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Dark recovery of the Chl *a* fluorescence transient (OJIP) after light adaptation: The q*T*-component of non-photochemical quenching is related to an activated photosystem I acceptor side

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Abstract

The dark recovery kinetics of the Chl *a* fluorescence transient (OJIP) after 15 min light adaptation were studied and interpreted with the help of simultaneously measured 820 nm transmission. The kinetics of the changes in the shape of the OJIP transient were related to the kinetics of the q*E* and q*T* components of non-photochemical quenching. The dark-relaxation of the q*E* coincided with a general increase of the fluorescence yield. Light adaptation caused the disappearance of the IP-phase (20–200 ms) of the OJIP-transient. The q*T* correlated with the recovery of the IP-phase and with a recovery of the re-reduction of $P700^+$ and oxidized plastocyanin in the 20–200 ms time-range as derived from 820 nm transmission measurements. On the basis of these observations, the q*T* is interpreted to represent the inactivation kinetics of ferredoxin-$NADP^+$-reductase (FNR). The activation state of FNR affects the fluorescence yield via its effect on the electron flow. The q*T* therefore represents a form of photochemical quenching. Increasing the light intensity of the probe pulse from 1800 to 15000 μmol photons m^{-2} s^{-1} did not qualitatively change the results. The presented observations imply that in light-adapted leaves, it is not possible to 'close' all reaction centers with a strong light pulse. This supports the hypothesis that in addition to Q_A a second modulator of the fluorescence yield located on the acceptor side of photosystem II (e.g., the occupancy of the Q_B-site) is needed to explain these results. Besides, some of our results indicate that in pea leaves state 2 to 1 transitions may contribute to the q*I*-phase.

Keywords: Chl *a* fluorescence; q*T*; qN_{sv}; OJIP-transient; 820 nm transmission; *Pisum sativum*

1. Introduction

Chl *a* fluorescence has been used extensively to monitor photosystem II (PSII) activity and the processes that affect it. The practical application of fluorescence is based on the principle that the redox state of Q_A modulates the fluorescence yield. In light-adapted leaves the fluorescence yield is also modulated by other factors of which the lumen pH is the most important one.

In 1981, Bradbury and Baker [1] proposed a method to separate the photochemical component (q*P*), related to the redox state of Q_A, and the non-photochemical component representing the processes unrelated to electron flow. By giving a pulse of light on top of the actinic light (light doubling) more reaction centers could be closed reducing the contribution of the photochemical component. The pulse amplitude modulated (PAM) fluorescence technique that was introduced shortly

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; F_o and F_m, fluorescence intensity measured when all photosystem II reaction centers are open or closed respectively; FNR, ferredoxin-$NADP^+$-reductase; F_s^l, fluorescence intensity measured under steady state conditions; $I_{820\ nm}$, photocurrent, a measure for the transmitted light at 820 nm; I-level, fluorescence intensity at ~30 ms; J-level, fluorescence intensity at ~3 ms; LA, light adaptation; LED, light emitting diode; LHCII, light harvesting complex II; O-level, fluorescence intensity at 20 μs; OJIP-transient, fluorescence induction transient defined by the names of its intermediate steps; P-level, the maximum fluorescence level; P680 and P700, reaction center pigments of photosystem II and I respectively; PC, plastocyanin; Q_A and Q_B, primary and secondary quinone electron acceptors of photosystem II respectively; q*E*, q*T*, q*I*, non-photochemical quenching components with respectively the fastest, medium and slowest relaxation rates; qN_{sv}, non-photochemical quenching calculated according to Stern–Volmer

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afterwards [2] allowed a more sophisticated application of the light doubling technique. In nearly all studies of photochemical and non-photochemical quenching that have been published since that time the PAM-method is used. A theoretical treatment of this approach can be found in [3]. Subsequently, a method was developed to analyse the different components of the non-photochemical quenching [4,5].

At least three components could be identified on the basis of their dark-relaxation kinetics. The fastest component, referred to as q*E*, relaxes during the first 100 s of dark adaptation. It is related to the pH of the lumen and sensitive to the presence of zeaxanthin. At moderate light intensities, the q*E* has the strongest effect on the fluorescence yield (e.g., [5,6]). The medium component, q*T*, has a dark-relaxation halftime of about 8 min. It has been associated with (de)phosphorylation of light harvesting complex II (LHCII) and state transitions on the basis of its time dependence [4] and its sensitivity to the phosphatase-inhibitor fluoride [5,7] although it was shown later that fluoride may have side effects [8]. Walters and Horton [6,8] using a combination of 77 K fluorescence meaurements and determinations of the relaxation of non-photochemical quenching further concluded that state transitions determined only a part of the q*T*. In spite of this, the relationship between state transitions and the q*T*-component has become a part of our accepted knowledge (e.g., [9,10]). The slowest component relaxing over many hours, q*I*, is thought to be related to processes like photoinhibition that necessitate the re-synthesis of the D1-protein although other slowly relaxing processes may also be involved (reviewed in [10]).

Here, we studied processes related to non-photochemical quenching using a direct (non-modulated) fluorescence technique. More precisely, the dark-adaptation kinetics of the shape of the fluorescence transient OJIP ([11] and reviewed in [12] also referred to as O–I_1–I_2–P [13]) after a light adaptation of 15 min were investigated. The observed dark-adaptation kinetics were interpreted with the help of simultaneously measured 820 nm transmission ($I_{820\ nm}$) transients. The obtained data suggest a close relationship between the q*T* and the activation state of ferredoxin-$NADP^+$-reductase (FNR). The consequences of this finding for the potential of light pulses to 'close' all PSII reaction centers in light-adapted leaves and the parameters q*P* and qN_{sv} are discussed.

2. Materials and methods

2.1. Plant material

For the 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.

2.2. PEA Senior instrument

The measurements presented in Figs. 1–4 and 7 and 8 were made using a dual channel PEA Senior instrument (Hansatech Instruments Ltd, King's Lynn, Norfolk, UK) with which transmission changes at 820 nm and Chl *a* fluorescence can be recorded simultaneously. The first reliable measuring point for fluorescence change was at 20 μs whereas the first measuring point for transmission change was at 400 μs. The time constant used for the transmission measurements was 100 μs. The light intensity used for all strong pulses was 1800 μmol photons m^{-2} s^{-1}. The light intensity to which the leaves were adapted was 540 μmol photons m^{-2} s^{-1}. Only in Fig. 7 several light intensities were used as indicated in the figure. The actinic light was produced by four 650 nm light emitting diodes (LEDs). The modulated (33.3 kHz) far-red measuring light was provided by an OD820 LED (Opto Diode Corp.) filtered at 830±20 nm. The transmission was measured with a gain of 50. The plants were adapted to dim light (~10 μmol photons m^{-2} s^{-1}) for at least 1 h before the start of the experiment. The measured leaves were dark adapted for 10 min before the start of each measurement.

In Fig. 1 a light-adaptation protocol is shown indicating that the PEA Senior instrument can be used to do a quenching analysis. The first strong pulse (1800 μmol photons m^{-2} s^{-1}) and the 3 strong pulses given during the light adaptation (after 300, 600 and 800 s of illumination) were 1 s long. The 6 pulses given during dark adaptation were 0.7 s long, spaced 200 s apart. The light adaptation intensity was 540 μmol photons m^{-2} s^{-1} in the experiment of Fig. 1 or as indicated.

In all cases the 820 nm transmission ($I_{820\ nm}$) transient was recorded simultaneously. Changes in $I_{820\ nm}$ reflect changes in the redox states of P700 and plastocyanin (PC) and ferredoxin (Fd) [14,15] and were used here to monitor electron flow through photosystem I (PSI).

Each strong pulse and all the recordings of the Chl *a* fluorescence emission and $I_{820\ nm}$ between the strong light pulses represent individual elements that were stored separately. The advantage of our measuring equipment is that for each element of the protocol the initial time resolution is high: (10–300 μs: 1 point per 10 μs; 300 μs–3 ms: 1 point per 100 μs, 3–30 ms: 1 point per ms etc.) as is demonstrated in (Figs. 3, 4 and 7). The fluorescence intensities at 20 μs, 2 ms and 30 ms are referred to as O, J and I independent of the state of the sample (dark-adapted, light-adapted or in between).

2.3. Handy PEA

The measurements shown in Figs. 5 and 6 were made using a Handy PEA instrument (Hansatech Instruments Ltd, King's Lynn, Norfolk, UK). For the measurements, a custom made head constructed by Hansatech Instruments Ltd was used containing a single 650 nm LED providing a maximum light intensity of more than 15000 μmol photons m^{-2} s^{-1}. Leaves were dark adapted for at least 1 h before the start of the experiment.

2.4. Definition of parameters used

To indicate dark and light-adapted states, the superscripts d (=dark-adapted), l (=light-adapted), td (=time in dark after light adaptation) and r (=recovery in the dark) are used.

The q*P* is calculated as $(F_m^l - F_s^l)/(F_m^l - F_o^d)$ [16]. The F_o^l measured 2 s after lights off was only 0.5% lower than F_o^d indicating that F_o-quenching can be ignored in pea confirming earlier data [17]. It can further be noted that a 1 s FR-pulse given immediately after lights off did not induce a further lowering of the Fo^l-level in our pea plants.

There are two ways to quantify non-photochemical quenching and its components. In [10] the term qN_C is suggested for the classical method where $qN_C = 1 - F_v^l/F_v^d$. Here, the second calculation method $qN_{sv} = NPQ = (F_m^d/F_m^l) - 1$ is used, where the subscript refers to the fact that it represents a Stern–Volmer approach to qN [10]. It has been shown that PSI emits little or no variable fluorescence at room temperature (e.g., [18]) and therefore the Stern–Volmer approach can be used here. For the qN_{sv} no knowledge of the F_o^l-value is needed. The F_m-intensity is proportional to k_F/k_N in which k_F and k_N are the rate constants for fluorescence emission and non-photochemical quenching, respectively [12]. Assuming that k_F is constant [3], qN_{sv} can be written as $(k_N^l - k_N^d)/k_N^d$, which represents the percentage change of k_N in a given (light-adapted) state relative to the dark-adapted state. Further, it is important to note that qN_{sv} is the sum of its components: $qN_{sv} = qE_{sv} + qT_{sv} + qI_{sv}$ [10]. The subscript 'sv' is only used when a reference is made to the calculated value, otherwise the terms q*E*, q*T* and q*I* are used.

The increases in the J-level in response to anaerobisis [19] as well as the slow dark-adaptation kinetics of the J-level after a saturating pulse [18] suggest a close relationship between the relative position of the J-level and the redox state of the PQ-pool. As the PQ-pool becomes more reduced, the fraction of Q_B-sites to which a PQ-molecule is bound will decrease. As a consequence the fraction of PSII reaction centers in which $Q_A$cannot be re-oxidized by forward electron transport increases. These centers will behave as if they were inhibited with DCMU (high

fluorescence level at 2 ms). See also [11] for a treatment of this point in the context of the JIP-test. The J-level can even be used as a quantitative indicator of the PQ-redox state (S.Z. Tóth, G. Schansker and R.J. Strasser, in preparation).

3. Results

3.1. The relaxation kinetics of the non-photochemical quenching

In Fig. 2 the relaxation kinetics of the F_m and F_o following a 15 min 540 μmol photons m^{-2} s^{-1} light adaptation are shown. At this light intensity two phases were observed: a fast phase that was completed within 100 s (q*E*-related) and a slower phase that was completed within about 800 s of dark adaptation (q*T*-related). The non-photochemical quenching of F_m that persisted for times longer than 1100 s was ascribed to the q*I*.

In the inset of Fig. 2 the calculated qN_{sv}-values are shown. Comparing both data sets demonstrates that the calculation of the qN_{sv} diminishes the contributions of the q*T* and q*I* relative to the raw F_m-data. The q*T*-phase represented under our experimental conditions 13% of F_v. Each point in Fig. 2 was derived from an OJIP-transient induced by a strong (1800 μmol photons m^{-2} s^{-1}) pulse of light.

3.2. Effects of qE, qT and qI on the fluorescence and $I_{820\ nm}$-transients

In Fig. 3A and B the Chl *a* fluorescence transients that form the basis of Fig. 2 are shown as a function of the dark-time following the light adaptation. In Fig. 3A several transients measured during the first 140 s of dark-relaxation are shown (q*E*-range). During this time interval the fluorescence transients recovered from a general decrease of their amplitude. The transients measured after 10 and 40 s of dark adaptation had an elevated J-level. This is typical for samples with a reduced PQ-pool [18,19]. A transient reduction of the PQ-pool on turning off the light after light adaptation has been reported before [20].

The simultaneously measured 820 nm transmission transients are shown in Fig. 3C. Initially (2 and 10 s traces), a transient increase of I_{820} is observed in the 20–200 ms time range representing a transient accumulation of reduced PC and P700 (cf. [18,21]). This points to a limitation on the acceptor side of PSI possibly due to a substrate limitation (e.g., a relatively reduced NADP-pool) at short times after turning off the light. At longer times after lights off (40–140 s), no such I_{820}-increase is observed and therefore in this time range there is no indication for a limitation on the acceptor side of PSI.

In Fig. 3B fluorescence transients measured after 100–1000 s of dark adaptation are shown (q*T*-range). During the 100–200 s time interval the J-level reached a minimum-value indicating that the PQ-pool was again re-oxidized. However, the most striking feature in the 100–1000 s interval is the recovery of the IP-phase. The accompanying transmission transients (Fig. 3D) indicate that the recovery of the IP-phase is paralleled by a recovery of the $I_{820\ nm}$-increase in the 20–200 ms time range. The $I_{820\ nm}$-transients measured after 720 and 1010 s of dark adaptation resemble the transients observed in dark-adapted leaves (cf. [18,21]).

In Fig. 4A, a comparison is made between a fluorescence transient measured before light adaptation and transients measured 1000–1150 s after the light was turned off. This residual non-photochemical quenching-phase has been called the q*I* although it may represent at least 2 different processes [10]. The OJIP-transients measured in this time-range still showed a decreased JI-amplitude and a somewhat lower J-level compared to the transients of dark-adapted leaves. This was not compensated by an increase in, e.g., the IP-phase and therefore resulted in a lower P-level. The transients shown in Fig. 4A indicate that the decrease of the JI-amplitude depended on the light-adaptation intensity. In Fig. 4B the ratio between the JI ($=F_{30\ ms}-F_{2\ ms}$) amplitude before and 1000–1150 s after 15 min light adaptation is shown. A more than 15% decrease of this ratio was already observed at relatively low light intensities and at the highest light adaptation intensity used the decrease approached 30%.

The data presented in Figs. 3 and 4 demonstrate that the q*E*, q*T* and q*I* affect the OJIP-transient in specific ways.

3.3. Light-intensity dependence and IP-phase

The data shown in Fig. 3B and D indicate that the relaxation of the q*T*-phase measured under our conditions is due to a recovery of the IP-phase. The recovery of the IP-phase in turn is closely paralleled by a recovery of the re-reduction of ferredoxin, $P700^+$ and PC^+ in the 20–200 ms range as demonstrated by the 820 nm transmission measurements. The transmission data indicate that q*T* may represent an incomplete reduction of the electron transport chain.

Over the last 25 years a body of evidence has been collected supporting the notion that in dark-adapted leaves FNR is responsible for a transient block on the acceptor side of PSI. Satoh [22–24] did a series of studies in which he showed that this transient block is located between ferredoxin and NADP, that $NADP^+$ addition to intact chloroplasts does not relieve the block and that it caused a lag in the reduction of $NADP^+$. In [25] it was shown that activation of the acceptor side of PSI depends on an alkalinization of the stroma. Characterizations of the biochemical properties of FNR, reviewed in [26], showed that FNR undergoes light and pH-induced conformational changes and that the pH-dependence of its activity indicates that it will become more active as the stroma becomes more alkaline. In [27], it is concluded that FNR is the initial limitation at the acceptor side of PSI since NADP-reduction had been shown to have a considerable lag and the Calvin–Benson cycle needs 20 s to be activated. These observations are in agreement with the 820 nm transmission data presented in [21]. In other words, the (in)ability of the light to reduce the whole electron transport chain could depend on the presence or absence of a block on the acceptor side of PSI (the activation state of FNR) as suggested in [18] in the case of MV-infiltrated pea leaves.

The data described above confirm earlier measurements of Schreiber et al. [28]. These authors used strong pulses of about 18000 μmol photons m^{-2} s^{-1} and showed that the IP-phase is no longer observed when spinach leaves adapted to light intensities as low as 20 μmol photons m^{-2} s^{-1} are probed. Nevertheless, we considered the possibility that the intensity of our 1800 μmol

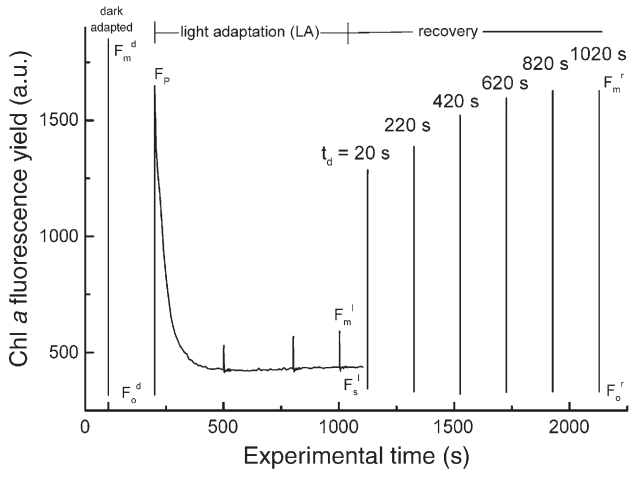

Fig. 1. Light-adaptation protocol for the experiments presented in Figs. 2–4 and 7. The figure shows that it is possible to obtain the information needed for a quenching analysis using a PEA Senior instrument. The transients indicated as spikes in the figure are shown at higher time-resolution in Fig. 7 for the light-adapted situation and in Figs. 3 and 4 for the transients measured during dark adaptation. Strong pulses (1800 μmol photons m^{-2} s^{-1}) were given 200 s before the light adaptation, after 300, 600 and 800 s of illumination and the last 6 pulses in darkness, spaced 200 s apart. The fluorescence levels related to the light adaptation (540 μmol photons m^{-2} s^{-1}) were corrected for the difference in light intensity by multiplying by a factor 3.33 (the ratio between the two light intensities). The 820 nm transmission kinetics were recorded in all cases simultaneously.

photons m^{-2} s^{-1} 650 nm pulses was insufficient to close all PSII reaction centers in light-adapted leaves. To test if an increase of the light intensity would make it possible to close all PSII reaction centers in the presence of active FNR (i.e., to eliminate the q*T*-phase) we did three types of experiments. In Fig. 5 OJIP-transients of dark-adapted pea leaves measured at different light intensities (3000–15000 μmol photons m^{-2} s^{-1}) are shown. The transients were normalized to the *P*-value, since the light intensities were high enough to saturate the P-level (=F_m) in all cases. The inset on the left side of Fig. 5 shows that there is an almost perfect linear relationship between P-level and light intensity indicating that the highest light intensities used did not affect the F_m. The higher light intensities resulted in a much steeper OJ-rise. On the other hand, the effect on the JIP-rise was limited with nearly overlapping JIP-transients for the highest three light intensities, confirming the 'thermal' (=biochemical) character of this part of the transient [13,29]. A striking effect of the high light intensities was the induction of a considerable dip just beyond the J-level. A transient limitation on the donor side associated with $P680^+$-formation is a possible cause for this phenomenon.

We were especially interested in the IP-part of the transients. In the inset on the right side of Fig. 5 the IP as a fraction of the variable fluorescence (this can also be written as 1-V_I) is given as a function of the light intensity. Increasing the light intensity suppresses the IP-phase, but the data also indicate that a full suppression cannot be achieved. The amplitude of the IP-phase levels off at about 14% of the variable fluorescence.

In an earlier publication [18] we have demonstrated that fluorescence transients from dark-adapted leaves infiltrated with methylviologen (MV, a compound that can catalyse the transfer of electrons from the FeS-centers of PSI to oxygen) lacked an IP-phase and that the maximum fluorescence level measured was lower than that of untreated leaves. In Fig. 5B this experiment is extended to higher light intensities (3000–15000 μmol photons m^{-2} s^{-1}). The measured transients indicate that at the highest light intensities used the IP-phase still has a strongly reduced amplitude and the maximum fluorescence level measured is still lower than that of untreated leaves.

In the experiment of Fig. 6, a 15 min light adaptation to 540 μmol photons m^{-2} s^{-1} was followed by a single 2 s 15000 μmol photons m^{-2} s^{-1} pulse at the indicated times. These data showed that the disappearance of the IP-phase and its recovery during a subsequent dark adaptation are still observed while using light pulses of very high intensity (15000 μmol photons m^{-2} s^{-1}), just as we observed in the case of the 1800 μmol photons m^{-2} s^{-1} pulses (Fig. 3B). The observed 100 s lag-phase and the 800 s recovery time for the IP-phase are very similar to the values that can be derived from Fig. 3B using pulses of 1800 μmol photons m^{-2} s^{-1}.

3.4. Disappearance of the IP-phase as a function of the light adaptation (LA) intensity

In Fig. 7A and C Chl *a* fluorescence and $I_{820\ nm}$ transients measured after 800 s of light adaptation to various light intensities (100 s before the lights were turned off, see Fig. 1) are shown. As the LA-intensity is increased, the amplitude of the fluorescence transients decreases reflecting an increase of the q*E*. To allow a better comparison between the different fluorescence transients, four transients were chosen and normalized between $F_{20\ \mu s}$ and F_I

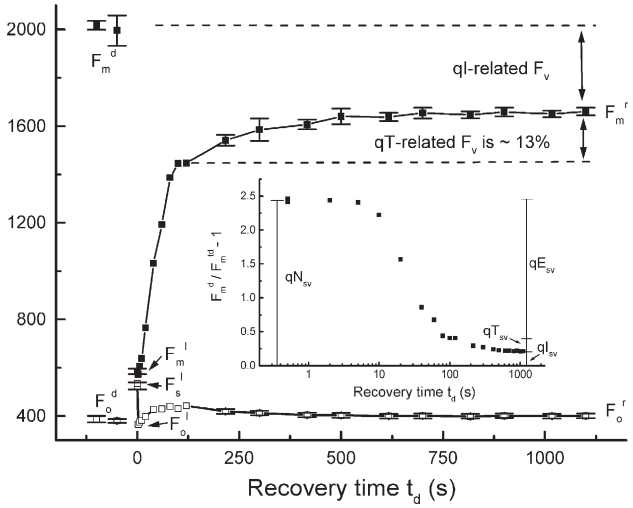

Fig. 2. Dark-relaxation kinetics of non-photochemical quenching after 15 min light adaptation to 540 μmol photons m^{-2} s^{-1}. In the main panel the dark recovery of F_o and F_m are shown and in the inset the dark-relaxation kinetics of qN_{sv} calculated from the F_m-values of the main-panel. For the inset a logarithmic time scale was used. The contributions of qE_{sv}, qT_{sv} and qI_{sv} to qN_{sv} are indicated in the inset, and the effects of q*T* and q*I* on the F_v are indicated in the main panel. The F_o^d and F_m^d are averages of 20 measurements. The same is true for recovery time points longer than 200 s where data from 5 different times were averaged. For the first 120 s of recovery averages of 4 independent experiments are shown.

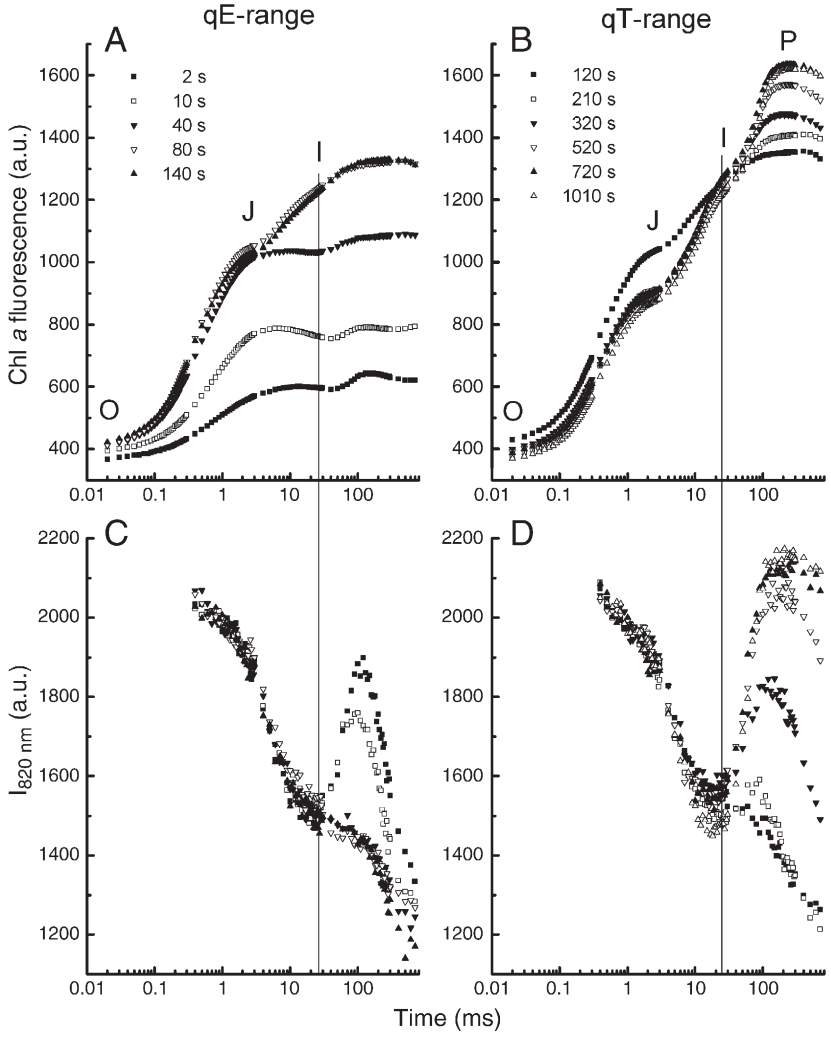

Fig. 3. Changes in the fluorescence rise and $I_{820\ nm}$-transients related to the relaxation of q*E* (panels A and C) and q*T* (panels B and D) after 15 min light adaptation to 540 μmol photons m^{-2} s^{-1}. For the q*E* time-range, transients measured during the first 140 s after lights off are presented. The q*T* time-range is defined here as the 100–1000 s time interval. To make the connection between Chl *a* fluorescence and transmission panels the 30 ms time point (I) has been marked by a line. The same symbols are used in panels A and C and B and D, respectively. The data represent the average of 4 independent experiments.

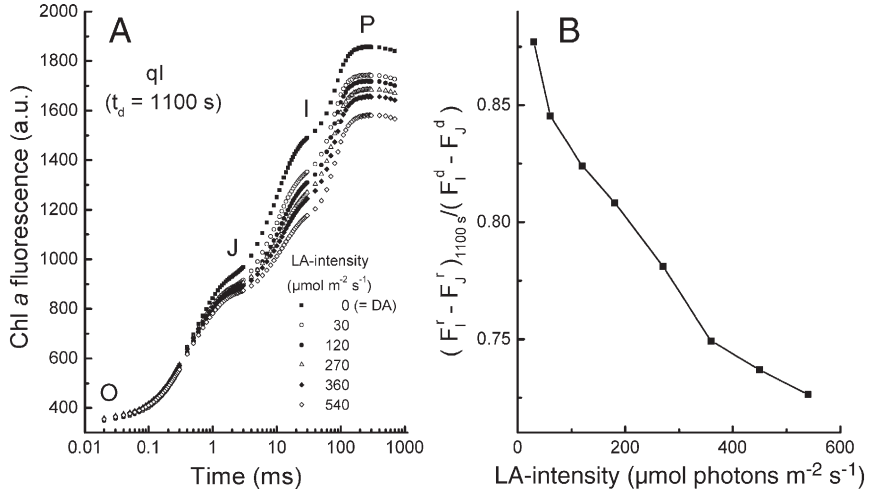

Fig. 4. Changes in the fluorescence rise transients related to the q*I*. Fluorescence transients measured 1010–1150 s after 15 min light adaptation with 30–540 μmol photons m^{-2} s^{-1} were averaged (n=23–26) and compared with the dark-adapted transients.

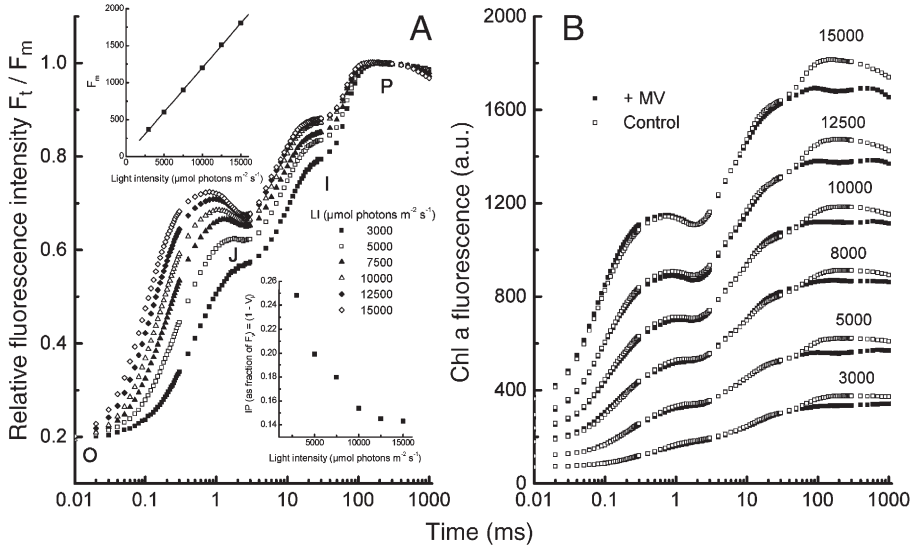

Fig. 5. Light intensity dependence of the Chl *a* fluorescence transients of dark-adapted leaves in the 3000–15000 μmol photons m^{-2} s^{-1} light intensity range. In panel A Chl *a* fluorescence transients measured at different light intensities normalized to the P-level (=F_m) to correct for the light intensity. The inset in the left upper corner of panel A demonstrates the near perfect correlation between the light intensity and the measured *P*-value. The inset in the right lower corner of panel A gives the relative amplitude of the IP-phase as a function of the light intensity. In panel B, a comparison of Chl *a* fluorescent transients measured in the presence and absence of methylviologen is made. The transients represent the average of 20–25 independent measurements.

where F_I is taken as the $F_{100\ ms}$ value (Fig. 7B). The normalized transients show that the effect of the q*E* (the dominant factor, see Fig. 2) on the rise kinetics between O and I is very limited. The main effect is a lowering of the fluorescence yield at all light intensities. It can also be noted that the higher J-level (an indicator of a reduction of the PQ-pool, see above) as observed in Fig. 3A is not observed under steady state conditions. At pulse-times longer than 100 ms light-intensity dependent differences are observed. Increasing the LA-intensity results in a smaller fluorescence rise between 100 ms and 1 s and a slowdown of the rise kinetics (Fig. 7B).

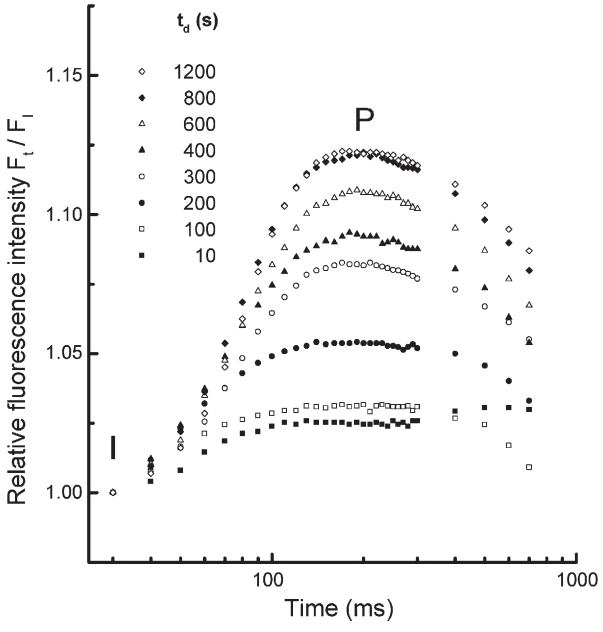

Fig. 6. Dark recovery of the IP-phase following a 15 min light adaptation to 540 μmol photons m^{-2} s^{-1} as detected by a single 15000 μmol photons m^{-2} s^{-1} pulse. The time between the light adaptation and the strong light pulse is indicated. The transients were normalized to F_I. The transients represent the average of 5–9 independent experiments.

The simultaneously measured $I_{820\ nm}$ transmission transients shown in Fig. 7C were graphically shifted relative to each other to allow a clear view of the individual transients. The initial $I_{820\ nm}$-decrease (0.4–30 ms) of the transients was very similar. Only in the case of the highest LA-intensity used, the amplitude was smaller indicating that there was some steady state oxidation of P700/PC induced by the light adaptation. In all the other cases P700 and PC were probably nearly completely reduced on switching from LA-light to 1800 μmol photons m^{-2} s^{-1}. The transmission kinetics between 30 ms and 1 s consisted of two rise or re-reduction phases separated by a dip. Only at the highest LA-intensity used a rise phase was almost completely missing. The multiphasic kinetics of this part of the transmission transients did not show up in the fluorescence rise transients. The IP-phase of dark-adapted leaves is characterized by parallel changes of fluorescence (PSII) and transmission (PSI) kinetics (e.g., [18]). The absence of this parallelism in this case can most easily be explained by assuming that the relatively small transmission changes around 100 ms were too strongly buffered by the electron acceptors that separate PSII and the site of the limitation to show up in the Chl *a* fluorescence transient.

In the inset of the Fig. 7B, a fluorescence parameter (the ratio between the highest fluorescence level around 1 s and F_I) is plotted as a function of an $I_{820\ nm}$-parameter (the difference between the transmission level at 1 s and the lowest transmission-level determined for the whole set of transients). There is a good correlation between these two parameters, which indicates that the fluorescence rise between 100 ms and 1 s depends on an accumulation of reduced PC and P700.

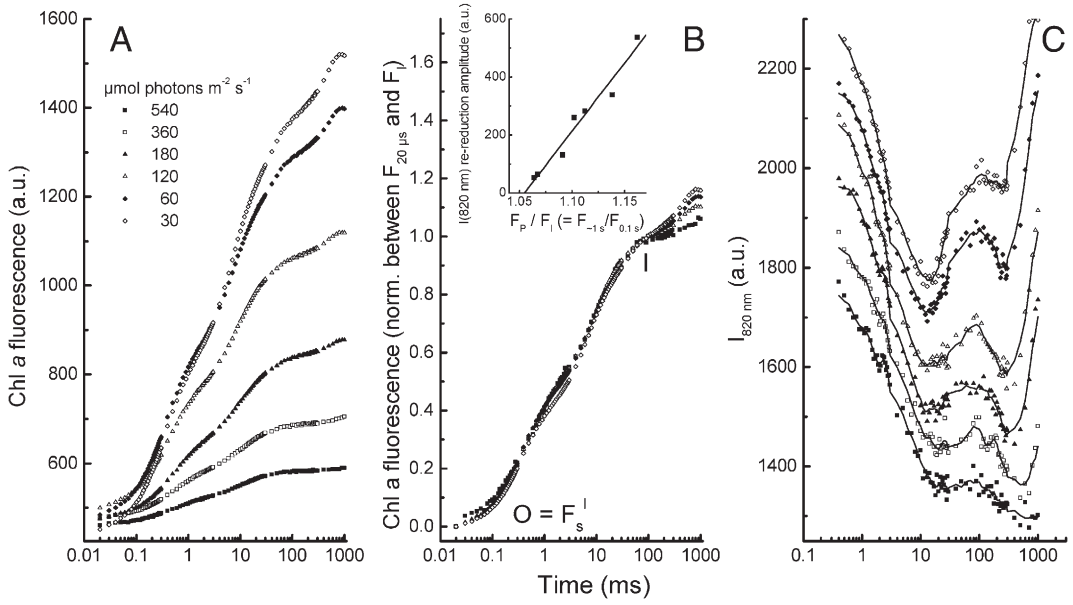

Fig. 7. Effect of the light-adaptation intensity on the fluorescence (panels A and B) and 820 nm transmission transients (panel C) induced by a strong pulse of light (1800 μmol photons m^{-2} s^{-1}). The transients were measured at the end (after 800 s) of the light adaptation to the indicated light intensities (see Fig. 1 for the protocol). In panel B, 4 transients taken from panel A were double normalized between $F_{20\ \mu s}$ and F_I (defined here as $F_{100\ ms}$). In panel C the transients were graphically shifted relative to each other to allow an easier comparison. In the inset of panel B a correlation between a fluorescence parameter (the ratio between F_p and F_I) and a transmission parameter (the difference between $I_{820\ nm}$ at 1 s and the lowest $I_{820\ nm}$-value determined for the whole set of transients) is shown. The transmission parameter is referred to as the $I_{820\ nm}$ re-reduction amplitude. The transients represent the average of 4 independent experiments.

3.5. Re-calculation of the qP and qN_{sv}-values

The results presented in Figs. 3–6 indicate that the q*T* is related to an incomplete reduction of the electron transport chain due to the outflow of electrons from PSI (blocked in dark-adapted leaves whereas the outflow is relatively free in light-adapted leaves). Therefore, in the context of the quenching analysis, q*T* represents a photochemical process and it should be taken into account when calculating the q*P*.

In Fig. 8, the q*P* and qN_{sv}-values were re-calculated taking into account that the q*T* represents a photochemical process. For the re-calculation, F_v^l had to be increased by the q*T*-contribution. For the determination of F_v^l we assumed that there was no F_o-quenching. The F_o^l measured 2 s after lights off was only 0.5% lower than F_o^d and it agrees with earlier observations on pea leaves [17]. The contribution of the q*T* to the variable fluorescence was estimated as the difference between the F_m measured 1100 s and 100 s after the light adaptation: $F_v\ (qT) = F_m^{1100s\ dark} - F_m^{100s\ dark}$. Hundred

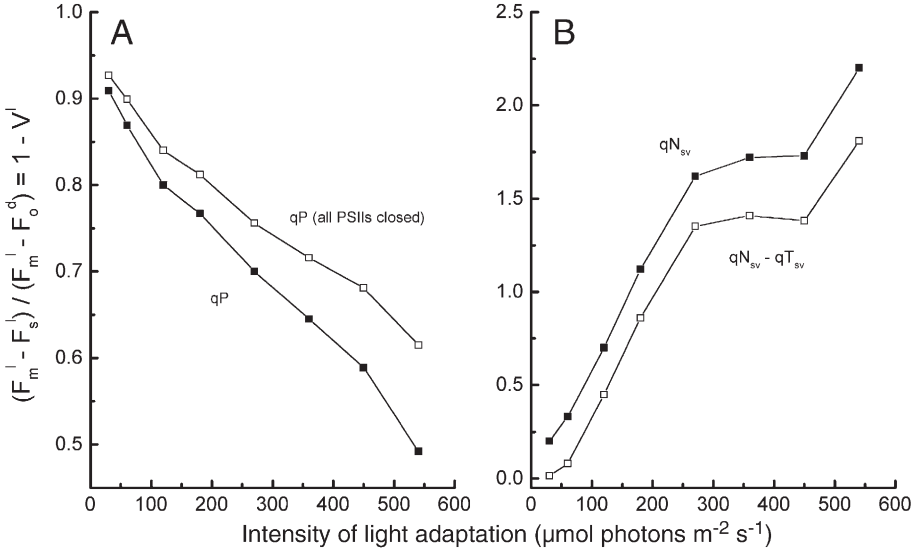

Fig. 8. Correction of the parameters q*P* (panel A) and qN_{sv} (panel B) for the q*T*. For a description of the correction-method, see the text. The data were derived from the average of 4 independent experiments.

seconds is enough for the relaxation of the q*E* and 1100 s is more than enough time for a full relaxation of the q*T* (see, e.g., Fig. 3). A straightforward addition of this fluorescence amplitude to F_v^1 gave unrealistic values and it seemed reasonable to assume that the contribution of the q*T* to F_v^1 was sensitive to the effect of energy quenching (q*E*). We took this into account by multiplying F_v (q*T*) by the ratio between the F_v determined in the light and after 100 s darkness (no q*E*): F_v (q*T*) * $(F_m^1 - F_o)/(F_m^{100\ s\ dark} - F_o)$. The obtained value was added to the F_m^1 and F_v^1-values and the q*P* and qN_{sv}-values were recalculated.

The re-calculated q*P*-values indicate that there is little effect of the re-attribution of the q*T* at low light intensities but that at higher light intensities there is a considerable underestimation of the q*P*. For the qN_{sv} the re-attribution of the q*T* leads essentially to a shift to lower values, whereby at the lowest light intensity (30 μmol photons m^{-2} s^{-1}) the qN_{sv} becomes nearly 0.

4. Discussion

4.1. Identification of the process reflected by the qT-phase

In 1988 two papers were published [4,5] that analysed the different components of the non-photochemical quenching. Both proposed that the intermediate phase, having a dark-relaxation halftime of 8 min, represented LHCII-phosphorylation. Horton and Hague [5] coined for this phase the term q*T*. In [30] it was observed that the q*T* saturated already at low light intensities. This is also a property of state transitions [31,32]. To link the q*T* to state transitions fluoride, an inhibitor of phosphatase, had been used which seemed to suppress the q*T*-phase [5,7]. In two subsequent papers [6,8] doubt was cast on the relationship. It was observed that fluoride also caused a slow down of linear electron transport. In addition, on the basis of combined fluorescence and 77K-measurement and q*T*-determinations it was shown, that there could only be a correlation between the q*T* and LHCII-phosphorylation at low light intensities.

This opened the door for the proposal of new mechanisms, but they never came. In the present paper we show that there is a close relationship between the IP-phase of the OJIP-curve and the q*T* during dark adaptation following a 15 min light adaptation (Figs. 2–4). Together with the I_{820nm} data we can now propose for the dark-adaptation kinetics of the fluorescence transients that the q*T* is a reflection of the activation state of FNR. The saturation of the q*T* at low light intensities [30] noted above can also be explained in the context of the present interpretation. In [28] it was shown that a light adaptation to 10 μmol photons m^{-2} s^{-1} is already enough to make most of the IP-phase disappear indicating that a full activation of FNR also occurs at low light intensities.

Figs. 3C and 7C indicate that under certain conditions substrate limitations may occur on the acceptor side of PSI that are unrelated to the activation state of FNR. In Fig. 7C an increase in the 820 nm transmission is observed between 300 and 1000 ms when a strong light pulse was given to leaves adapted to relatively low light intensities. The effect diminishes as the LA-intensity increases and is absent at the highest light intensity used (540 μmol photons m^{-2} s^{-1}). A similar phenomenon is observed at short times after lights off (Fig. 3C) although in this case the transmission increase takes place between 20 and 200 ms like in dark-adapted leaves. The transient transmission increase observed at short times after lights off (Fig. 3C), coincides with a transient reduction of the PQ-pool (high J-level in Fig. 3A). Both processes seem to be related to a rather reduced NADP-pool.

The fact that the transmission increase observed in the light is slower (300–1000 ms compared with 20–200 ms) indicates that in the light a slightly different mechanism is at work. A possible explanation for this slower transmission increase in the light may be found in the ferredoxin/thioredoxin system that regulates the activity of several enzymes on the stroma side of PSI (e.g., the Calvin–Benson cycle enzymes fructose-1,6-biphosphatase and sedoheptulose-1,7-biphosphatase) [33,34]. The ferredoxin/thioredoxin system is thought to continuously adjust the activity of its target enzymes in response to changes in the redox state of the stroma [33,34]. Down-regulation of these target enzymes at lower light intensities may lead to a complete reduction of the NADP-pool after a sudden shift to much higher light intensities (strong pulse). This would explain the observed 820 nm transmission increase in the 300–1000 ms time range. Such limitations are not observed in the q*T* time domain indicating that the NADP-pool is oxidized enough to accept all electrons produced by the strong pulse.

4.2. The qE and qI

The transients shown in Fig. 3A indicate that the q*E* represents a general decrease of the fluorescence yield. This is expected for a process that is thought to increase the probability that excitonic energy is lost as heat (reviewed by [35]).

The q*I* as observed in our experiments (Fig. 4) has a different character. The main effect is on the JI-phase of the fluorescence induction curve. This is the part of the transient that runs kinetically parallel to the reduction of the PQ-pool [36]. In other words, this phase is sensitive to the electron transport capacity of PSII reaction centers that share a PQ-pool.

The relationship between the JI-phase and the electron transport capacity of PSII was, e.g., shown in [37] making use of treatments that affected the donor side of PSII. In the case of photoinhibition individual PSII reaction centers are inactivated leading at the same time to a loss of their variable fluorescence ([10] and references therein). This would diminish the electron donation capacity of the PSII reaction centers that share a PQ-pool and thus slowdown the reduction of the PQ-pool and at the same time lead to a lowering of the F_m-level. However, a down-regulation of the donor side of PSII that has been proposed (e.g., [38]) would have a similar effect. State transitions could represent a third process that can explain the observed phenomena. A smaller PSII antenna size could slow down the reduction of the PQ-pool and also would result in a lower F_m-level. State transitions form a very good candidate to explain the q*I* induced at low light intensities (more than 15% smaller JI-amplitude at 120 μmol photons m^{-2} s^{-1}, see Fig. 4).

The rather long lasting effect of the light adaptation on the JI-phase was quite reproducible. However, a more extensive study

is needed to establish the relationship between the form of the OJIP-transient and the q*I* in more detail and to establish if state 2 to 1 transitions in pea leaves occur with slower kinetics than expected.

4.3. Activation and inactivation of FNR

In [28] two possible explanations for the observed loss of the IP-phase were considered. The first explanation considered was that the loss of the IP-phase was due to a light-induced reduction of the PQ-pool accompanied by a removal of PQ-pool quenching. This explanation can be discarded for several reasons. Even at the highest light intensities used in this study the PQ-pool is in a quite oxidized state at the end of the light-adaptation period. Using the J-level as an indicator for the redox state of the PQ-pool (see Materials and methods) a transient reduction of the PQ-pool was observed during the first 200 s of the dark-adaptation period, but there was no correlation with the recovery of the IP-phase (Fig. 3). The recovery of the IP-phase occurs after and independent of the re-oxidation of the PQ-pool (Fig. 3). We have also shown recently that PQ-pool quenching probably does not occur in leaves at all [39].

The second explanation that was considered in [28] was an incomplete closure of all reaction centers as a consequence of the activation of the acceptor side of PSI. This explanation is supported by our data. The data in Figs. 3–7 provide experimental support for a lowering of the maximum attainable fluorescence level related to an outflow of electrons from PSI up to the highest pulse intensity used. We had observed the same phenomenon before in dark-adapted leaves infiltrated with MV [18]. Here, this observation is extended to very high light intensities (Fig. 5B). Both approaches clearly suggest that the maximum fluorescence level can only be reached in the presence of a blocked PSI acceptor side.

On a transition from light to dark the enzyme FNR is inactivated again putting an end to the outflow of electrons from PSI. In other words the inactivation of FNR in the dark will restore the typical $I_{820\ nm}$ re-reduction kinetics in the 20–200 ms time range and in parallel the IP-phase of the OJIP-transient. As shown in Figs. 3–7 this process parallels the q*T*-phase of the non-photochemical quenching. This means that the inactivation kinetics of FNR can be determined by analysing the kinetics of the q*T*. Looking at the recovery kinetics of the IP-phase, it can be observed that during the first 100–150 s there is nearly no recovery of the IP-phase. This indicates that the inactivation of FNR starts only after a lag of 100 to 150 s. Subsequently, it takes another 250 s to inactivate about 50% of the FNR and 900–1000 s after the light was turned off the inactivation was complete.

The relaxation of the q*T* induced by a variety of light intensities (30–540 μmol photons m^{-2} s^{-1}) was studied, but little indication for a light-intensity dependence was found (data not shown). It seems that the inactivation of FNR is independent of the LA-intensity. The lag-phase that we observe before the q*T* started to relax is of importance for the analysis of the non-photochemical quenching. It is commonly analysed assuming that dark adaptation of all processes that determine the non-photochemical quenching start immediately following lights off (e.g., [8,10,17]). On the basis of that assumption a simple 3-exponential fit can be used. Our data indicate that the lag-phase of the q*T* should be taken into account too.

4.4. Consequences for qP and qN_{sv}-determinations

In Fig. 8 re-calculated q*P* and qN_{sv}-values (taking into account that q*T* has a photochemical character) are shown. For the q*P* the major effect is that its value is underestimated at higher light intensities. At the lowest light intensities the re-attribution of the q*T* had little effect. As shown in Fig. 5A, an increase of the light intensity from 1800 to 15000 μmol photons m^{-2} s^{-1} suppresses the IP-phase by 40%. This light intensity dependent effect will diminish but not eliminate the underestimation of the q*P*. The re-attribution of the q*T* resulted essentially in a downward shift of the qN_{sv}-values with a value of approximately 0 for the lowest light intensities used. This means that the extent to which the non-photochemical quenching is induced is strongly overestimated at low light intensities.

4.5. The closure of all PSII reaction centers in light-adapted leaves

We have demonstrated in this paper that raising the light intensity to 15000 μmol photons m^{-2} s^{-1} does not lead to a full disappearance of the IP-phase (Fig. 5) and more importantly that the highest light intensities used are saturating in this respect. Further we have shown that using a 15000 μmol photons m^{-2} s^{-1} probe pulse we still observe a suppression of the IP-phase after a 15 min light adaptation (Fig. 6) and the same is true for MV-treated leaves (Fig. 5B). It has been shown in the past that the IP-phase is related to PSI-activity [18,22,40]. Taking these 3 observations together we conclude that it is impossible to close all PSII reaction centers in light-adapted leaves with a strong pulse of light.

This would seem to be in disagreement with a recent study [41]. There, the criterion for saturation was a fluorescence level that could not be further increased by a further increase of the light intensity. However, the process that is described here prevents saturation but does not show up in the analysis used in [41]. The maximum attainable fluorescence level behaves as a Blackman phenomenon. This means that the maximum response (in this case the maximum attainable fluorescence level) can be further increased as another limitation (here the activation state of FNR) is changed.

4.6. Determinants of the fluorescence yield

The saturating pulse method is based on the assumption that in light-adapted leaves all PSII reaction centers can be 'closed' by a pulse of light of sufficient intensity (see [42] where the principles of the method are discussed). Since the paper of Duysens and Sweers [43] the redox state of Q_A has been thought to be the major if not only determinant of the fluorescence yield; therefore, the assumption that the maximum obtainable fluorescence yield can be reached if a sufficiently high light intensity was used seemed to be a reasonable one.

However, over time, several authors have thought about modifying this concept to explain certain heterogeneities for example by assuming that in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) two quinone molecules Q_1 and Q_2 are active [44,45]. During the last 5–10 years the feeling

that the assumption of Q_A as the dominant quencher is too absolute has become stronger. In [46] the possibility that Q_B might also play a role was considered, an idea that these authors subsequently tried to prove experimentally [47]. The idea was elaborated on by Schreiber [48] who tried to envision possible mechanisms by which Q_B could play a role as quencher. If the occupancy state of the Q_B-site modifies the fluorescence yield, it would explain the observation that in the presence of DCMU the F_m-level is reached within 2 ms, whereas in its absence the J-level saturates at a value considerably below the F_m-level. A value of about 53% was found for intact spinach chloroplasts [13] and for pea leaves the maximum value observed is 66% (Fig. 5).

Independent of the mechanism by which Q_B can modulate the fluorescence yield, a role for Q_B, a molecule that is in a dynamic equilibrium with the PQ-pool introduces dark-limitations in the kinetics of the fluorescence rise transients and raises therefore questions with respect to the ability of light pulses to 'close' all reaction centers under all conditions. Our data indicate that both for light-adapted leaves (Figs. 3 and 6) and for leaves treated with MV (Fig. 5B) the flow of electrons out of PSI even at light intensities that saturate the J-level prevents a full reduction of the PQ-pool. A possible explanation for this phenomenon could be that as the PQ-pool becomes more reduced (at the end of the JI-phase), the limiting step of the electron transport chain may shift from the re-oxidation of PQH_2 to the binding of PQ to the Q_B site. This shift will prevent the full reduction of the PQ-pool. If the re-oxidized PQ-molecules keep arriving at the Q_B-site in a random fashion, oxidized PQ-molecules would be bound to the Q_B-site of *some* PSII reaction centers at all times. This would prevent a full reduction of the acceptor side of all PSII reaction centers even at very high light intensities.

The argument can also be reversed. In showing that in MV-treated or light-adapted plant leaves fluorescence cannot be saturated (q*P* remains larger than zero) we think that we have obtained a small piece of evidence pointing to a role of Q_B or the occupancy of the Q_B-site as a modulator of the fluorescence yield independent of its role as an electron acceptor. An alternative explanation is provided by the concept of PQ-pool quenching, but as noted above, we were recently able to show that in leaves such a role for the PQ-pool is unlikely [39]. In addition, the data presented here indicate that the transient reduction of the PQ-pool observed during the first 200 s after lights off is unrelated to the disappearance and the recovery of the IP-phase (Fig. 3B).

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