$ |

## Introduction

Photosynthesis begins with the absorption of light by photosystem II (PS II) and PS I complexes that span thylakoid membranes. Light induces the oxidation of water at a catalytic site within the PS II complex, in a unique reaction that occurs at a thermodynamic driving force needing only a low over-potential:

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^-$$

Because of its water-splitting ability, PS II is the subject of intensive and extensive studies (Wydrzynski and Satoh 2005). In addition, PS II is 'intrinsically suicidal' (van Gorkom and Schelvis 1993) during its normal function (Ewart 1896; Powles 1984; Krause 1988; Barber 1995; Adir et al. 2003; Vass and Cser 2009), and needs to be repaired after photoinactivation (Kyle et al. 1984; Prásil et al. 1992; Aro et al. 1993; Chow 1994; Melis 1999; Andersson and Aro 2001; Chow and Aro 2005). For this reason, it is often the most vulnerable component under environmental stress.

Photoinactivation of PS II is sometimes termed chronic photoinhibition to distinguish it from dynamic photoinhibition in which regulated energy dissipation in the form of heat helps in photoprotection (Osmond and Grace 1995). Both chronic and dynamic photoinhibition lead to a decline in photochemical efficiency. Photoinactivation of PS II has been thought to be initiated when the light absorbed by chlorophyll (Chl) and other accessory pigments is in excess of that needed for the prevailing rate of photosynthesis; this is called the excess-energy mechanism of photoinactivation of PS II (Ögren et al. 1984; Demmig and Björkman 1987, Osmond 1994; Kato et al. 2003). Another school of thought has proposed that a two-step mechanism leads to photoinactivation of PS II: the initial step is the absorption of light by the Mn cluster that catalyzes water-splitting within PS II, resulting in Mn being dislodged from the catalytic site; subsequent absorption of light by Chl molecules then results in photoinactivation of PS II (Hakala et al. 2005; Ohnishi et al. 2005; see also Takahashi and Badger 2011). These two mechanisms, however, need not be mutually exclusive. Indeed, both mechanisms can be observed when leaves are illuminated by different coloured lights that are differentially absorbed by Chl and Mn (Oguchi et al. 2009). Similarly, the photoinhibition gradient inside a leaf exposed to high light of different colours can be explained only when both mechanisms are involved (Oguchi et al. 2011.

The quantum yield of photoinactivation of PS II is of the order of 1 PS II photoinactivated after leaf tissue has absorbed $10^7$ photons (Park et al. 1995b), varying somewhat according to environmental conditions and plant species (Chow et al. 2005). While this quantum yield appears small, a square metre of leaf area (containing about 1 μmol PS II) may receive $>2 \times 10^7$ μmol photons during a sunny day. Therefore, the entire population of PS II may undergo photoinactivation during a sunny day. If the rate of repair cannot keep up with the rate of photoinactivation, net loss of functional PS II ensues. In that case, light is still absorbed by non-functional PS II complexes which do not split water, resulting in an overall loss of quantum efficiency of photosynthesis. Not surprisingly, therefore, there is a continuing interest in measuring or monitoring the functionality of PS II in vivo.

PS II and PS I work in series in linear electron transport, but PS I also drives cyclic electron flow. Given the extreme lateral heterogeneity in the distribution of the two photosystems (Andersson and Anderson 1980), there is no reason to expect equal or fixed numbers of the two photosystems. Melis and Brown (1980) first reported variable ratios of the two photosystems. Indeed, the photosystem stoichiometry is adjusted by the spectral quality of the growth light environment, thereby optimizing the quantum yield of oxygen evolution in that particular light environment (Chow et al. 1990a). In general, either the antenna size of each photosystem or the content of each photosystem, or both, may vary depending on growth conditions; in steady limiting light when the quantum efficiency of both

photosystems is maximal, the quantum efficiency of linear electron flow will be optimal when the antenna size of PS II multiplied by the PS II content equals the antenna size of PS I multiplied by the PS I content. Notwithstanding the variability of the photosystem stoichiometry, an accurate value for the stoichiometry of the two photosystems is needed, for example, for predicting the input of electrons to and the output of electrons from the cytochrome (Cyt) *bf* complex on applying single-turnover flashes. Thus, there is also a need to quantify the stoichiometry of the two photosystems, particularly since the stoichiometry has been a controversial topic (Fan et al. 2007).

In this mini-review, the advantages and deficiencies of four approaches for quantifying or monitoring functional PS II or the stoichiometry of the two photosystems in situ in leaf tissue will be discussed. These involve measurements of (1) Chl fluorescence, (2) the oxygen yield per single-turnover, saturating flash applied repetitively, (3) the cumulative delivery of electrons from PS II to PS I after a single-turnover, saturating flash and (4) the electrochromic signal (ECS) induced by a single-turnover, saturating flash. All are in vivo measurements, so they reflect PS II functionality in situ, without any potential complication associated with isolation of thylakoids. This mini-review is largely based on work done in Canberra over a number of years, mostly during separate visits of most of the co-authors. Emphasis will be given to the approximations made to arrive at the values, and the pitfalls to beware.

## Approach 1. Chlorophyll fluorescence parameters

At physiological temperature, Chl *a* fluorescence is predominantly emitted by PS II, with relatively little contribution from PS I, with the exception of $F_o$ measurement (Pfündel 1998) where $F_o$ is the Chl fluorescence yield when all PS II traps are open. The ratio of variable to maximum Chl fluorescence yield ($F_v/F_m$) gives the photochemical efficiency of PS II in the dark relaxed state (Demmig and Björkman 1987). As PS II is progressively photoinhibited, there is a gradual accumulation of non-functional PS II complexes which, although still absorbing light, are unable to perform useful photochemical conversion. Therefore, the overall photochemical efficiency is reduced. The decline in $F_v/F_m$ is correlated with the ability of PS II in pre-photoinhibited leaf segments to evolve oxygen as revealed by the oxygen yield per repetitive single-turnover flashes (Fig. 1, taken from Losciale et al. 2008), a technique based on the assumption that each functional PS II is able to evolve one $O_2$ molecule after four flashes (see "Approach 2. Flash-induced oxygen evolution" section); however, the correlation varies among the various plant species examined, such that a combined plot is highly

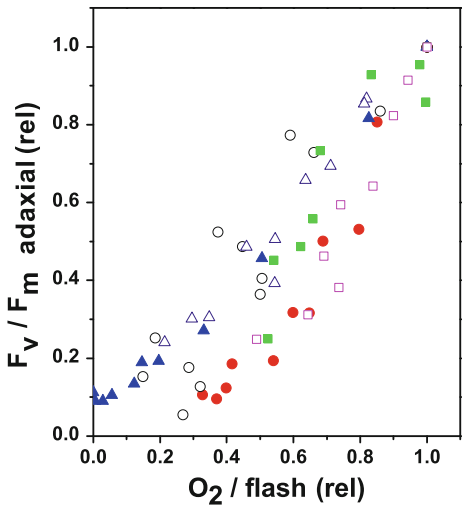


**Fig. 1** Approach 1. Correlation of $F_v/F_m$ (measured on the adaxial side of leaf segments) with the oxygen yield per single-turnover repetitive flash (relative) representing the functional fraction of PS II. Leaf segments from various plant species, representing C3 and C4 plants, monocots and dicots, herbaceous and woody plants, and a wild-type and Chl *b*-less mutant, were photoinhibited to various extents so as to vary the parameters. Data are re-plotted from Losciale et al. (2008). The plants were nectarine (*filled circle*), *Arabidopsis* (*open circle*), wild-type barley (*open triangle*), Chl *b*-less barley (*filled square*), capsicum (*filled triangle*) and *Flaveria bidentis*, a C4 species (*open square*)

scattered and sometimes curvilinear (Fig. 1). Nevertheless, for a given plant species, the ratio $F_v/F_m$ is a sensitive and convenient parameter to quantify photoinactivation of PS II, being able to discern, for example during photosynthetic induction, (1) the exacerbation of photoinactivation of PS II and (2) the absence of reciprocity of irradiance and duration of illumination, i.e. equal light dose does not necessarily give the same extent of photoinactivation (Shen et al. 1996).

Havaux et al. (1991) and Walters and Horton (1993) advocated the use of the parameter $1/F_o - 1/F_m$ as representing charge separation in PS II. The parameter $1/F_o - 1/F_m$ could indeed be linearly correlated with the oxygen yield per repetitive single-turnover flash (Park et al. 1995a; Kim et al. 2001; Lee et al. 2001; He and Chow 2003). Unfortunately, the linear regression seldom passed through the origin, with the rare exception of *Capsicum* on occasions (Lee et al. 2001; He and Chow 2003). Nevertheless, once the linear regression line is known for a particular plant species grown in a given set of conditions, the parameter $1/F_o - 1/F_m$ can be used to infer the functional PS II content when the oxygen yield per flash cannot be used. For example, in conditions where PS I is preferentially photoinhibited, the plastoquinone (PQ) pool is more chemically reduced during repetitive-flash illumination, such that $Q_A$ (the primary quinone acceptor in PS II) is also somewhat reduced. In such conditions, the oxygen yield

per flash under-estimates the functional PS II content, and Kim et al. (2001) had to rely on $1/F_o - 1/F_m$ as an alternative measure of PS II content in cucumber leaf segments photoinactivated under conditions of moderately low light and chilling temperature.

Because of the ease of measurement, $1/F_o - 1/F_m$, once calibrated against oxygen yield per repetitive flash, could be conveniently used to monitor the recovery of PS II from photoinactivation in a large number of leaf segments. By contrast, measurement of the oxygen yield per repetitive flash with a gas-phase Clark electrode is typically very time-consuming. Thus, it was relatively easy to investigate the time course of recovery using $1/F_o - 1/F_m$ measured in a large number of *Capsicum* leaf segments, and to derive the rate coefficients of photoinactivation ($k_i$) and of repair ($k_r$) (He and Chow 2003). In particular, it was shown that $k_i$ was directly proportional to irradiance (Lee et al. 2001; He and Chow 2003), in confirmation of observations made by others (Tyystjärvi and Aro 1996; Lee et al. 1999; Kato et al. 2003) and according to expectation arising from the reciprocity law (Lee et al. 1999). Further, it was shown that $k_r$ was already high at a low irradiance, increasing further at moderately high irradiance, but decreasing at much higher irradiance (He and Chow 2003) probably due to oxidative stress (Nishiyama et al. 2001).

Photoinactivation of PS II is frequently accompanied by an increase in $F_o$. This fluorescence parameter indicates PS II reaction centre dysfunction (Franklin et al. 1992; Park et al. 1995a). Trissl and Lavergne (1995) expressed $F_o$ in terms of the rate coefficients of (1) loss of excitation energy in the antenna and (2) forward and backward electron transfers within PS II. For example, a decrease in the rate coefficient for charge separation ($k_a$) or charge stabilization ($k_b$) increases $F_o$ (symbols as used by Fan et al. 2009). However, in applying the expression of Trissl and Lavergne (1995) for $F_o$ to photoinhibited leaves, Chow and Park (1995) assumed that there was a uniform population of 'average' PS II with the 'average' rate coefficients, although in fact there was a mixture of functional and non-functional PS II complexes with separate sets of rate coefficients. This assumption needs to be borne in mind when interpreting changes in $F_o$ after photoinhibition.

Although modern instrumentation has greatly facilitated the measurement of Chl fluorescence yield, a fundamental limitation exists in regard to sampling of the signal in leaf tissue. The excitation light is readily attenuated as it penetrates leaf tissue, such that the fluorescence signal predominantly comes from relatively shallow depths. Further, any fluorescence signal emitted by Chl *a* molecules in deeper tissue is likely to be re-absorbed on its way to the detection light guide located near the leaf surface. Therefore, the detected fluorescence signal is only representative of chloroplasts at a certain depth. Unfortunately, the detection depth is not even fixed. For example, as PS II is progressively photoinhibited at shallow depths, the contribution of the signal from greater depths becomes relatively more significant, as photoinhibited chloroplasts in shallow tissue contribute less to the fluorescence intensity; consequently, the detection depth increases continually during the time course of photoinhibition (Oguchi et al. 2011). Notwithstanding these shortcomings, however, the Chl fluorescence technique allows microfiber insertion into the leaf tissue, enabling the measurement of PS II activity at various depths and demonstrating the highly heterogeneous photosynthetic activity within an intact leaf.

Nevertheless, this fundamental problem of the localized nature of the Chl fluorescence signal in leaves makes it difficult to compare Chl fluorescence measurement with gas exchange measurements which involve the whole leaf tissue. For example, there was at first a promising curvilinear relationship between the photochemical yield of PS II ($\phi_{PS\ II}$) and the quantum yield of oxygen evolution among a number of plant species grown under various conditions (Seaton and Walker 1991; Öquist and Chow 1992). Such a curvilinear curve, if universal, could be used to obtain the quantum yield of oxygen evolution, and hence the rate of oxygen evolution when the irradiance is known, from Chl fluorescence measurements. However, correlations for some plants deviated from the 'universal' relationship, e.g. those of wild-type barley and a Chl *b*-less mutant of barley. The differential sampling of the chloroplast population within the barley leaf tissues by the two measurements of oxygen evolution and Chl fluorescence may be partly responsible for the deviation from a universal relationship (Öquist and Chow 1992).

To sum up, Chl fluorescence in leaves is an information-rich but complex signal. It is conveniently measured with a variety of commercial instruments, but the localized signal from a specific region of photosynthetic tissue makes it hard to compare with parameters measured for the whole tissue.

## Approach 2. Flash-induced oxygen evolution

The repetitive-flash technique of quantifying functional PS II via oxygen evolution was originally applied to algae (Emerson and Arnold 1932; Myers and Graham 1983) and cyanobacteria (Kawamura et al. 1979; Myers et al. 1982). Later on, the technique began to be applied to leaf segments of higher plants in $CO_2$-enriched air using a gas-phase Clark-type electrode. The gross rate of oxygen evolution was obtained by algebraically subtracting the dark drift from the rate during repetitive-flash illumination; it was then divided by the flash frequency to obtain the oxygen evolution per flash (Chow et al. 1988, 1989a, 1991; Jursinic and Pearcy

1988). A small heating artifact due to flash illumination which can be revealed by substitution of green fabric for a leaf segment, if significant, should be taken into account. The technique of determining functional PS II by gross oxygen evolution relies on the reasonable assumption that each functional PS II evolves one $O_2$ molecule after four single-turnover flashes. Provided photorespiration is suppressed (as is the case in $CO_2$-enriched air), the assumption seems a reasonable approximation. Subsequently, this technique was applied to examine the loss of functional PS II after photoinhibition treatment (Chow et al. 1989b; Öquist et al. 1992; Park et al. 1995a, b, 1996a, b, c; Russell et al. 1995; Sinclair et al. 1996). A more sophisticated $O_2$ measurement, using a zirconium-oxide analyzer, allowed Oja and Laisk (2000) to measure oxygen evolution from leaves in response to individual single-turnover flashes, provided that the background $[O_2]$ was kept low (2 %).

Each PS II, whether functional or non-functional, contains one binding site for $Q_B$, the secondary quinone acceptor in PS II, when the binding site has not been photodamaged. $Q_B$-binding sites can be quantified using $^{14}$C-labelled inhibitors that bind to the site in isolated thylakoids (Tischer and Strotmann 1977; Graan and Ort 1986; Chow et al. 1990b). A comparison between binding sites in thylakoids for 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and functional PS II content in leaf segments determined by the $O_2$ yield per flash showed that the former exceeds the latter by about 12 % in spinach (Chow et al. 1991), and by <15 % among leaves of a number of species (Chow et al. 1989a). Atrazine-binding sites were slightly (about 16 %) less abundant than DCMU-binding sites (Chow et al. 1990b), the number of atrazine-binding sites being closer to the number of functional PS II complexes obtained from the $O_2$ per single-turnover flash. Thus, there is reasonable agreement between $Q_B$-binding sites in thylakoids and functional PS II content in leaf segments. Any discrepancy could be due to either a miss-factor (Chow et al. 1991) or non-functional PS II that are still able to bind the inhibitor (Graan and Ort 1986). Indeed, during progressive photoinhibitory treatment of spinach leaf segments at 25 °C, both the functional PS II content in vivo and the atrazine-binding sites on isolated thylakoids declined by the same proportion (Osmond and Chow 1988). It was only when the photoinhibitory treatment of thylakoids was carried out at 0 °C (to answer Barry Osmond's question of whether '$Q_B$ or not $Q_B$' was the primary site of damage that necessarily accompanies the loss of PS II function) that the functional PS II content declined ahead of that of the atrazine-binding sites (Osmond and Chow 1988).

Significantly, there is also reasonable agreement between the $O_2$ per flash obtained using leaf segments and that using thylakoids isolated from the same batch of leaves; the latter was about 94 % of the former when assayed with flashes at 4 Hz (Chow et al. 1991). Allowing for some potential damage upon isolation of thylakoids, the agreement is excellent. At a higher flash frequency (20 Hz), however, the $O_2$ per flash in a thylakoid suspension was about 20 % less than that at 5 Hz (Chow et al. 1991), an observation which should be borne in mind when making in vitro measurements. The isolation of thylakoids could have de-stablized PS II to such an extent that PS II electron-transfer steps became limiting at 20 Hz.

When the frequency of repetitive flashes was varied between 1 and 40 Hz, the functional content of PS II in spinach leaf segments, obtained from the $O_2$ evolved per flash, was essentially constant at 2.9 mmol PS II (mol Chl)$^{-1}$ or 345 Chl (PS II)$^{-1}$ (Fig. 2a). The constancy of the value is reassuring, but at the same time curious, because of the following observations. The quantum yield of $O_2$ evolution measured in steady low light using the method of Björkman and Demmig (1987) and Evans (1987) was linearly correlated with functional PS II content in wild-type *Arabidopsis* obtained from the $O_2$ evolved per flash, when both quantities were varied by photoinhibitory pre-treatment of leaf segments; the regression line could be comfortably constrained to pass through the origin (Fig. 2b which has been re-plotted from Chow et al. 2002; see also the linear correlation of Öquist et al. (1992) for a few plant species). Such a linear regression is easily understandable, since any non-functional PS II still absorbed the limiting steady light without performing useful photochemistry.

However, it is known from work with *Eucalyptus* leaves that mitochondrial respiration decreases from a magnitude in the dark to one that is measurably less in the light, and that the decrease occurs even in limiting light in which the quantum yield of $O_2$ evolution is often measured (Atkin et al. 2000). If this observation is generally true, the quantum yield of $O_2$ evolution in limiting continuous light may be over-estimated slightly. Similarly, the gross $O_2$ evolution rate during flash illumination (e.g. 10 Hz) may also be over-estimated slightly since the gross rate is obtained as the algebraic difference between flash illumination and dark. This is because PS II complexes in a leaf segment, present at approximately 1 μmol m$^{-2}$, receive for example 10 flashes s$^{-1}$, equivalent to being exposed to steady light of absorbed irradiance 20 μmol m$^{-2}$ s$^{-1}$ where the two photosystems share the light equally. Therefore, the functional content of PS II, obtained from the gross $O_2$ evolved per flash, may also be over-estimated, the extent of over-estimation expected to increase somewhat with increase in flash frequency (equivalent to increasing the irradiance of steady low light). If so, why is the observed functional content of PS II constant over the range of flash frequencies in Fig. 2a? Could there be a factor which decreases with increase in flash frequency, and which compensated for the increasing over-estimation associated with lower

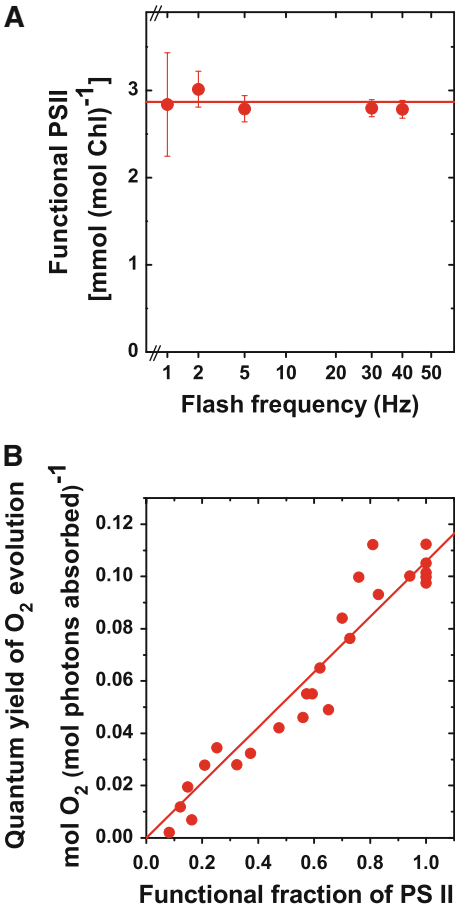


**Fig. 2** Approach 2. **a** The content of functional PS II in spinach leaf segments as a function of the frequency of single-turnover flashes used to assay the gross $O_2$/flash in $CO_2$-enriched air. Re-plotted from Chow et al. (1991). **b** Linear correlation of the quantum yield of oxygen evolution in limiting steady light with the functional fraction of PS II in *Arabidopsis* leaf discs assayed by the gross $O_2$/flash after photoinhibition pre-treatment. Measurements were made in $CO_2$-enriched air. The linear regression is constrained to pass through the origin. Re-plotted from Chow et al. (2002)

mitochondrial respiration induced by increasing light associated with increasing flash frequency? This factor cannot be the Kok model miss parameter ($\alpha \approx 0.10$) which is rather constant over the range of flash frequencies from 2 Hz to at least 30 Hz in both *Chlorella* and *Spirulina* (Ananyev and Dismukes 2005). Perhaps an artifact compensated for the effect of mitochondrial respiration varying with flash frequency. For example, as the flash frequency was increased excessively, there could be insufficient time to fully recharge the capacitor, resulting in sub-saturating flash intensity and a tendency to under-estimate the gross $O_2$ evolved per flash. If so, a more accurate estimation of functional PS II is obtained at a lower flash frequency, but obviously the signal:noise ratio is poorer, as indicated by the larger standard error at low flash frequencies (Fig. 2a).

Another curious point, made by Myers and Graham (1983), is that the repetitive-flash technique of quantifying functional PS II via gross oxygen evolution should not work when PS II is more abundant than PS I. In leaves of higher plants, the photosystem stoichiometry (the ratio of PS II reaction centers to PS I reaction centers) is considerably greater than unity (see section below on the photosystem stoichiometry). If the flash is single-turnover for both photosystems, more electrons are delivered to the PQ pool than exiting from PSI. In that case, one expects that repetitive-flash illumination would over-reduce the PQ pool and, therefore, chemically reduce $Q_B$ and $Q_A$ to some extent; if so, PS II complexes containing $Q_A^-$ will not be able to perform further charge stabilization, and the oxygen yield per flash will under-estimate the functional PS II content. Fortunately, $Q_A$ seems to be practically completely oxidized during flash illumination at 10 Hz (photochemical quenching parameter $qP = 0.99$), at least after a leaf segment has undergone induction (Chow et al. 1991; Kim et al. 2001). A reason for the nearly complete oxidation of $Q_A$ may be that PS I turns over more than once during the time of a xenon flash (Myers and Graham 1983). An attempt to test this hypothesis was made using isolated thylakoids. It was found that, in the presence of DCMU, $NaN_3$ and superoxide dismutase, about 3.2 mmol $e^-$ per mol Chl per flash (of duration ca. 3 μs at half peak height) were donated from durohydroquinone to methyl viologen, a value approximately twice the concentration of PS I centers (Chow et al. 1989a). Therefore, each PS I seems to turn over more than once during a xenon flash, and each PS II seems to be returned as an open trap before the next flash is applied.

To sum up, the gross $O_2$ yield per flash in leaf segments seems to be a most direct way of obtaining an absolute measure of the functional PS II content, though it is time-consuming and may over-estimate the content at high frequencies of saturating flashes.

## Approach 3. The use of the $P700^+$ signal to assay PS II

With the availability of commercial instruments, the absorbance change at 830 nm associated with the photo-oxidation of P700 in PS I can be measured with excellent time resolution and signal-to-noise ratio (Klughammer and Schreiber 1994; Laisk et al. 2010). Far-red light, even at a low irradiance, oxidizes P700 to the extent of 80–90 %, being preferentially absorbed by PS I. Complete photo-oxidation of P700 in far-red light is not observed, however. This could be because a small fraction of P700 is maintained in the reduced state by (1) reducing equivalents from the chloroplast stroma, (2) slight excitation of PS II by far-red light and/or (3) any cyclic electron flow in far-red light. Nevertheless, superposition of a saturating single-turnover

flash on steady background far-red light transiently photo-oxidizes the remaining P700. Following a flash, as electrons from PS II arrive at $P700^+$, there is a tendency to reduce $P700^+$, while the steady far-red light brings the oxidation level back to the steady state. Hence the 'dip' in the oxidation level of P700 reflects the number of electrons that arrive from PS II per flash; progressive photoinactivation of PS II gave an increasingly shallow dip (See Fig. 3a, re-plotted from Losciale et al. 2008).

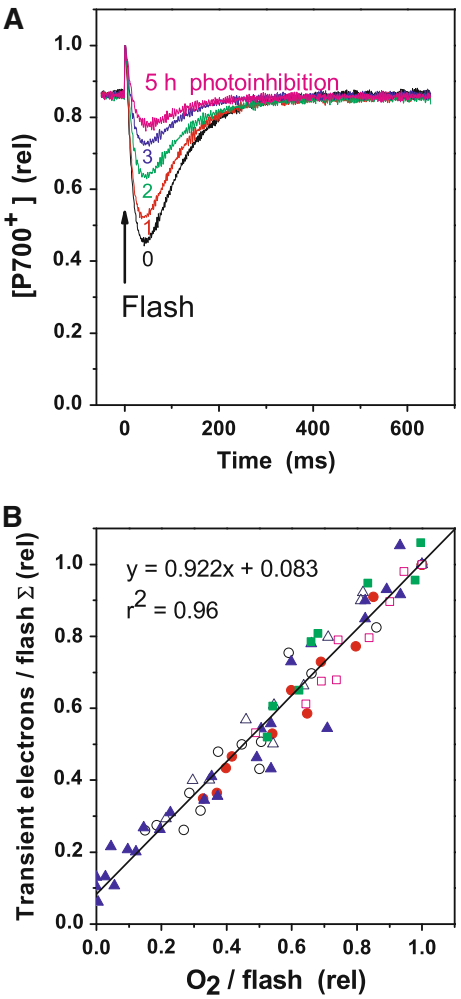


**Fig. 3** Approach 3. **a** Transient changes in the redox state of P700 on adding a single-turnover flash (at time 0) to steady far-red light given to nectarine leaf segments. The leaf segments had been photoinhibited for various durations (0–5 h) prior to measurement. Re-plotted from Losciale et al. (2008). **b** Estimation of the cumulative delivery of electrons from PS II ($\Sigma$) to $P700^+$ following a single-turnover flash, plotted against the functional fraction of PS II. Leaf segments from various plant species, representing C3 and C4 plants, monocots and dicots, herbaceous and woody plants, and a wild-type and Chl *b*-less mutant, were photoinhibited to various extents so as to vary the parameters. Data are re-plotted from Losciale et al. (2008). The plants were nectarine (*filled circle*), *Arabidopsis* (*open circle*), wild-type barley (*open triangle*), Chl *b*-less barley (*filled square*), capsicum (*filled triangle*) and *Flaveria bidentis*, a C4 species (*open square*)

An attempt was made to quantify the number of flash-induced electrons that arrive from PS II at $P700^+$, normalized to the total photo-oxidizable P700 (Losciale et al. 2008). In this approximate analysis, it was assumed that (1) the rate of photo-oxidation of P700 is directly proportional to [P700] and (2) the feeding of reducing equivalents to the PQ pool occurs at a constant rate during illumination with steady far-red light. The integrated transient flow of electrons from PS II ($\Sigma$) that arrive at $P700^+$ after a flash is essentially given by the area between the dipping curve and the horizontal line corresponding to the steady state. $\Sigma$ declined linearly with the loss of the functional fraction of PS II in a photoinhibition pre-treatment, though the straight line did not exactly pass through the origin (Fig. 3b, re-plotted from Losciale et al. 2008). Significantly, the one straight line was obtained for a number of plant species representing C3 and C4 plants, monocot and dicot plants, herbaceous and woody species and wild-type and a Chl *b*-less mutant of barley. That is, the 'calibration' straight line was apparently independent of leaf anatomy.

By contrast, a plot of $F_v/F_m$ against the functional fraction of PS II was much more scattered (Fig. 1, re-plotted from Losciale et al. 2008) than the plot of $\Sigma$ against the functional fraction of PS II, even though the same samples were used in both measurements. The difference between the two plots in terms of scattered data is almost certainly due to the fact that $F_v/F_m$ is a localized signal detected from a certain (variable) depth of the leaf tissue. By contrast, $\Sigma$ obtained from the $P700^+$ signal and functional PS II obtained from flash-induced oxygen evolution are both whole-tissue measurements. For example, the area between the dipping curve and the horizontal line corresponding to the steady-state oxidation level of P700 is practically the same when measured in the reflection mode either from the adaxial side or the abaxial side (Oguchi et al. 2011). Therefore, the small scatter in Fig. 3b results from a comparison of two parameters both of which are whole-tissue measurements.

$\Sigma$, being normalized to the total photo-oxidizable P700, should give the ratio of PS II to PS I reaction centers (i.e. the photosystem stoichiometry), but unfortunately it did not. Instead, $\Sigma$ was typically only about 0.7 for control leaves (Losciale et al. 2008). The true ratio of PS II to PS I centers in higher plants should be considerably greater than unity (see "Approach 4. The use of the electrochromic signal to determine the photosystem stoichiometry" section). Therefore, $\Sigma$ as derived by the approximate method is only a semi-quantitative measure of the ratio of PS II to PS I. Another complication may arise if some of the electrons originating in water photo-oxidation in PS II are either intercepted on their way to PS I (Cleland and Grace 1999), or are lost in charge recombination with the S-states of the Mn cluster in PS II. Then the cumulative delivery of

electrons to $P700^+$ will be an under-estimate of the functional PS II content. For these reasons, further study of the flash-induced cumulative delivery of electrons from PS II to PS I is warranted.

To sum up, given the convenience of measuring the $P700^+$ signal that represents the whole-tissue, there are advantages in using this PS I signal to monitor PS II functionality. Further, the instrument has potential for portability for use in the field.

### Approach 4. The use of the electrochromic signal to determine the photosystem stoichiometry

The electrochromic signal (ECS) reflects trans-membrane charge transfer through the thylakoid membrane (Witt 1975). Significantly, it can be measured in leaf segments, thereby allowing in vivo monitoring of trans-membrane charge transfer events (Kramer and Crofts 1989, 1990, 1996; Klughammer et al. 1990; Chow and Hope 1998, 2004), and acting like a 'voltmeter'. Further, it is a whole-tissue measurement because the measuring light (wavelength 520 nm) is scattered and transmitted through the leaf. In flash-induced ECS, a very fast rise (<1 ns) is followed by a slow rise in the ms time scale (Joliot and Delosme 1974; Crowther et al. 1979; Hope and Morland 1980); both phases then undergo a very slow relaxation to a baseline in the dark (Fig. 4a). The fast rise is attributed to charge separation across the thylakoid membrane at both PS II and PS I. The slow rise is attributed to trans-membrane charge transfer (electrons outwards and/or protons inwards) at the Cyt *bf* complex in leaf discs (Chow and Hope 2004), *Chlorella* (Joliot and Joliot 1998) and *Chlamydomonas* (Zito et al. 1998; Deniau and Rappaport 2000; Joliot and Joliot 2001; Finazzi 2002). These charge transfers soon result in the deposition of protons in the lumen and hydroxide anions on the stromal side of the thylakoid membrane. Both $H^+$ and $OH^-$ ions are free to diffuse in their respective compartments, such that the electric potential difference across the thylakoid membrane is rapidly delocalized; therefore, regardless of where the charge transfer across the membrane occurs, each transfer is sensed equally by the pigments, Chl *b* and carotenoids, in the membrane (see Witt 1975).

We made use of the magnitude of fast ECS rise to investigate the separate contributions from the two photosystems (Chow and Hope 1998; Fan et al. 2007). With progressive photoinactivation of PS II in a pre-treatment, the magnitude of the fast phase declined linearly with the decline in functional PS II content. Extrapolation to zero functional PS II (see Fig. 4b) gave a residual signal which represented the sole contribution by PS I ($ECS_r$), whereas the maximum loss of the fast phase ($ECS_l$) represented

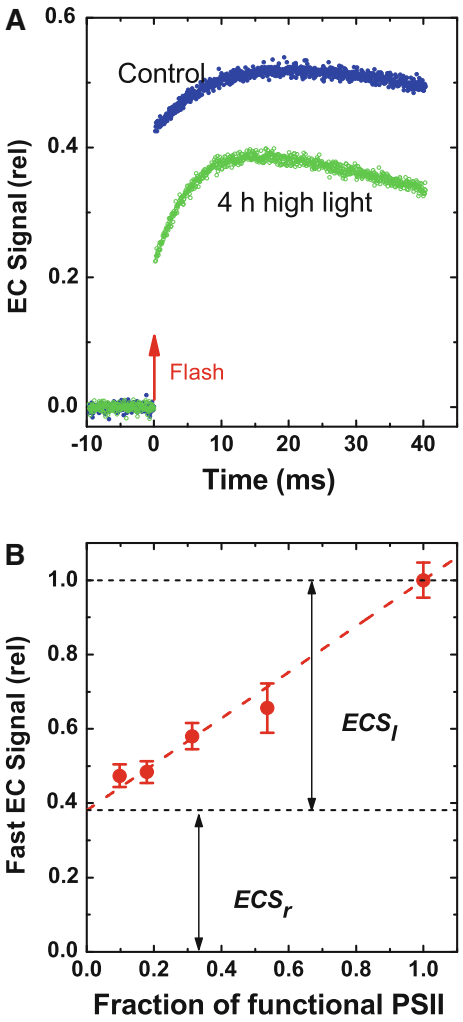


**Fig. 4** Approach 4. **a** The electrochromic (EC) signal induced by a single-turnover flash applied at time 0. Market spinach leaf segments were used either without exposure to high light (Control) or after 4 h exposure to 1200 µmol photons $m^{-2}$ $s^{-1}$ in the presence of lincomycin. Each trace is an average of 25 scans given at 0.2 Hz. Re-plotted from Fan et al. (2007). **b** Linear correlation of the fast rise of the EC signal with the functional fraction of PS II in *Populus deltoides* leaves previously subjected to photoinhibition treatment so as to vary the parameters. Mean values (± SE) are for four leaf discs. Extrapolation to zero functional PS II gives the separate contributions of the two photosystems to the fast-rise signal. Re-plotted from Fan et al. (2007)

contribution by PS II. It is reasonable to assume that extensive photoinactivation of PS II could be brought about without affecting the charge-separation capability of PS I: when sun and shade leaves of several plant species from a neotropical forest were exposed to excessive light, PS II suffered a great extent of photoinactivation, but the potential P700 photo-oxidation activity was unaffected (Barth et al. 2001). Therefore, the ratio $ECS_l/ECS_r$ should equal the stoichiometry of the two photosystems. In market spinach, the ratio $ECS_l/ECS_r$ was 1.61 (Table 1). For

**Table 1** Stoichiometry of the two photosystems in market spinach assayed by various methods

| Method | PS II/PS I stoichiometry |
|---|---|
| Progressive photoinactivation of PS II in leaf segments (EC signal) | 1.61[a] |
| EPR measurements on isolated thylakoids | 1.66[b] |
| Functional PS II in leaf segments, P700 content in isolated thylakoids | 1.46 ± 0.11 ($n = 3$) |
| Suppression of PS I in leaf segments via P700 photo-oxidation by far-red light, (EC signal) | 1.54 ± 0.12 ($n = 6$) |

Values are means ± SE, taken from Fan et al. (2007)

[a] This value was obtained by linear extrapolation to zero functional PS II using 28 leaf discs which had been photoinhibited to various extents

[b] This value was obtained from one preparation of thylakoids from market spinach; a preparation from fresh garden spinach gave a slightly higher value by the same method (data not shown)

comparison, the PS II/PS I ratio was 1.66 from electron paramagnetic resonance (EPR) measurements made with isolated thylakoids, and 1.46 ± 0.11 obtained from (1) quantification of PS II via the $O_2$ yield per single-turnover flash in leaf segments and (2) quantification of PS I via photo-oxidation of P700 in a thylakoid suspension containing detergent in a cuvette (Table 1). In general, the agreement between $ECS_l/ECS_r$ and the photosystem stoichiometry determined by other methods is good. Freshly-harvested spinach leaves gave a slightly higher $ECS_l/ECS_r$ ratio compared with market spinach (Fan et al. 2007).

A variation of this ECS method is to suppress charge separation in the PS I reaction centre by illumination with background far-red light which photo-oxidizes the majority of P700. The remaining P700 (reduced form) can be ascertained by transient photo-oxidation with a saturating flash. Allowing for the contribution of charge separation in the remaining PS I complexes that have open traps, the contribution of PS II to the fast phase can be determined and used to estimate the PS II/PS I ratio (Fan et al. 2007). For market spinach, the ratio so obtained was 1.54 ± 0.12, which compares well with the values shown above (Table 1).

To sum up, the fast rise in the EC signal is contributed by charge separation in both photosystems. Hence, the separate contributions by PS II and PS I in leaf tissue can be conveniently obtained to yield the stoichiometry of the two photosystems.

## Conclusions

We have reviewed four approaches for quantifying/monitoring PS II functionality in leaf segments, all except one approach being whole-tissue measurements. Refinements of the methodologies are still needed, but the approaches show great promise in providing a ready measure of active PS II at work in leaf tissue. In particular, the development of portable instruments for kinetic spectrophotometric measurements of P700 and the EC signal in the field will be an important feature for future research in photosynthesis.

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