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Quantification of non-Q_B-reducing centers in leaves using a far-red pre-illumination

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Abstract

An alternative approach to quantification of the contribution of non-Q_B-reducing centers to Chl *a* fluorescence induction curve is proposed. The experimental protocol consists of a far-red pre-illumination followed by a strong red pulse to determine the fluorescence rise kinetics. The far-red pre-illumination induces an increase in the initial fluorescence level ($F_{25\ \mu s}$) that saturates at low light intensities indicating that no light intensity-dependent accumulation of Q_A^- occurs. Far-red light-dose response curves for the $F_{25\ \mu s}$-increase give no indication of superimposed period-4 oscillations. $F_{25\ \mu s}$-dark-adaptation kinetics following a far-red pre-pulse, reveal two components: a faster one with a half-time of a few seconds and a slower component with a half-time of around 100 s. The faster phase is due to the non-Q_B-reducing centers that re-open by recombination between Q_A^- and the S-states on the donor side. The slower phase is due to the recombination between Q_B^- and the donor side in active PS II reaction centers. The pre-illumination-induced increase of the $F_{25\ \mu s}$-level represents about 4–5% of the variable fluorescence for pea leaves (~2.5% equilibrium effect and 1.8–3.0% non-Q_B-reducing centers). For the other plant species tested these values were very similar. The implications of these values will be discussed.

Abbreviations: Chl – chlorophyll; DCMU – 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; $F_{25\ \mu s}$ – initial fluorescence, for dark adapted leaves equal to F_o; F_{pl} – intermediate fluorescence induction level when measured at low light intensity; F_v – variable fluorescence here used as maximum variable fluorescence; LED – light emitting diode; OJIP curve – fluorescence induction transient defined by the names of its intermediate steps, O-level is fluorescence-level at 25 μs, J-level is fluorescence-level at ~2 ms, I-level is fluorescence level at ~30 ms and P-level is F_m, the maximum fluorescence; Q_A and Q_B – primary and secondary quinone electron acceptors of Photosystem II

Introduction

PS II reaction centers are (among other things) characterized by their heterogeneity in terms of antenna size and function. On the basis of differences in antenna size, α- and β-centers have been defined and on the basis of acceptor side function, Q_B-reducing and non-Q_B-reducing centers or in the terminology of Lavergne and Briantais non Q_B-transferring (Black et al. 1986; Lavergne and Briantais 1996). The non-Q_B-reducing centers are incapable of transferring electrons from the electron acceptor Q_A to the secondary electron acceptor Q_B. In such centers Q_A^- can only be re-oxidized by a back reaction with the donor side of PS II (Lavergne and Briantais 1996, and references therein).

Forbush and Kok (1968) reasoned that if the excitation rate of PS II were much lower than the rate of forward electron transfer, no Q_A^- would accumulate (as long as the plastoquinone pool was not yet reduced). For the fluorescence rise F_o–F_{pl} this condition seemed to be met and therefore it should, according to these authors, be unrelated to 'normal' photochemistry. Forbush and Kok (1968) also presented data showing that the dark-recovery of the F_o–F_{pl} phase was biphasic. Melis (1985) proposed on the basis of the effects of various electron acceptors on the F_o–F_{pl} phase that it represented the closing of the PS II β-centers (he defined PS II β-centers the way non-Q_B-reducing centers are defined here). Melis assumed that the biphasic kinetics observed by Forbush and Kok (1968) were the result of an artefact caused by the isolation of the chloroplasts. Chylla and Whitmarsh (1989) repeated the analysis of the dark-recovery kinetics of the F_o–F_{pl} phase. They fitted their data with a single exponential function despite a considerable deviation from the fit around a dark time interval of 10 s that probably would have justified a two-component fit. It should be noted that both Melis (1985) and Chylla and Whitmarsh (1989) used 40–50 μmol green light $m^{-2}\,s^{-1}$ for their experiments. The F_o–F_{pl} method aroused considerable interest, but also criticism. Hsu and Lee (1991) using a mathematical approach showed that the F_o–F_{pl} increase did not arise solely from non-Q_B-reducing centers but that a part could be attributed to the equilibrium between Q_A and Q_B in functional reaction centers. In the rest of the text we will refer to this contribution as the equilibrium effect. Lavergne and Leci (1993) showed that period-4 oscillations also contributed to this initial fluorescence rise and Tomek et al. (2003) by a modelling approach showed that down to very low light intensities (2 μmol photons $m^{-2}\,s^{-1}$) it was not possible to separate unambiguously contributions from non-Q_B-reducing centers and functional reaction centers to the fluorescence induction curve.

The contribution of period-4 oscillations could be removed by destroying the manganese cluster by e.g. hydroxylamine (Lavergne and Leci 1993). This method also limited the number of stable charge separations to 1 and thus prevented the light intensity-dependent accumulation of Q_A^- in functional reaction centers. Apart from the potential artefacts induced by a chemical treatment of PS II, the method is difficult to apply to leaves and it would still be necessary to separate the contributions of the non-Q_B-reducing centers and the equilibrium effect to the fluorescence induction curve.

Here, we present a different approach for determining the contribution of non-Q_B-reducing centers to the fluorescence induction curve. Far-red light (718 nm) causes a very slight excitation of PS II. A 10 s far-red pulse with an intensity of 80 μmol photons $m^{-2}\,s^{-1}$ induces a small $F_{25\ \mu s}$-increase (~4–5% of F_v) that is insensitive to a further increase of the far-red light intensity. The far-red-induced $F_{25\ \mu s}$-increase is shown to consist of contributions of non-Q_B-reducing centers and Q_A^- related to the equilibrium effect. These two contributions can be separated by analysis of the dark-adaptation kinetics of the far-red light-induced $F_{25\ \mu s}$-increase.

Materials and methods

Plant material

For most measurements mature leaves of 2–3 week-old pea plants (*Pisum sativum* L. cv. Ambassador) were used. Plants were grown in a greenhouse where the temperature was 20–25 °C during the day and 14–16 °C at night. The other plants used (*Ficus benjamina*, vine, Amaryllis, barley, kiwi, Schefflera) were grown in the greenhouse or near the laboratory. Measurements for each data-set were taken from single leaves that were left attached to the plant with approximately 15 min dark-adaptation time between measurements.

Chl a *fluorescence measurements*

The Chl *a* fluorescence measurements were recorded with a PEA Senior instrument (Hansatech Instruments, King's Lynn, Norfolk, UK). The red light intensity used for all experiments was 1800 μmol photons $m^{-2}\,s^{-1}$, produced by four 650 nm LEDs (light-emitting diodes). The far-red source was a QDDH73520 LED (Quantum Devices Inc.) filtered at 720 ± 5 nm. In the inset of Figure 1A, the emission spectrum of the far-red LED is shown (scan rate 100 nm/min, slit width 0.5 mm, Jobin Yvon H 10 vis monochromator).

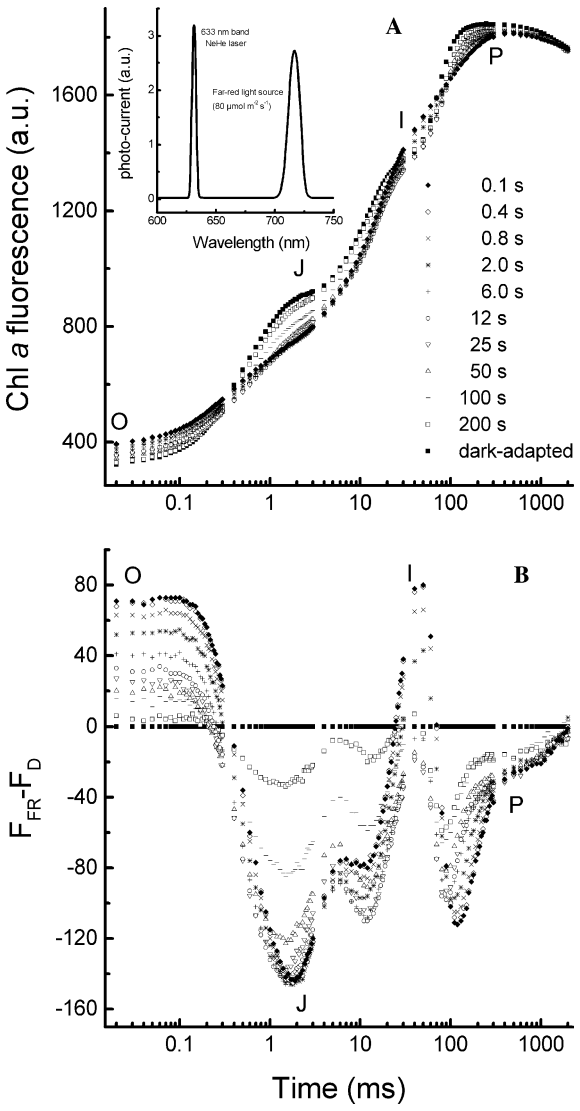

Figure 1. Effect of a far-red pre-illumination on OJIP-fluorescence induction curves measured at different times after a far-red pulse. In (A) the measured transients are shown and in (B) the transient measured in the absence of a far-red pre-illumination was subtracted from the other transients. The pre-illumination consisted of a 10 s 80 µmol m^{-2} s^{-1} far-red light pulse. The results of a representative data set are shown. The inset of (A) shows the emission spectrum of the far-red LED and of the 633 nm light of a NeHe laser that was used as a reference.

Using the 633 nm band of a NeHe laser as reference, we determined the emission peak of the far-red LED to be at 718 nm with a bandwidth at half peak height of 9.5 nm. The inset also shows that emission below 700 nm is negligible. The far-red light intensity is indicated in the figures. The modulated (33.3 kHz) far-red measuring light was provided by an OD820 LED (Opto Diode Corp.) filtered at 830 ± 20 nm. The 830 nm light is not photosynthetically active.

Results

Effect of far-red light on the form of the fluorescence induction curve

In Figure 1, the effects of a 10 s far-red pre-illumination on the fluorescence induction curve are shown. The far-red light caused a small (4–5% of F_v) increase of the $F_{25\ \mu s}$-level and a considerable depression of the J-level. It can also be observed that after the far-red pre-illumination, more time is needed to reach the maximum fluorescence level. To visualize these differences better, the control transient measured in the absence of a far-red pre-illumination was subtracted from the transients measured at various times after the far-red pre-illumination, and the resulting curves are shown in Figure 1B. The differences show three minima around 2, 12 and 85 ms. The 820 nm transmission data that were measured in parallel indicated that the valley at 85 ms may be related to the extra time needed to reduce plastocyanin and P700 oxidized by the far-red light (not shown). The minima at 2 and 12 ms could be related to the occupancy of the Q_B-site and the redox state of the plastoquinone pool, respectively. We also tested if the far-red light pulses induce more structural changes in the electron transport chain. A protocol consisting of a 10 s far-red pulse followed by two red pulses spaced 3 s apart was used for this purpose. The results showed that the second red pulse strongly depended on the first red pulse, but it was insensitive to the far-red pulse or to the time between the far-red pulse and the first red pulse (not shown). It indicates that the far-red light induces mainly changes in the redox state of the electron transport chain.

Far-red dose effect

In Figure 2 the far-red dose effect on the $F_{25\ \mu s}$ increase is demonstrated. Figure 2A shows that for pea, *Ficus benjamina* and kiwi leaves, the effect of a 10 s far-red light pulse on the $F_{25\ \mu s}$-level saturates near 50–80 µmol m^{-2} s^{-1}. Figure 2B shows the

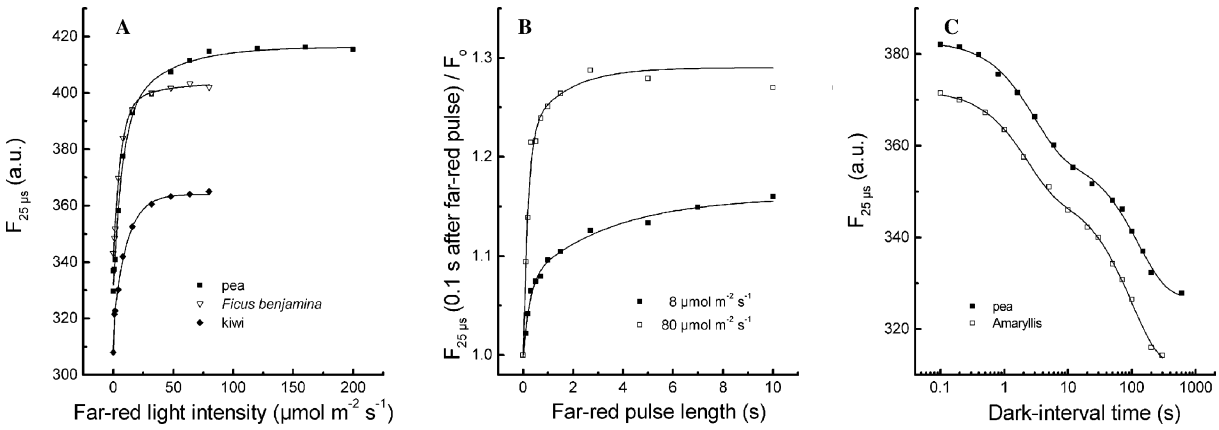

Figure 2. Light and dark-adaptation of the $F_{25\ \mu s}$-level as a function of a far-red pre-illumination. In (A) and (B) far-red-light-dose response curves are shown. The dose was varied either by changing the far-red light intensity (A) or by changing the pulse length (B). In (C) the $F_{25\ \mu s}$-level as a function of the dark-time interval after a 10 s 80 μmol m^{-2} s^{-1} far-red light pulse is shown. The $F_{25\ \mu s}$-levels in (A) and (B) were determined 100 ms after the far-red pre-illumination. The far-red pulse length in (A) and (C) was 10 s. In (B) only data obtained from pea leaves are shown. For the different panels data from 3–5 independent measurements were averaged.

far-red pulse length dependence of the $F_{25\ \mu s}$-increase for 8 and 80 μmol photons m^{-2} s^{-1} pulses for pea leaves. The saturation of the $F_{25\ \mu s}$-increase indicates that the far-red light used does not cause a light intensity dependent accumulation of Q_A^-. On the other hand, the far-red light does induce a large accumulation of Q_A^- in leaves that were pre-treated with DCMU. Far-red illumination of DCMU-inhibited leaves led to a strong increase of $F_{25\ \mu s}$ of up to 70–80% of total F_v in fully inhibited leaves (data not shown). It is also worth noting that we did not observe any period-4 oscillations superimposed on the dose response curves shown in Figure 2.

Separation of the equilibrium effect from the non-Q_B-reducing centers

The equilibrium effect can simply be separated from fluorescence changes related to non-Q_B-reducing centers by making use of the differences in the recombination kinetics. Q_A^- in non-Q_B-reducing centers should disappear by recombination with the donor side of PS II with decay times of a few seconds (Lavergne and Etienne 1980; Rutherford and Inoue 1984). The concentration of Q_A^- related to the equilibrium effect disappears at a much slower rate (decay time of 30 s or more) (Rutherford et al. 1984; Vermaas et al. 1984).

In Figure 2C this principle is demonstrated for pea and Amaryllis leaves. The dark-adaptation kinetics of the $F_{25\ \mu s}$ -level are biphasic and can be fit quite well with two exponential functions as shown in Figure 2C. The faster of the two dark-adaptation phases has a lifetime of 2–3 s and the slower phase has a lifetime of ~100 s.

Plant species dependent variability

In Table 1, the estimated contributions of the non-Q_B-reducing centers and the equilibrium effect for several plant species are given on the basis of three measurements: $F_{25\ \mu s}$ measured after dark adaptation ($= F_o$) and 0.1 and 15 s after a far-red pulse

Table 1. Contributions of non-Q_B-reducing centers and the equilibrium effect (sharing of an electron between Q_A and Q_B) as a percentage of the maximum variable fluorescence for several plant species

	Non-Q_B-reducing centers	Equilibrium effect
	Percentage of variable fluorescence	
Pea (summer)	3.0 ± 0.6	1.8 ± 0.5
Pea (autumn)	1.8 ± 0.5	2.5 ± 0.3
Barley	2.8 ± 0.3	1.9 ± 0.5
Schefflera	2.8 ± 0.4	2.9 ± 0.4
Vine	2.5 ± 0.3	1.0 ± 0.3
Amaryllis	1.6 ± 0.1	2.7 ± 0.3

For pea and Amaryllis the values were determined as demonstrated in Figure 2 and for the other three plant species estimated on the basis of three-point measurements. See the text for a description of the estimation-method. The averages of data from 3–7 leaves with their standard deviation are given.

(cf. Figure 2C). The values are very similar for the different plants measured. It is worth noting that pea leaves measured in autumn (lower ambient light levels) contained fewer non-Q_B-reducing centers (1.8%) than pea leaves measured in summer (3.0%).

Discussion

Kinetic separation of the non-Q_B-reducing centers and the equilibrium effect

Non-Q_B-reducing centers are blocked in the state Q_A^- and cannot transfer electrons towards PSI for structural reasons. This in contrast to the PS II reaction centers described by Strasser et al. (2004) that lack substrate (oxidized PQ-molecules). Re-oxidation of Q_A^- in non-Q_B-reducing centers centers occurs via a charge recombination with the donor side of PS II. It has been shown that the recombination between Q_A^- and the S_2 state of the manganese cluster occurs with a half-time of a few seconds (Lavergne and Etienne 1980; Rutherford and Inoue 1984). In contrast, recombination reactions in case of the equilibrium effect only very slowly diminish the concentration of Q_A^-. Vermaas et al. (1984) using pea thylakoids and Rutherford et al. (1984) using spinach leaf discs observed that the recombination between Q_B^- and S_2 occurred with a halftime of about 30 s and the recombination between Q_B^- and S_3 had a half-time of 80–150 s. The difference is related to the availability of Q_B^- (Rutherford et al. 1984). In PS II membranes, the recombination pattern can be inverted by pre-treating the samples with ferricyanide, which slowly oxidizes the non-heme iron. After a ferricyanide pre-treatment, the S_3 state recombines faster than the S_2 state (Schansker, unpublished data). Therefore, the faster phase of the $F_{25\ \mu s}$-dark recovery in Figure 2C is expected to be related to the non-Q_B-reducing centers and the slower phase to the equilibrium effect.

The equilibrium effect

Diner (1977) determined the reversibility of electron transport between Q_A and Q_B. He calculated that an electron shared between Q_A and Q_B spends 95% of its time on Q_B and 5% of its time on Q_A. Robinson and Crofts (1984) determined this equilibrium constant and pH dependence. They observed that for pea thylakoids this constant varied between 95 at pH 6 and 3.5 at pH 8. At pH 7, the calculated value of 20 equalled the value determined by Diner (1977). At lower pH values, protonation of the semiquinone stabilized the electron on Q_B. The far-red light occasionally induces a charge separation in the functional PS II reaction centers, by which after a while 50% of the active centers are in the semiquinone form and 50% of the reaction centers have an empty Q_B site or a Q_B site occupied by an oxidized Q_B molecule. Lavergne and Leci (1993) calculated that cooperativity or grouping between PS II reaction centers would lead to a fluorescence intensity factor 2.25 lower than expected on the basis of the amount of Q_A reduced. In our case, it would mean that 2.5% of F_v would equal ~5.6% Q_A^- for a sample in which almost all PS II reaction centers are open. If the assumption of Lavergne and Leci (1993) is correct, we would have to assume, that the stroma pH in our dark adapted leaves would be close to 7.5 (Robinson and Crofts 1984). A stroma pH of 7.5 in darkness would be in agreement with Hauser et al. (1995) who calculated an initial stroma pH in darkness of ~7.6 for sunflower and cabbage leaves on the basis of bicarbonate uptake experiments.

Quantification of the non-Q_B-reducing centers

We have determined the dark-adaptation kinetics of the $F_{25\ \mu s}$-increase for several plant species. In all cases, the fluorescence amplitude that could be attributed to the non-Q_B-reducing centers was in the order of 1.6–3.0% of F_v. Lavergne and Leci (1993) argued that since the absorbance increase on the first flash/F_o-jump is also observed in PS II membranes (Dekker et al. 1984; Lavergne 1991; Schansker et al. 2002), non-Q_B-reducing centers would be located in the grana stacks. However, Triton-X, the detergent used to isolate PS II membranes, is known to affect the Q_B-site of photosystem II (Renger et al. 1986, 1988). Therefore, it cannot be excluded that the non-Q_B-reducing centers observed in PS II membranes were caused by the isolation procedure. Another problem in comparing fluorescence data of leaves and thylakoids is the maximum fluorescence yield that is often lower in thylakoid membranes relative to leaves (lower F_v/F_m). This could have to do

with a partial destacking and better mixing of PS II and PSI (more spillover) as a consequence of the isolation of the thylakoid membranes. If the non-Q_B-reducing centers were located in the stroma lamellae, their fluorescence yield would not be affected by destacking, and their relative contribution to the F_v would increase.

Lavergne and Leci (1993) argued that their non-Q_B-reducing centers behaved like α-centers and thus form a part of a network of functional granal PS II reaction centers. However, they did not correct their measurements for the equilibrium effect. Therefore, the α-center behavior can probably also be explained on the basis of the contribution of the functional reaction centers to the fraction of non-Q_B-reducing centers they determined.

It has been suggested that non-Q_B-reducing centers form an intermediate step in the synthesis of PS II reaction centers (Guenther and Melis 1990). This would mean that under normal circumstances, in which the turnover of PS II reaction centers is relatively low, it would be inefficient for the plant to have a high percentage of their reaction centers in this state. In this respect, the 1.6–3.0% of F_v that we observe would be a very reasonable number for such a function. It is worth noting that the γ-phase of the fluorescence induction transient (nomenclature Hsu et al. 1989) measured at high light intensities in the presence of DCMU represents about 2% of F_v (Tóth, personal communication). This may point to equivalence between the non-Q_B-reducing centers measured here and the PS II reaction centers of the γ-phase observed in the presence of DCMU.

Plant species dependent variability

The estimation of the fraction of non-Q_B-reducing centers in several plant species presented in Table 1 indicates that there is very little variability with respect to this value. There seems to be a seasonal effect though. The ambient light intensity in the greenhouse was much lower in autumn than in summer. In autumn, we consistently measured values of approximately 1.8% (of F_v) non-Q_B-reducing centers, whereas in summer this value was around 3.0%. Table 1 also shows that there was a considerable amount of variability with respect to the equilibrium effect (for plants measured during the summer). Values much lower than 2.5% of F_v could be avoided by dark-adapting the plants overnight before the measurement. This could mean that higher ambient light intensities affect the stroma pH relatively long term (making the stroma more acid). Alternatively, it could mean that the fraction of PS II reaction centers that contain Q_B^- after a dark-adaptation was higher for the plants measured in summer. This would lead to a measured (but not real) decrease of the equilibrium effect. We noted that older leaves contained a lower fraction of non-Q_B-reducing centers (data not shown).

Far-red light and the J-level

The transients shown in Figure 1A demonstrate clearly that the far-red pre-illumination caused a significant decrease of the J-level. Before, we have noted that far-red light has two effects. The slightly actinic effect with respect to PS II leads to a state in which approximately 50% of the centers will have a semi-reduced Q_B-molecule (in equilibrium with Q_A). A second effect is the complete oxidation of the plastoquinone pool. This in turn will lead to a higher occupancy state of the remaining Q_B-sites. A missing piece of information is the occupancy state of PS II reaction centers in dark-adapted pea leaves. Reaction centers to which no Q_B is bound will initially behave like DCMU-inhibited centers. Binding of a Q_B-molecule to an empty site takes 1–2 ms (Crofts et al. 1984; Diner et al. 1991). It may be that the larger number of centers that need 2–3 electrons instead of 1 to achieve a transiently stable Q_A^- (until the reduced Q_B-molecule has been replaced) is sufficient to explain the lower J-level after a far-red pre-illumination. A link between far-red induced J-level-changes and the plastoquinone pool is the 10 s lag-time and a half-time of about 100 s observed for the dark recovery of the J-level. Figure 1B shows two minima at approximately 2 and 12 ms. The 12 ms minimum falls in the time-window where one would expect the reduction of the plastoquinone pool. A graph of both minima as a function of each other yields a linear correlation for dark interval times between 5 and 300 s (data not shown). Therefore we propose as a working hypothesis that the effects of a far-red pre-illumination on the redox state of the plastoquinone pool and the related occupancy state of the Q_B-site are responsible for the observed decrease of the J-level.

Conclusion

With the help of a far-red pre-illumination it is possible to obtain a reliable estimate of the contribution of the population of non-Q_B-reducing centers to the fluorescence induction curves in leaves. For the range of leaves we measured, the contribution of the non-Q_B-reducing centers was about 1.6–3.0% of the maximum variable fluorescence.

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