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Toth, Szilvia Zita; Schansker, Geert; Strasser, Reto

ORIGINAL ARTICLE

A non-invasive assay of the plastoquinone pool redox state based on the OJIP-transient

Szilvia Z. Tóth · Gert Schansker · Reto J. Strasser

Abstract The plastoquinone (PQ) pool of the photosynthetic electron transport chain becomes reduced under anaerobic conditions. Here, anaerobiosis was used as a tool to manipulate the PQ-pool redox state in darkness and to study the effects of the PQ-redox state on the Chl-a fluorescence (OJIP) kinetics in pea leaves (*Pisum sativum* L.). It is shown that the F_J (fluorescence intensity at 3 ms) is linearly related to the area above the OJ-phase (first 3 ms) representing the reduction of the acceptor side of photosystem II (PSII) and F_J is also linearly related to the area above the JI-phase (3–30 ms) that parallels the reduction of the PQ-pool. This means that F_J depends on the availability of oxidized PQ-molecules bound to the Q_B-site. The linear relationships between F_J and the two areas indicate that F_J is not sensitive to energy transfer between PSII-antennae (connectivity). It is further shown that a ~94% reduced PQ-pool is in equilibrium with a ~19% reduction of Q_A (primary quinone acceptor of PSII). The non-linear relationship between the initial fluorescence value ($F_{20\,\mu s}$) and the area above the OJ-phase supports the idea that $F_{20\,\mu s}$ is sensitive to connectivity. This is reinforced by the observation that this non-linearity can be overcome by transforming the $F_{20\,\mu s}$-values into $[Q_A^-]$-values.

Based on the F_J-value of the OJIP-transient, a simple method for the quantification of the redox state of the PQ-pool is proposed.

Abbreviations

Chl	Chlorophyll
DCMU	3-(3′,4′-dichlorophenyl)-1,1-dimethylurea
FNR	Ferredoxin-NADP$^+$-reductase
F_0	Fluorescence intensity at 20 µs when all reaction centres are open
$F_{20\,\mu s}$	Fluorescence intensity measured at 20 µs, also called apparent F_0
F_J	Fluorescence intensity at ~3 ms
$F_{J\text{-}ox}$	F_J-value after 10 s of far-red pre-illumination
F_I	Fluorescence intensity at ~30 ms
F_p	The maximum measured fluorescence intensity
F_m	Fluorescence intensity when all PSII reaction centres are closed
$I_{820\,nm}$	A measure for the transmitted light at 820 nm
$I_{820\,nm}$-10s FR	Transmission value obtained after 10 s of far-red illumination
OJIP-transient	Fluorescence induction transient defined by the names of its intermediate steps
P680 and P700	The primary electron donors of photosystems II and I, respectively
PC	Plastocyanin
PQ	Plastoquinone

Szilvia Z. Tóth and Gert Schansker contributed equally to this study.

S. Z. Tóth · G. Schansker (✉) · R. J. Strasser
Laboratory of Bioenergetics, University of Geneva, Chemin des Embrouchis 10, CH-1254 Jussy, Geneva, Switzerland
e-mail: Gert.Schansker@bioveg.unige.ch

Present Address:
S. Z. Tóth
Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, P.O. Box 521, 6701 Szeged, Hungary

Q_A and Q_B	Primary and secondary quinone electron acceptors of photosystem II, respectively

Introduction

The plastoquinone (PQ) pool forms the functional connection between photosystems II (PSIIs) and cytochrome b_6/f-complexes (cyt b_6/f) in the photosynthetic electron transport chain. The redox state of PQ plays a role in the regulation of several processes. A reduced PQ-pool causes the activation of a kinase that is responsible for the phosphorylation of several PSII-subunits (reviewed by Bennett 1991; Allen 1992). The interaction of reduced PQ with the cyt b_6/f-complex is thought to activate another kinase capable of the phosphorylation of a part of the PSII light-harvesting complex (LHCII) (Vener et al. 1995; 1997). The redox state of the PQ-pool in conjunction with the redox state of the ferredoxin/thioredoxin pool is also thought to play a role in the regulation of the transcription of several genes related to photosynthetic electron transport (e.g. Allen 1993; Escoubas et al. 1995; Pfannschmidt et al. 1999; Trebitsh and Danon 2001). The PQ-pool plays a role in linear electron transport, in chlororespiration, cyclic electron transport around photosystem I (PSI) and the Q-cycle (Heber and Walker 1992; Kramer and Crofts 1993; Bennoun 2001; Haldimann and Tsimilli-Michael 2002; Joët et al. 2002). The PQ-pool forms a buffer between PSII and the rest of the electron transport chain. It has been suggested that the occupancy state of the Q_B-site (that depends directly on the redox state of the PQ-pool) may play a role in the determination of the Chl-a fluorescence yield (Samson et al. 1999; Schreiber 2002; Yaakoubd et al. 2002). On the other hand, the suggested PQ-pool quenching of fluorescence by oxidized PQ-molecules was recently shown not to occur in intact leaves (Tóth et al. 2005).

Despite the central role of the PQ-pool, an accurate and experimentally tested non-invasive assay to determine its redox state in leaves is still lacking. Although it has to be noted that Kruk and Karpinski (2006) recently introduced an invasive, HPLC-based assay for the PQ-pool redox state. Two Chl-a fluorescence parameters have been used for the determination of the PQ-pool redox state: the area above the fluorescence transient (Bennoun 1982, Bennoun 2001) and the initial fluorescence (apparent F_0) value (Joët et al 2002; Groom et al. 1993; Harris and Heber 1993; Feild et al. 1998; Munekage et al. 2002). The use of the apparent F_0 is based on the equilibrium between Q_A and the PQ-pool (Velthuys and Amesz 1973; Diner 1977). It is very sensitive to pre-illumination, connectivity (energy transfer between PSII antennae) (Joliot and Joliot 1964; Strasser 1978; Strasser and Greppin 1981; Strasser et al.

2004) and changes in the redox equilibrium between Q_A and the PQ-pool (e.g. after heat stress, Ducruet and Lemoine 1985). The area above the entire fluorescence transient has as a disadvantage that the reduction of the ferredoxin pool (Terashima and Inoue 1985; Holtgrefe et al. 2003) on the acceptor side of photosystem I considerably contributes to it (Joliot and Joliot 2002).

The kinetics of the Chl-a fluorescence (OJIP) transient are thought to be largely determined by changes in the redox state of Q_A (for a recent review see Lazár 2006) but at the same time, the OJIP-transient reflects the reduction of the photosynthetic electron transport chain (e.g. Schansker et al. 2005). It was suggested that the OJ-phase represents a single charge separation (e.g. Strasser et al. 1995; Schreiber 2002). This interpretation is based on the similarity of the kinetics of the OJ-phase in the presence and absence of the inhibitor DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea) (e.g. Schreiber 2002; Strasser and Stirbet 2001). However, it is also known that in response to a light intensity of 3,000 µmol photons m^{-2} s^{-1} (a standard intensity for fluorescence measurements), each PSII reaction centre is excited approximately once every 200 µs (cf. Schreiber and Neubauer 1990) and 3 ms–where the J step is situated–equates more than 10 potential charge separations. In addition, in heat-treated samples where only one single stable charge separation can occur, fluorescence has a maximum at 300–400 µs (K peak, e.g. Srivastava et al. 1997) and not at ~3 ms. Furthermore, Petrouleas and Crofts (2005) concluded that a pair of electrons needs about 2 ms to reach the PQ-pool. Simulations give similar indications (e.g. Strasser and Stirbet 2001; Lazár 2003; Zhu et al. 2005 (although it has to be noted that Zhu et al. call the 2–3 ms point I)). These observations suggest that the OJ-phase represents the reduction of the PSII acceptor side (reduction of Q_A and Q_B if the site is occupied by a quinone/semiquinone) as suggested by Schansker et al. (2005). This can also be derived from the light-intensity dependence of the OJIP-transients: below 200–300 µmol photons m^{-2} s^{-1} the J-level disappears (e.g. Strasser et al. 1995; Tomek et al. 2001; Schansker et al. 2005). On the basis of the excitation rate calculated by Schreiber and Neubauer (1990) this is understandable, because below 200–300 µmol photons m^{-2} s^{-1} the excitation rate drops below 1 per 2–3 ms. This means that the excitation rate becomes slower than the exchange of PQH_2 for PQ. In other words, the F_J represents the moment when the reduction of the PQ-pool starts.

If the OJ-phase represents the reduction of the acceptor side of PSII and the IP-phase represents the reduction of the acceptor side of PSI (Munday and Govindjee 1969; Schansker et al. 2005; Ilík et al. 2006; Schansker et al. 2006) then it follows that the JI-phase represents the (partial) reduction of the PQ-pool. This agrees with

observations of Schreiber et al. (1989) and is corroborated by the observation that in the presence of DBMIB (it blocks the re-oxidation of PQH_2 by the cyt b_6/f-complex) the IP-phase disappears and the JI-phase increases in amplitude (Schansker et al. 2005).

Anaerobiosis is an excellent tool to reduce specifically the PQ-pool. Oxygen-depletion inhibits the terminal oxidase that normally keeps the PQ-pool in an oxidized state (Cournac et al. 2000; Carol and Kuntz 2001). As a result, constitutive chlororespiratory activity (Joët et al. 2002; Groom et al. 1993; Harris and Heber 1993; Feild et al. 1998; Munekage et al. 2002) leads to a reduction of the PQ-pool. Intermediate states can be obtained by varying the length of the treatment. The acceptor side of PSI remains in the oxidized state (e.g. Kautsky et al. 1960).

The effects of anaerobiosis on the OJIP-transient are well described (e.g. Kautsky et al. 1960; Schreiber and Vidaver 1974; Haldimann and Strasser 1999). In this study, we have used anaerobiosis to study the responses of different parameters derived from the OJIP-transient to a gradual reduction of the PQ-pool. This analysis was used to develop a non-destructive, in vivo assay for the determination of the PQ-pool redox state.

Materials and methods

Plant material

Measurements were carried out on mature leaves of 2–4 weeks old pea plants (*Pisum sativum* L. cv. Ambassador). Plants were grown in a greenhouse where the temperature was 20–25°C during the day and ~14°C at night.

Anaerobiosis treatment

Plants were dark-adapted overnight before the treatment in order to achieve complete Q_A^- and Q_B^- re-oxidation. Leaves were cut off and placed in leaf clips of which the sponges were moistened in order to avoid desiccation during the treatment. These leaf clips were put in a plastic bag. The head of the measuring equipment (Handy PEA or PEA Senior, see below) was also placed in there but the control unit was outside the bag. To achieve anaerobiosis, N_2-gas was blown into the bag for 1–10 min; some outflow was allowed. The measurements were carried out in the N_2-atmosphere. We note that the Chl-*a* fluorescence transients were completely recovered in air within 20 min following a 10-min N_2-gas treatment (data not shown).

DCMU-treatment

Plants were put in darkness for about 1 h before the DCMU-treatment, and then pairs of leaves were placed in small trays (without detaching them from the plant) filled with 10 ml DCMU solution (containing 200 µM DCMU and 1% ethanol that was used to dissolve DCMU). The treatment was carried out in complete darkness and lasted ~14 h. Following the treatment, the leaves were removed from the DCMU-solution (still not detached and in darkness), wiped and left in the air for about 1 h before the measurements were made. This treatment does not damage the leaves and changes in the F_m and F_0-values were avoided (Tóth et al. 2005).

Measuring equipment

Chl-*a* fluorescence emission was measured with a Handy PEA instrument (Hansatech Instruments Ltd, UK). Samples were illuminated with continuous light (650 nm peak wavelength, ~3,000 µmol photons $m^{-2}s^{-1}$ light intensity) provided by three light-emitting diodes (LEDs) and the fluorescence was measured at wavelengths longer than 700 nm. The first reliably measured point of the fluorescence transient is at 20 µs, which is taken as F_0 in the case of aerobic dark-adapted leaves.
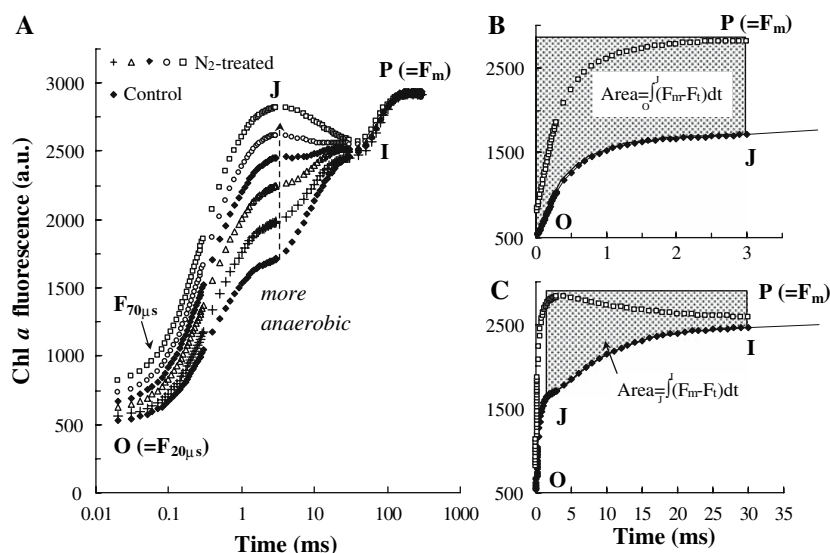
820 nm transmission ($I_{820\ nm}$) and Chl-*a* fluorescence were measured with a PEA Senior instrument (Hansatech Instruments Ltd, UK). The excitation light intensity was ~1,800 µmol photons $m^{-2}\ s^{-1}$, produced by four LEDs (650 nm peak wavelength). Far-red light (718 nm peak wavelength, light intensity of 200 µmol photons $m^{-2}\ s^{-1}$) and modulated FR measuring light (820 nm peak wavelength) were provided by two additional LEDs. Further technical details are described by Schansker et al. (2005, 2006).

Results

Effects of anaerobiosis

Figure 1 demonstrates the effects of anaerobiosis (i.e. the reduction of the PQ-pool) on the Chl-*a* fluorescence (OJIP) transient. Anaerobiosis induces an increase of $F_{20\ µs}$, a strong increase of F_J, approaching F_m under the most anaerobic conditions (after 10 min of N_2-treatment) and a strong decrease of the areas above the OJ and JI-phases of the transient (Figs. 1B, C). The F_m and the IP-phase remained nearly unaffected by the anaerobiosis treatment. The anaerobiosis-treatment also caused an increase of the initial slope (Figs. 1A, B). In the most anaerobic samples, the fluorescence rises during the OJ-phase as in DCMU-treated samples (Haldimann and Strasser 1999), where the Q_B-site is occupied by a DCMU-molecule and not by an oxidized PQ-molecule.

Fig. 1 Effects of anaerobiosis (1–10 min N_2-gas treatment) on the Chl-*a* fluorescence (OJIP) transient of pea leaves measured at 3,000 µmol photons m^{-2} s^{-1}. (**A**) OJIP transients presented on a logarithmic time scale. (**B**) the first 3 ms of the OJIP transients presented on a linear time scale; the area above the OJ-phase is indicated for the control sample. (**C**) the first 30 ms of the OJIP transients presented on a linear time scale; the area above the JI-phase is indicated for the control sample. Each transient is an average of ~15 measurements; the data are presented without any normalization

It is known from other studies that F_J is very sensitive to changes in the redox state of the PQ-pool induced either by anaerobiosis or a preceding saturating light pulse (Haldimann and Strasser 1999; Haldimann and Tsimilli-Michael 2002). This is demonstrated in Fig. 1. The time needed to reach F_J is nearly independent of the Q_A redox state at the start of the measurement (see e.g. Fig. 2 in Schansker et al. 2005) and therefore the J-step is not expected to be affected by the initial Q_A^- concentration. As mentioned in the Introduction, it is very likely that during a saturating light pulse the acceptor side of PSII is largely reduced at the J step and the J step may be the starting point for the reduction of the PQ-pool. Therefore, F_J could be an indicator of the PQ-pool redox state *at the start* of the saturating light pulse. In the following this hypothesis was evaluated.

Analysis of the area above the fluorescence transient

The area above the fluorescence transient is generally assumed to be a measure for the number of electrons that have to flow through the electron transport chain in order to reach F_m (e.g. Bennoun 1982; Strasser and Strasser 1995; Joliot and Joliot 2002). However, the area above the fluorescence transient can only be used as an indicator for the PQ-pool redox state under special conditions (e.g. in the case of mutants that show little electron transport beyond the PQ-pool (Bennoun 1982, 2001). Otherwise the area above the IP-phase, representing the filling up of the Fd-pool on the acceptor side of PSI (Schansker et al. 2005) contributes considerably to the total area (Joliot and Joliot 2002). Therefore we treated the areas above the different phases separately.

The area above the OJ-phase is likely to represent the number of electrons needed for the reduction of the PSII acceptor side (see Introduction). As the PQ-pool becomes more reduced (longer anaerobiosis treatment) fewer Q_B-sites will be occupied by an oxidized PQ-molecule and fewer electrons are needed to reduce the acceptor side of PSII. The area above the JI-phase may be proportional to the number of electrons needed for the reduction of the PQ-pool. Figure 2 shows that both areas (calculated by taking into account the changes in the data acquisition of the fluorescence signal and using a linear time-base) were linearly related to the F_J-value. The linear relationship between F_J and the area above the OJ-phase is an indication that the F_J-value is linearly related to the number of electrons needed to reduce the acceptor side of PSII. This implies at the same time that the F_J-value is not sensitive to connectivity. This agrees with the study of Strasser and Stirbet (2001) who showed that connectivity only affected the first few hundreds of µs of the fluorescence rise but disagrees with Baker and Oxborough (2004) and Kramer et al. (2004) who assumed that the whole fluorescence rise (OJIP) was affected by connectivity. The linear relationship between F_J and the area above the JI-phase indicates that changes in F_J follow the reduction of the PQ-pool very closely. The transients measured on the more anaerobic leaves show that during the JI-phase the area above the transient is due to a decrease of the fluorescence value. The fluorescence decrease is caused by a partial re-oxidation of the PQ-pool and electron transfer towards PSI (e.g. Kautsky et al. 1960; Schreiber and Vidaver 1974). As a result fewer electrons are needed during the IP-phase to reduce the acceptor side of PSI and this is observed as a decrease of the area above the IP-phase as a function of F_J (data not shown).
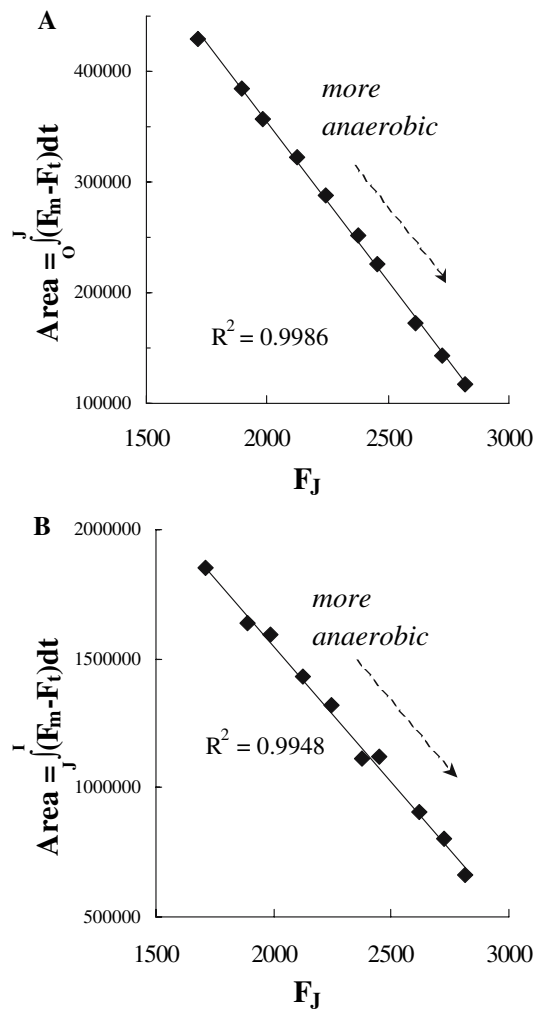
Fig. 2 Areas above the OJ (**A**) and JI (**B**) phases of the OJIP transients plotted as a function of F_J. The values are derived from transients like the ones presented in Fig. 1. Each point corresponds to an average of ~15 measurements

The OJ-rise

A basic requirement for using the F_J as an indicator of the PQ-pool redox state would be that there is no change in the PQ-pool redox state during the first 2–3 ms of the strong light pulse. On the basis of published data there is a strong experimental support for this (see Introduction), but we looked for further evidence investigating the initial part of the OJIP-transient in relation to the F_J-value. The apparent F_0-value ($F_{20 \mu s}$ in our case) has been used as an indicator of the PQ-pool redox state (see Introduction). In Fig. 3A the relationship between F_J and the $F_{20 \mu s}$ values is shown. The relationship between the two parameters was hyperbolic. If the $F_{20 \mu s}$-value would be affected by connectivity and the F_J-value not (as suggested in the previous paragraph) a hyperbolic relationship between the $F_{20 \mu s}$ and F_J-values would be expected (Strasser et al. 2004). Another parameter representing the first part of the fluorescence transient is the

initial slope (($F_{70 \mu s}$–$F_{20 \mu s}$)/50 μs). It was assumed that the two time points ($F_{20 \mu s}$ and $F_{70 \mu s}$) were so close to each other that they were affected to the same extent by connectivity, so the difference ($F_{70 \mu s}$–$F_{20 \mu s}$) should be free from it. Fig. 3B demonstrates that there is a linear relationship between the initial slope and F_J. This indicates that both the F_J and the initial slope are affected in the same way by changes in the PQ-pool redox state. The control value in Fig. 3B did not fall on the regression line. Although we do not know the reason for this deviation we may speculate that non-Q_B-reducing centres are insensitive to the PQ-redox state. Their contribution to the fluorescence rise could be an explanation for the observed deviation (see Schansker and Strasser 2005 and references therein).

In Fig. 4 a more elaborate approach was used to determine the effect of the PQ-pool redox state on $F_{20 \mu s}$ in the absence of connectivity. If the non-linearity between the $F_{20 \mu s}$-values and F_J is really due to connectivity, the relationship between the fraction Q_A^- and F_J should be linear. In DCMU-treated samples (Fig. 4A) the Q_A-reduction kinetics can be obtained by determining the area-growth kinetics of the complementary area above the Chl-a fluorescence transient (Malkin and Kok 1966; Murata et al 1966; Joliot and Joliot 1979; Melis and Schreiber 1979). Melis and Schreiber (1979) showed that the area growth, C–550 (electrochromic band-shift in response to changes in the Q_A redox state) and A320 (direct measure of the redox state of Q_A) kinetics are strictly proportional. Since the two maxima of the normalized area growth are defined (all Q_A either oxidized or reduced), the fraction Q_A^- can be obtained by normalizing the area growth (Fig. 4B) between 0 and 1. In Fig. 4C the raw fluorescence kinetics are plotted versus the normalized area growth. We assumed that this curve could be used to determine the fraction Q_A^- at $F_{20 \mu s}$ in the absence of DCMU (see the arrows from Fig. 4A to the x-axis of Fig. 4C). The observation that the normalized area growth of the OJ-phase is nearly identical in the presence or absence of DCMU (Tóth 2006) supports this assumption. It is important to note that the F_0 and F_m-values were the same for DCMU-treated and control leaves (see Tóth et al. 2005 for details).

In Fig. 4D the fraction of Q_A^- at the start of the measurement is given as a function of F_J. There is a very good linear relationship between the two parameters indicating that both parameters are affected in the same way by changes in the PQ-pool redox state. It supports the premise that F_J can be used as an indicator of the PQ-pool redox state before the illumination. It may be interesting to note that under the most anaerobic conditions (~94% of the PQ-pool reduced) Q_A is reduced in about 19% of the reaction centres (Fig. 4D).

So far, we have only made use of fluorescence parameters. To confirm the relationship between F_J and redox state
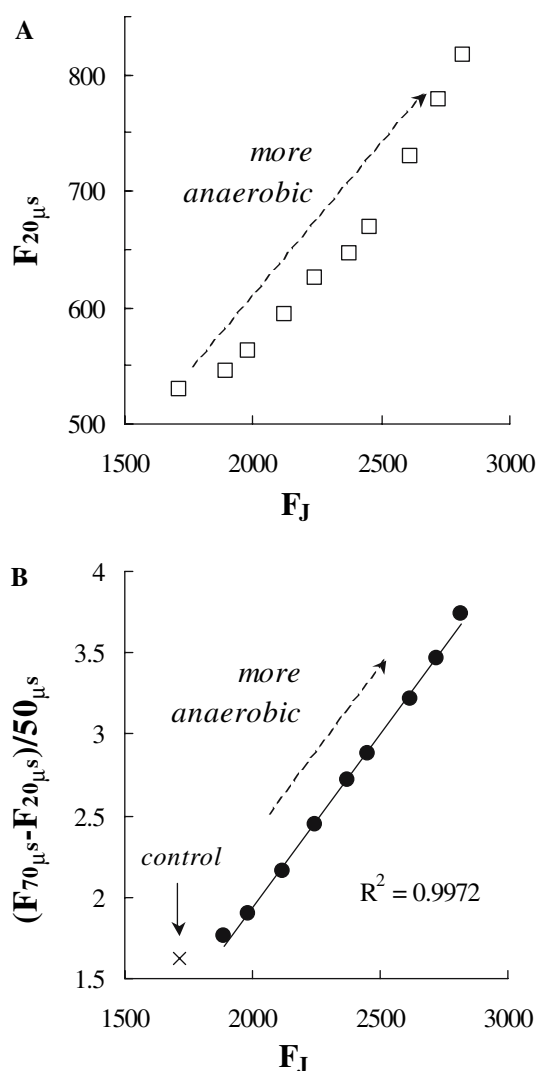
A

$F_{20\mu s}$

more anaerobic

F_J

B

$(F_{70\mu s} - F_{20\mu s})/50\mu s$

more anaerobic

control

$R^2 = 0.9972$

F_J

Fig. 3 $F_{20\mu s}$-values (**A**) and initial slopes (($F_{70\mu s}$–$F_{20\mu s}$)/50 μs) (**B**) plotted as a function of F_J. The values are derived from transients like the ones presented in Fig. 1. Each point corresponds to an average of ~15 measurements

of the PQ-pool we searched for a completely independent parameter. 820 nm transmission ($I_{820\ nm}$) measurements can be used to monitor electron flow through PSI since changes of $I_{820\ nm}$ reflect changes in the redox states of PC, P700 and ferredoxin (Fd) with Fd being a minor contributor in pea leaves (Klughammer and Schreiber 1991; Schansker et al. 2003). Figure 5A shows that a 10 s FR light induces less and less oxidation of P700 and PC as leaves become more anaerobic (seen as a decrease in $I_{820\ nm}$). Longer FR illuminations lead to a levelling off of $I_{820\ nm}$ approximately at the values measured after 10 s (data not shown). In dark-adapted pea leaves the ferredoxin-NADP$^+$-reductase (FNR) is inactive and the re-oxidation of the Fd-pool occurs with a decay time of about 0.4 s (Schansker et al. 2003). This means that under aerobic conditions 10 s is enough for several reduction-oxidation cycles. It should be noted that

there was little indication of Mehler-type reactions under our experimental conditions. This may be due to a pH-gradient-dependence of this reaction pathway (Hormann et al. 1994). As the development of anaerobiosis proceeds, the stroma becomes more and more reduced and the re-oxidation of Fd more and more difficult. This leads to a limited oxidation of PC and P700 (Fig. 5A). Therefore, the extent of PC and P700 oxidation by FR light probably forms an indicator for the extent of anaerobicity of the sample. In other words, the linear relationship between F_J and $I_{820\ nm}$ after 10 s FR light ($I_{820\ nm}$ (10s FR)) indicates that F_J is linearly related to the extent of anaerobicity of the leaf.

Assay for the determination of the PQ-pool redox state

To calculate the PQ-pool redox state not only the F_J-value of the sample but also the F_J-values of the leaf in the presence of a fully reduced and a fully oxidized PQ-pool have to be determined. For leaves with a fully reduced PQ-pool, it can be assumed that F_J equates approximately F_m as it is known that at F_m the whole electron transport chain including the PQ-pool is reduced (e.g. Schansker et al 2003; Schansker et al 2005) and at short times (0.1–5 s) after a saturating pulse F_J is very close to the F_m-value (Fig. 6D).

To obtain an F_J-value for the fully oxidized PQ-pool (F_{J-ox}) a second measurement is needed. To oxidize the PQ-pool at short times after the measurement of the first OJIP-transient, FR light was used. Fig. 6A shows that the FR pulse (200 μmol photons m^{-2} s^{-1}) has to be at least 5 s long to obtain a minimum F_{J-ox}-value. The relatively long (5 s) FR pulse is probably necessary because a pool of stromal electrons (Asada et al. 1992; Schansker et al. 2003) has to be depleted by the FR pulse first, to maintain the PQ-pool in the oxidized state for some time. Fig. 6B shows that the second measurement has to be made within 5 s after the FR pulse (the FR pulse length was 10 s in the case of Fig. 6B) to obtain a reliable F_{J-ox}-value. At longer times after the FR-pulse non-photochemical re-reduction of the PQ-pool leads to a significant increase of the F_J-value.

On the basis of the data in Figs. 6A and B a protocol for the determination of the PQ-pool redox state can be designed: the measurement of two OJIP-transients with a FR pulse in between. The first transient gives the F_J and F_m-values. Subsequently, the minimum $F_{J–ox}$-value, representing a fully oxidized PQ-pool is obtained by giving a FR pulse (200 μmol photons m^{-2} s^{-1}) of at least 5 s duration followed within seconds (1 s in this case) by a second fluorescence measurement. The fraction of reduced PQ then equates (F_J–F_{J-ox})/(F_m–F_{J-ox}) as indicated in Fig. 6C. Our method does not depend on an external reference: the extremes of the scale (0 and 1 that correspond to F_{J-ox} and F_m) are determined separately for each sample.

Fig. 4 Determination of the fraction of Q_A^- at $F_{20\ \mu s}$ by taking connectivity between PSII antennae into account. (**A**): Chl-*a* fluorescence transients of untreated (control), anaerobic (10 min treatment in N_2-gas) and DCMU-treated samples are shown. (**B**): Normalized area growth curve of a DCMU-treated sample, which is assumed to be proportional to $[Q_A^-]$. (**C**): Chl-*a* fluorescence transient as a function of area growth of a DCMU-treated sample was used as a reference curve to read the fraction of Q_A^- at $F_{20\ \mu s}$ of the anaerobic sample. (**D**): Fraction of Q_A^- as a function of the fraction of reduced PQ-pool. The circled square represents the anaerobic sample of panel A
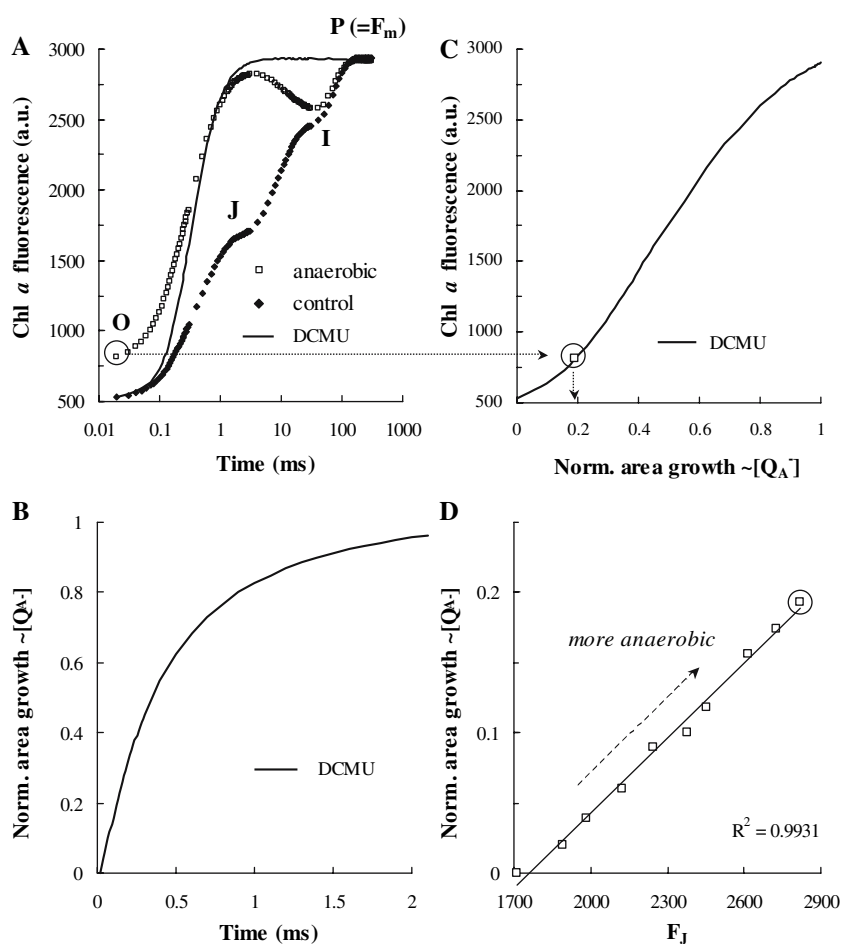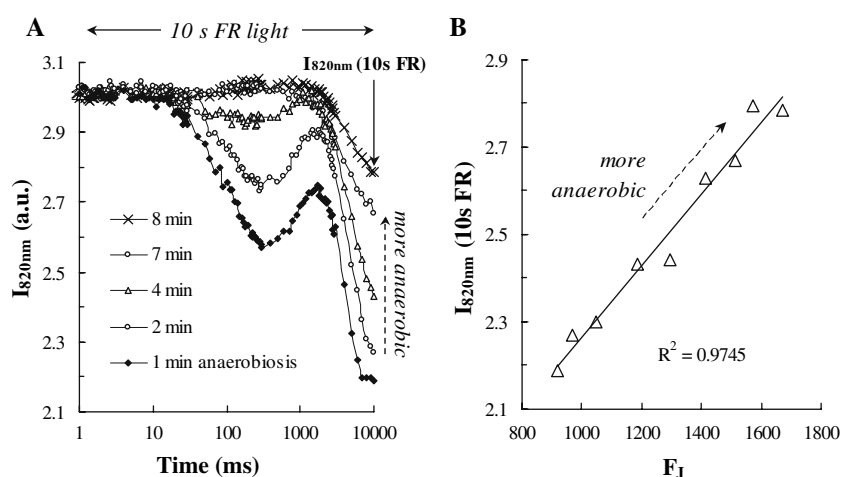

Fig. 5 (**A**): FR-light induced changes in the $I_{820\ nm}$-value of leaves pre-exposed to 1–8 min anaerobiosis treatments. (**B**): Relationship between the $I_{820\ nm}$-change induced by 10 s FR light ($I_{820\ nm}(10s\ FR)$) and F_J. The intensity of the FR light was 200 μmol photons $m^{-2}\ s^{-1}$. Each transient is an average of ~15 measurements; the data are presented without any normalization

In Fig. 6D an example of an application is given: the re-oxidation kinetics of the PQ-pool of pea and camellia were followed after a saturating pulse by giving a second light pulse at various times after the first one. In dark-adapted (well-developed) pea leaves approximately 14% of the PQ-pool was in the reduced state and 100 ms after the first saturating pulse the PQ-pool was ~94% reduced. The τ-value for the re-oxidation of the PQ-pool was ~60 s. The re-oxidation of the PQ-pool in camellia was much faster but heterogeneous with a τ ~9 s explaining two thirds of the kinetics. Depending on the plant species, up to 100-fold differences can be found (MG Ceppi and G Schansker, unpublished data). This is most probably caused by the difference in the activity of the terminal oxidase that re-oxidizes the PQ-pool in darkness (Cournac et al. 2000; Carol and Kuntz 2001).
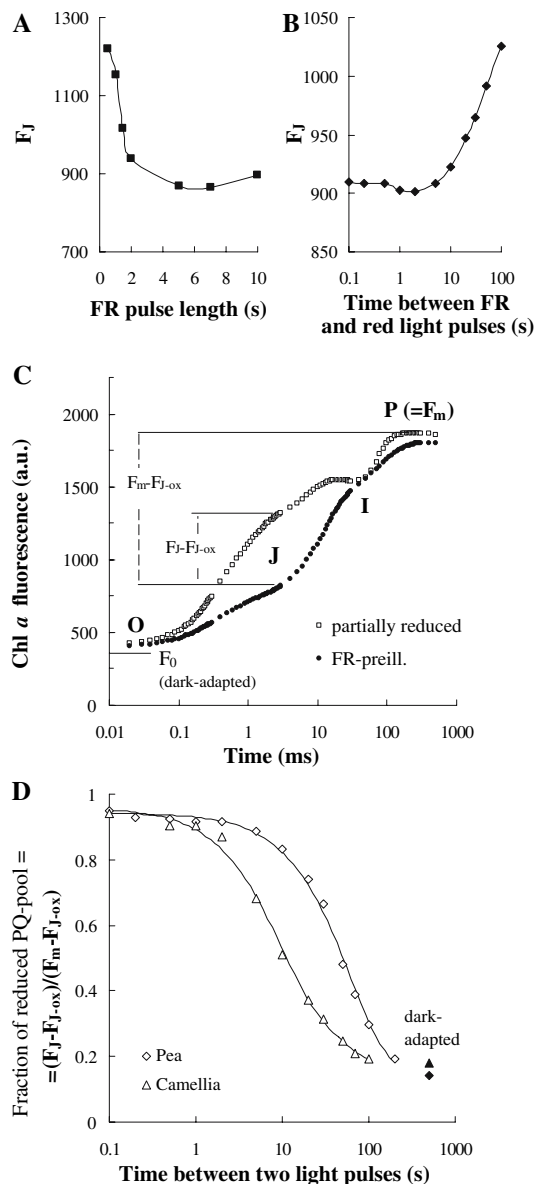
Fig. 6 Determination of the PQ pool redox state. (**A**) Effect of FR-pulse length on the measured F_J-value. (**B**) Effect of the dark-interval between a 10 s FR-pulse and the Chl-*a* fluorescence measurement on the F_J-value. (**C**) Parameters needed for the calculation of the PQ-pool redox state. The transient with the open symbols represents the sample of which the fraction of reduced PQ-pool has to be determined (in this case a leaf that had been pre-illuminated with red light). This transient gives the F_J and F_m-values. A second transient is measured 1 s after a 10 s FR-pulse (closed symbols). It gives the minimum F_J-value (F_{J-ox}), representing the oxidized PQ-pool. The fraction of reduced PQ then equates $(F_J-F_{J-ox})/(F_m-F_{J-ox})$. See text for details. (**D**): example of an application: the re-oxidation kinetics of the PQ-pool of pea and camellia leaves were monitored in darkness following a saturating light pulse. The intensity of the red and FR-pulses was 1,800 and 200 μmol photons m^{-2} s^{-1} respectively. The data represent averages of four (**A** and **B**) or 2 (**C** and **D**) measurements

Therefore, its activity can be studied by the regeneration of the $(F_J-F_{J-ox})/(F_m-F_{J-ox})$ value following a saturating light pulse.

Discussion

The principle underlying F_J as an indicator of the PQ-pool redox state

The relationship between F_J and the redox state of the PQ-pool may be explained by the sensitivity of F_J to the occupancy state of the Q_B-site (the fraction of Q_B-sites containing an oxidized PQ-molecule). If the PQ-pool is completely reduced there are no oxidized PQ-molecules available that can bind to the Q_B-sites and the fluorescence rises nearly to F_m in 3 ms like in the case of DCMU-treated leaves (see Figs. 1A, 4A). Robinson and Crofts (1983) have suggested that the binding constants of PQ and PQH_2 for the Q_B-site are the same. This assumption is necessary to explain the linear relationship between the occupancy state of the Q_B-site and the redox state of the PQ-pool. If both binding constants are the same, the fraction of Q_B-sites to which PQ-molecules are bound will be independent of the redox state of the PQ-pool.

Possible factors affecting the calculation of the PQ-pool redox state

If the first red light pulse (i.e. OJIP-transient) is too long (longer than 1–2 s), FNR will become activated. This will lead to a lower F_m-value of the second OJIP-transient. If the FR pulse is longer than 1–2 s, PC and P700 will become oxidized. As a consequence, more electrons are needed to reduce the electron transport chain and reach F_m during the second light pulse. In this case FNR may become activated before F_m is reached (Schansker and Strasser 2005). In both cases the result is a lower F_m-value for the second OJIP-transient. However, this F_m-value is not used for the calculation of the PQ-pool redox state and the activation state of the acceptor side of PSI has no effect on the F_J-value (Schansker et al. 2005).

Far-red light excites PSII slightly (e.g. Pettai et al. 2005; Schansker and Strasser 2005), causing an increase of $F_{20\ \mu s}$ due to some Q_A-reduction (Schansker and Strasser 2005). As argued above the F_J-value is not very sensitive to the Q_A^- concentration at the start of the measurement and therefore this effect can be ignored as well.

Potential limitations of the assay

This study is based on measurements of unstressed plants. There are a few practical limitations of the formula $(F_J-F_{J-ox})/(F_m-F_{J-ox})$ that have to be considered. Determination of a correct F_m-value is critical for the assay. In severely heat-treated samples it is not possible to close all PSII RCs with a strong light pulse (Tóth et al. 2007) and therefore the assay cannot be quantitatively used. The same limitation

applies to light-adapted leaves (Schansker et al. 2006). In light-adapted leaves an additional problem would be to obtain an F_{J-ox}-value without affecting the light-adapted state. The F_J also responds to changes in the PQ-pool redox state in light adapted leaves (Schansker et al. 2006) and changes in the F_J-value can be used as a qualitative indicator.

The PSII electron donation capacity can become smaller because of photoinhibition, UV-damage, partial inhibition at the Q_B-site, etc. Under these conditions the unaffected reaction centres will function normally. As long as the remaining PSII centres are able to reduce the electron transport chain and all the centres can be closed, only the number of PQ-molecules per PSII will increase. Therefore the $(F_J-F_{J-ox})/(F_m-F_{J-ox})$ value will not be affected. Indeed, partial (0–60%) inhibition of PSII reaction centres by DCMU did not affect the calculation of the redox state of the PQ-pool in dark-adapted leaves (SZ Tóth, unpublished data).

A third factor that can be considered is the excitation rate of the antenna. Both light intensity and antenna size affect the excitation rate and thereby the electron transport activity. By decreasing the light intensity, the limitation due to the exchange of PQ at the Q_B-site becomes less important (lower J-value) whereas the IP-phase increases in size (Strasser et al. 1995; Tomek et al. 2001; Schansker et al. 2005). In isolated thylakoid membranes a large (10-fold) increase in the Chl-concentration led to a lower F_J-value (Sušila et al. 2004). A less saturated F_J would indicate a less reduced PSII acceptor side at F_J. This could lead to some overlap in the reduction of the PSII acceptor side and the reduction of the PQ-pool, which would cause an underestimation of F_{J-ox}. However, stress factors rather lead to a decrease in Chl-content and not to an increase; therefore the risk of an underestimation of F_{J-ox} is limited. In specific cases it may be useful to determine the light intensity dependence of the calculated PQ-pool redox state.

A fourth potential problem would be a limitation on the acceptor side of PSI. As shown in Fig. 5, under anaerobic conditions the flow of electrons through PSI is limited, which may limit the oxidation of the PQ-pool by a FR light pulse and therefore the F_{J-ox}-value may be overestimated.

In summary, taking into account the limitations discussed above, the proposed assay should give a good estimate of the PQ-pool redox state.

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