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Photosynthetic electron transport activity in heat-treated barley leaves: The role of internal alternative electron donors to photosystem II

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Abstract

Electron transport processes were investigated in barley leaves in which the oxygen-evolution was fully inhibited by a heat pulse (48 °C, 40 s). Under these circumstances, the K peak ($\sim F_{400\ \mu s}$) appears in the chl *a* fluorescence (OJIP) transient reflecting partial Q_A reduction, which is due to a stable charge separation resulting from the donation of one electron by tyrozine Z. Following the K peak additional fluorescence increase (indicating Q_A^- accumulation) occurs in the 0.2–2 s time range. Using simultaneous chl *a* fluorescence and 820 nm transmission measurements it is demonstrated that this Q_A^- accumulation is due to naturally occurring alternative electron sources that donate electrons to the donor side of photosystem II. Chl *a* fluorescence data obtained with 5-ms light pulses (double flashes spaced 2.3–500 ms apart, and trains of several hundred flashes spaced by 100 or 200 ms) show that the electron donation occurs from a large pool with $t_{1/2} \sim 30$ ms. This alternative electron donor is most probably ascorbate.

Keywords: Alternative electron donor; Chlorophyll *a* fluorescence; Heat pulse; OJIP-transient; Photosystem II; 820 nm transmission

1. Introduction

Exposure of plants to high temperatures has two opposite effects on the photosynthetic electron transport chain. Photosystem I (PSI) is stimulated by heat (measured as the reduction rate of $P700^+$), which is related to an enhanced reduction of the plastoquinone (PQ) pool by either ferredoxin (Fd) or NADPH [1,2]. In contrast, photosystem II (PSII), and especially the oxygen evolving complex (OEC) is inactivated at relatively low temperatures (e.g. [3,4]). A further heat-induced effect is a shift of the redox equilibrium between Q_A and Q_B, increasing the residence time of an electron on Q_A relative to Q_B [5–7] that could lead to back flow of electrons from the PQ-pool to PSII [8,9].

The chl *a* fluorescence (OJIP) transient is a very sensitive indicator of photosynthetic electron transport processes. Light-dependent changes in the chl *a* fluorescence yield are thought to be determined by changes in the redox state of Q_A (reviewed in e.g. [10–14]). At the same time, it has been shown that the OJIP transient reflects the reduction of the photosynthetic electron transport chain (e.g. [15]). The OJ phase is the photochemical part of the transient. Its kinetics depend strongly on the light intensity [16,17]. The JI phase has been shown to parallel the reduction of the PQ-pool [18] and the IP phase depends on PSI activity representing the reduction of the ferredoxin (Fd) pool in

Abbreviations: Asc, ascorbate; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Fd, ferredoxin; F_0, initial fluorescence (measured at 20 μs), all PSII RCs are open; F_J, fluorescence intensity at ~3 ms (at 3000 μmol photons m^{-2} s^{-1}); F_M, maximum fluorescence, all PSII RCs are closed; FNR, ferredoxin-$NADP^+$-reductase; F_P, maximum measured fluorescence intensity; FR, far-red light; I_{820nm}, transmission at 820 nm; I, step in the OJIP transient at 30 ms; J, step in the OJIP transient at ~3 ms (at 3000 μmol photons m^{-2} s^{-1}); K, peak in the OJIP transient at ~400 μs (at 3000 μmol photons m^{-2} s^{-1}); MV, methylviologen, 1,1′-dimethyl-4,4′-bipyridinium-dichloride; OEC, oxygen evolving complex; OJIP, chl *a* fluorescence transient defined by the names of its intermediate steps; PpBQ, phenyl-p-benzoquinone; P680, primary electron donor of PSII; P700, primary electron donor of PSI; PC, plastocyanin; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; Q_A, primary electron acceptor quinone of PSII; Q_B, secondary electron acceptor quinone of PSII; Tyr_Z, tyrozine Z; φ_{Po}, maximum quantum yield for primary photochemistry

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the presence of inactive ferredoxin-NADP$^+$-reductase (FNR) [15]. The shape of the OJIP transient drastically changes after a heat treatment, especially if oxygen evolution is completely inhibited: the J and I steps disappear and the K peak develops at 300–400 μs if the measurements are carried out at a standard, 3000 μmol photons m^{-2} s^{-1} light intensity [19]. After the K peak the fluorescence intensity decreases. The K peak is thought to result from the only stable charge separation possible when the OEC is completely destroyed [20,21] because Tyr_Z can provide only one electron. However, in leaves in the 0.2–2 s time range another peak appears in the fluorescence transient [22,23], which might represent a second phase of Q_A^- accumulation.

In this study, we investigated the origin of the fluorescence increase in the 0.2–2 s time range in leaves in which the oxygen evolution was fully inhibited with a heat pulse (by immersing the leaves for 40 s in a water bath of 48 °C). This treatment induces typical heat stress effects, but causes virtually no visible symptoms and secondary effects [23]. (A similar method, a strong but short pulse instead of a longer, weaker treatment has been used for inducing UV-A stress in order to separate inactivation of PSII and repair [24].) We used simultaneous 820 nm transmission and chl *a* fluorescence induction measurements, and also recorded chl *a* fluorescence transients induced by short (5 ms), repetitive light pulses. It was found that alternative electron sources provide electrons to the donor side of PSII with $t_{1/2}$ ~30 ms. We also show that the electrons originate from a large pool of donors (probably ascorbate molecules).

2. Materials and methods

2.1. Plant material and growth conditions

Measurements were carried out on 7-day-old barley seedlings (*Hordeum vulgare* cv. Triangel). Plants were grown in a greenhouse where the temperature was 20–25 °C during the day and ~14 °C at night.

2.2. Heat pulse

The heat pulse was given by submerging the leaves of barley seedlings. The pots with seedlings were turned upside down into a water bath of 48 °C (if not stated otherwise) for 40 s. In the case of the DCMU+heat treatment and the MV+heat treatment leaf segments were used and submerged in 48 °C water for 40 s. In our previous study [23], we used a heat pulse of 50 °C to inactivate all OECs. In this case, 48 °C was enough to achieve the same effect, which is probably due to the lower growth temperature of the plants. We verified that this heat pulse fully inactivated the OECs. The B thermoluminescence band [25] (that is a reflection of recombination reactions between Q_B^- and the S_2 or S_3 states of the OEC) was completely eliminated; this was confirmed by oxygen-evolution measurements carried out on thylakoids isolated from heat-treated leaves using PpBQ (phenyl-p-benzoquinone) as an electron acceptor (data not shown).

2.3. DCMU+heat treatment

Leaf segments of ~2 cm long were submerged in DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea) solution (200 μM, containing 1% ethanol to dissolve DCMU) or 1% ethanol for 5 h. Half of the leaf segments treated with DCMU and half of the leaf segments kept in 1% ethanol were given a heat pulse (48 °C for 40 s in water bath). There were four types of samples: DCMU-treated, DCMU+heat-treated, heat-treated (treated in 1% ethanol before the heat treatment) and control (1% ethanol treated). It was important to carry out the DCMU treatment *before* the heat pulse otherwise the F_0 value of leaves became very high (~78% of F_M, data not shown).

2.4. Methylviologen (MV)+heat treatment

For MV treatments, a 200 μM MV (1,1′-dimethyl-4,4′-bipyridinium-dichloride, Fluka) solution was applied to both sides of the leaf with a fine brush. The leaves were not detached and the plants were kept overnight in complete darkness before the measurements (see also [15]). Following the MV treatment, leaf segments were prepared and half of them were given a heat pulse (48 °C, 40 s). Control plants were kept overnight in darkness.

2.5. Chl a fluorescence and 820 nm transmission measurements

Samples were kept in darkness after the heat pulse and measured within 30–60 min. In the case of fluorescence measurements carried out by the Handy-PEA (Hansatech Instruments Ltd, UK), the leaf samples were illuminated with continuous red light (650 nm peak wavelength, the spectral line half-width is 22 nm; the light emitted by the LEDs is cut-off at 700 nm by NIR short-pass filters) at the indicated light intensities for 1–5 s. The light was provided by an array of three light-emitting diodes focused on a circle of 5 mm diameter of the sample surface. The first reliably measured point of the fluorescence transient is at 20 μs, which was taken as F_0. Chl *a* fluorescence emission is measured at wavelengths longer than 700 nm (Kopp Corning RG-9 long-pass filter).

For some of the experiments (Figs. 2 and 3) we made use of a "fast" version of the Handy-PEA instrument that allows reducing the pulse length to 300 μs. Either two, or trains of several hundred 5-ms light pulses were applied. The dark-time intervals between the light pulses were 2.3, 9.6, 16.9, 31.5, 38.8, 53.4, 75.3, 100, 200 or 500 ms.

A special high-intensity measuring head was used to induce chl *a* fluorescence at a maximum intensity of 15000 μmol photons m^{-2} s^{-1} produced by a single 650 nm LED (Hansatech Instruments Ltd, UK).

820 nm transmission measurements parallel to chl *a* fluorescence were carried out using a PEA Senior instrument (Hansatech Instruments Ltd, UK). The excitation light intensity used was 1800 μmol photons m^{-2} s^{-1}, produced by four 650 nm LEDs. Far-red light (718 nm peak wavelength, 200 μmol photons m^{-2} s^{-1} light intensity) and modulated far-red measuring light (820 nm peak wavelength) were provided by two additional LEDs. Further technical details and applications are described in several papers by Schansker et al. [15,26,27].

3. Results

3.1. Chl a fluorescence transients of heat-treated barley leaves measured at different light intensities

In Fig. 1 several aspects of chl *a* fluorescence transients of heat-treated barley leaves are demonstrated. In Fig. 1A, a comparison is made between chl *a* fluorescence transients measured at light intensities of 5000 and 15000 μmol photons m^{-2} s^{-1}; the transients are presented without normalization. In Fig. 1B, the fluorescence transient of heat-treated samples are presented, measured between 200 and 3000 μmol photons m^{-2} s^{-1} and the transients were normalized to F_0. By increasing the light intensity from 200 to 15000 μmol photons m^{-2} s^{-1}, the intensity of the K peak increased and its peak position gradually shifted from ~3 ms to 0.16 ms which is in agreement with its photochemical character [21]. In heat-treated samples measured at high light intensities (3000–15000 μmol photons m^{-2} s^{-1}) the fluorescence intensity decreased after the K peak to a level that was almost equal to F_0. This is mainly due to forward electron transport to Q_B but $P680^+$ accumulation may explain

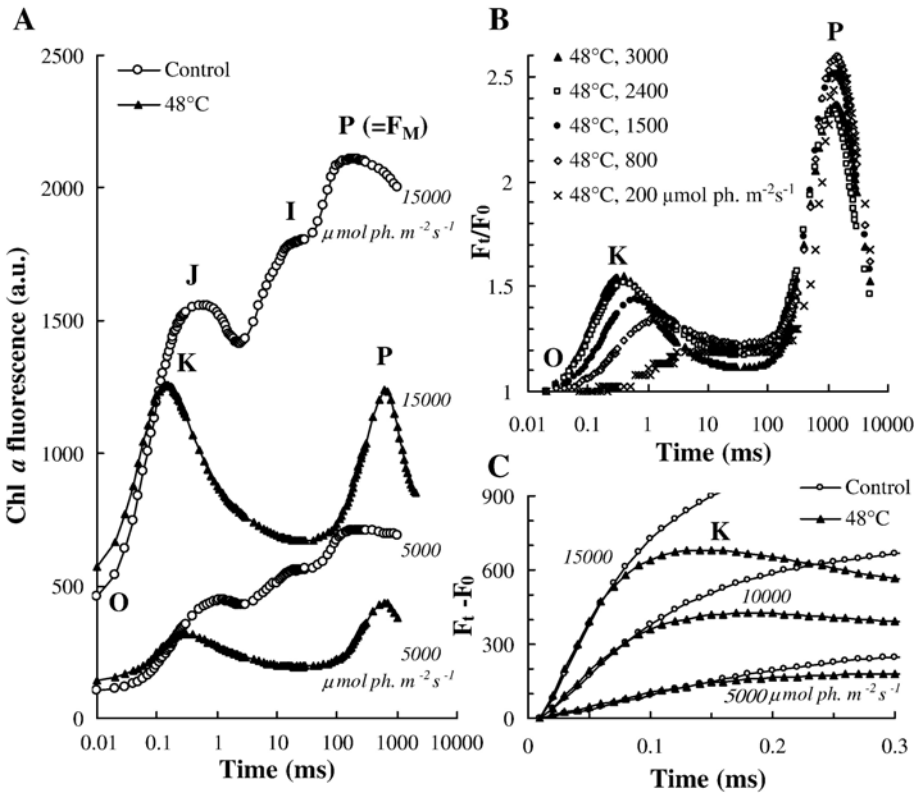

Fig. 1. Chl *a* fluorescence transients of untreated and heat-treated (48 °C, 40 s) barley leaves measured at 5000 and 15000 µmol photons m^{-2} s^{-1} (A) and 200–3000 µmol photons m^{-2} s^{-1} (B) light intensities. Transients of panel B were normalized to F_0. (C) F_t–F_0 kinetics (in the 0–0.3 ms time interval) of fluorescence transients measured at 5000, 10 000 and 15 000 µmol photons m^{-2} s^{-1} light intensities. The excitation light was produced by 650 nm LEDs and chl *a* fluorescence emitted at wavelengths >700 nm was measured.

part of the fluorescence decrease [21] because $P680^+$ is a strong fluorescence quencher [28–30].

Following this phase, a second peak emerged indicating further Q_A reduction. Its maximum was reached at ~1 s which gave the *P* value of the fluorescence transient. The position and the intensity of the normalized P level did not depend on the light intensity (Fig. 1B). The *P* value decreased strongly by increasing the temperature of the heat pulse from 48 to 54 °C (data not shown) but it did not disappear completely.

In Fig. 1C, F_t–F_0 kinetics of heat-treated and control leaves measured at three different light intensities are plotted. The F_0-increase caused by the heat pulse was rather small, ~6% of the F_V of the control sample and FR pre-illumination did not decrease the F_0 value (data not shown). This means that there was only a very limited amount of Q_A^- at the onset of illumination in the heat-treated plants. This made a comparison of the initial fluorescence kinetics of the heat-treated and control plants possible. The kinetics of the initial rise of the heat-treated and control samples were very similar at all light intensities. This indicates that the first charge separation was not affected by the heat pulse (Fig. 1C).

Hydroxylamine (NH_2OH) reduces and thereby destroys the manganese cluster. Depending on the concentration, it also acts as an electron donor [31,32]. In thylakoids, at low (0.1 mM) NH_2OH-concentration the K peak appears, but there is no further fluorescence increase. However, at 1 mM NH_2OH the fluorescence transient is very similar to the transient of our heat-treated samples presented in Fig. 1 [33]. Therefore, processes on the donor side of PSII seem to explain many of the heat pulse-induced changes in the fluorescence induction.

The Q_A^- reduction, as reflected by the fluorescence increase in the 0.2–2 s time range, suggested that not only the one electron of Tyr_Z was available (emergence of the K peak) but that the PSII units had access to further, alternative electron donors replacing water as electron donor to PSII. This hypothesis was tested in a series of experiments.

3.2. Detection of alternative electron donors with short repetitive light pulses

Regeneration of the K peak was studied by giving two 5-ms light pulses (3000 µmol photons m^{-2} s^{-1} light intensity) spaced 2.3–500 ms apart. In this way, the availability of electrons for the re-reduction of Tyr_Z^+ can be studied. Re-reduction of Tyr_Z^+ enables a further reduction of Q_A that is reflected by the regeneration of the K peak on the second light pulse.

After the shortest, 2.3 ms time interval (Fig. 2A) there was almost no variable fluorescence. This confirms that the OEC was completely inactivated by the heat pulse, because the donation time to Tyr_Z^+ by active OECs is 0.1–1 ms [34]. Indeed, in control leaves, fluorescence largely recovered within 2.3 ms (Fig. 2A).

On the basis of this experimental approach the $t_{1/2}$ for the regeneration of the K peak was determined to be ~30 ms (Figs. 2B and C). In Fig. 2D the results of a long series of light pulses (a

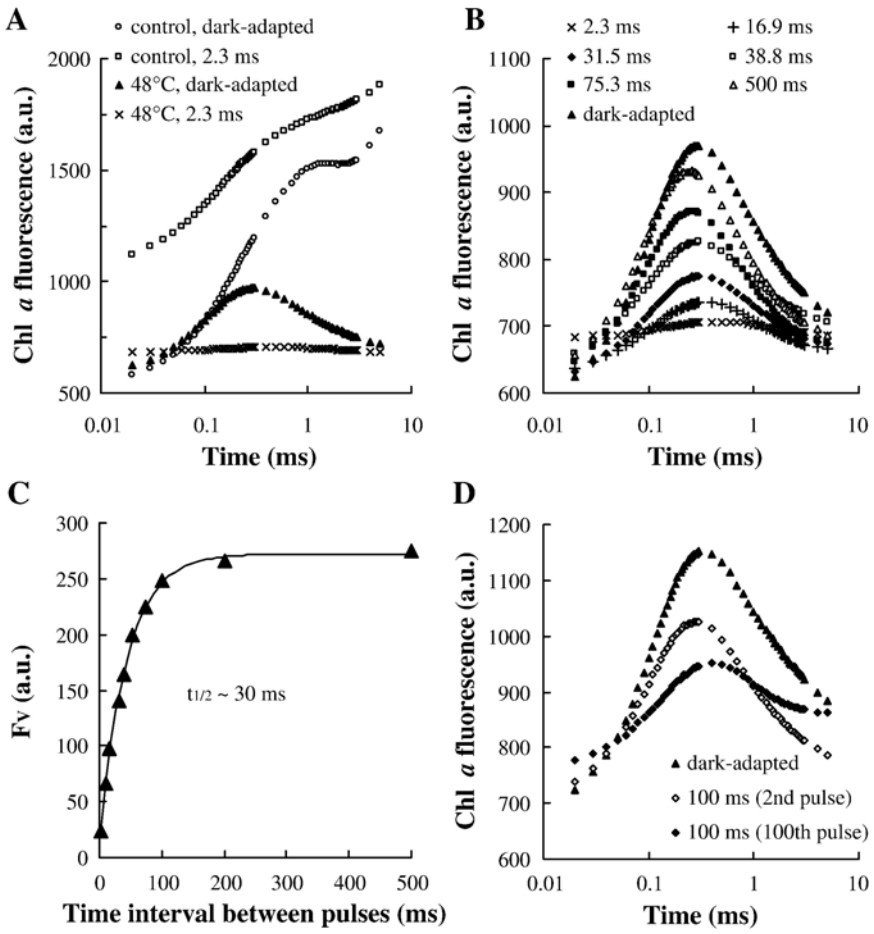

Fig. 2. Chl *a* fluorescence transients measured during 5-ms light pulses on heat-treated (48 °C, 40 s) and untreated barley leaves. (A) chl *a* fluorescence transients measured on untreated and heat-treated leaves induced by two 5-ms light pulses, spaced 2.3 ms apart. (B) Two 5-ms light pulses were spaced 2.3–500 ms apart (heat-treated leaves) (C) Regeneration kinetics of the K peak derived from measurements like the ones that are presented in panel B. (D) A train of one hundred 5-ms light-pulses were given spaced 100 ms apart (heat-treated leaves). The excitation light was produced by 650 nm LEDs and chl *a* fluorescence emitted at wavelengths > 700 nm was measured.

train of one hundred 5-ms-light-pulses spaced 100 ms apart) are presented: even in the case of the 100th light pulse, 30–40% of the original K peak was regenerated. Since the 5-ms flashes were nearly saturating for the K step with respect to a single charge separation (Fig. 2A shows that in untreated samples, several charge separations can occur in 5 ms), this experiment shows that the electrons originated from a large pool. In the order to estimate the pool size, the heat-treated leaves were illuminated with a series of 5-ms pulses spaced 200 ms apart. Half-depletion of the pool occurred after about 400–500 light pulses; further, a few minutes in darkness allowed full regeneration of the electron donor pool (data not shown).

3.3. Regeneration of the K peak after heat pulses of 50–54 °C

The regeneration of the K peak was also investigated after heat pulses of 50, 52 and 54 °C. The transients of Fig. 3 show that even after a 54 °C heat pulse, the intensity of the K peak and its regeneration after a 31.5 ms time interval were similar to those of a heat pulse at 48 °C (Fig. 2). We note, however, that the fluorescence decrease after the K peak becomes slightly slower by increasing the temperature of the treatment. This may be explained by a gradual change of the redox equilibrium between Q_A and Q_B (e.g. [5]).

3.4. Chl a fluorescence transients and 820 nm transmission kinetics of DCMU + heat-treated leaves

Forward electron transport beyond Q_A can be blocked by DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea). In DCMU + heat-treated leaf segments the P level was close to the F_M value of untreated leaves (Fig. 4A). This indicates that in heat-treated leaves (in the absence of DCMU) at the maximum of the peak in the 0.2–2 s time range (P) not all PSII reaction centers were closed, and thus the F_V/F_M was low. This, however, does not necessarily mean that the maximum quantum yield of PSII was very much reduced in heat-treated leaves (cf. Fig. 1C).

The F_0 and F_M values of the fluorescence transient are used to determine the maximum quantum yield of primary photochemistry ($\varphi_{Po}=F_V/F_M=k_P/(k_P+k_N)$, where k_P and k_N are rate constants of photochemistry and other losses of excitation energy, respectively; [35]). A requirement for the use of the F_V/F_M value as the maximum quantum yield of primary photochemistry is that at F_M all PSII reaction centers are

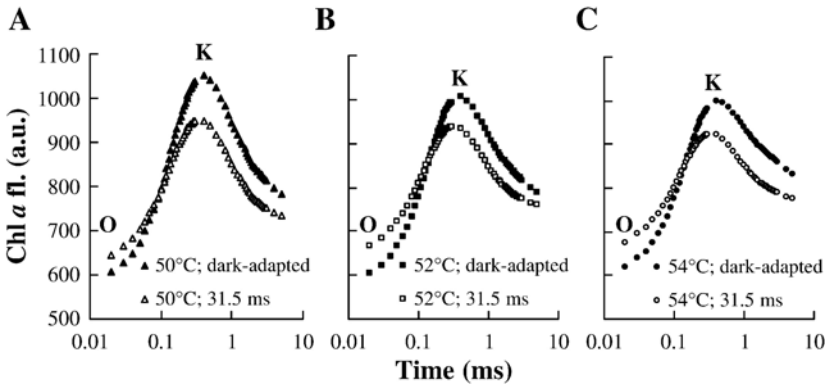

Fig. 3. Regeneration of the K peak after heat treatments at 50 °C (A), 52 °C (B) or 54 °C (C). The light pulses were 5 ms long and the time interval was 31.5 ms between the first (dark-adapted) and second light pulse. The excitation light was produced by 650 nm LEDs and chl *a* fluorescence emitted at wavelengths > 700 nm was measured.

closed. This requirement is not fulfilled in the case of heat-treated samples (Fig. 4A). For this reason, the F_V/F_M value should not be used as an indicator of maximum quantum yield of primary photochemistry in such samples.

We also note that in the case of non-heat-treated samples the F_M values were almost identical in the presence or absence of DCMU. This confirms an earlier study in which it was shown that PQ-pool quenching does not occur if DCMU is allowed to diffuse slowly into intact leaves [36].

The shape of the chl *a* fluorescence transient of DCMU + heat-treated leaf segments was biphasic: a large fast and a smaller slow phase can be distinguished. This may suggest that the phenomenon is related to α and β centers [37]. However, the $t_{1/2}$ of this slow phase is in the order of tens of ms, which is much slower than the closure of the β centers at this light intensity [38]. We explain the very slow phase of the heat-treated samples by assuming that charge recombinations between Q_A^- and Tyr_Z^+ slow down the reduction of Q_A^-. These recombination reactions may be gradually blocked when the alternative electron donors re-reduce Tyr_Z^+ (Fig. 2).

In Fig. 4, simultaneously measured Chl *a* fluorescence transients (Fig. 4A) and 820 nm transmission kinetics (Fig. 4B) are presented. Transmission changes at 820 nm reflect the redox states of P700, plastocyanin (PC) and Fd, with Fd being a small component [15,26,39]. Transmission changes were used here to monitor electron flow through PSI. In control leaves, a red light pulse induced an initial oxidation of P700 and PC (decrease in the transmission level) followed by a re-reduction when electrons arrive from PSII [26,36]. Re-reduction occurred also in the case of heat-treated samples, but it happened later (after 20 ms for the control and 80 ms for the heat-treated samples) and it was also much slower ($t_{1/2}$ of ~40 ms for the control and ~140 ms for the heat-treated leaves). This indicates that in the case of the heat-treated samples the electrons responsible for $P700^+$ and PC^+ reduction arrived much more slowly than in the control samples. This is in agreement with the data showing that

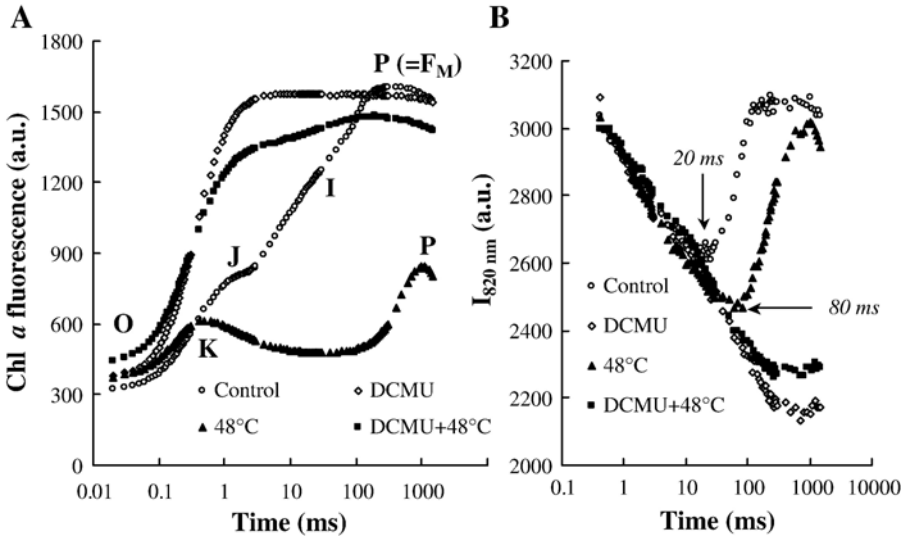

Fig. 4. Chl *a* fluorescence (A) and simultaneously measured 820 nm transmission kinetics (B) of heat-treated, DCMU-treated, DCMU + heat-treated and untreated barley leaves. The excitation light was produced by 650 nm LEDs and chl *a* fluorescence emitted at wavelengths > 700 nm was measured.

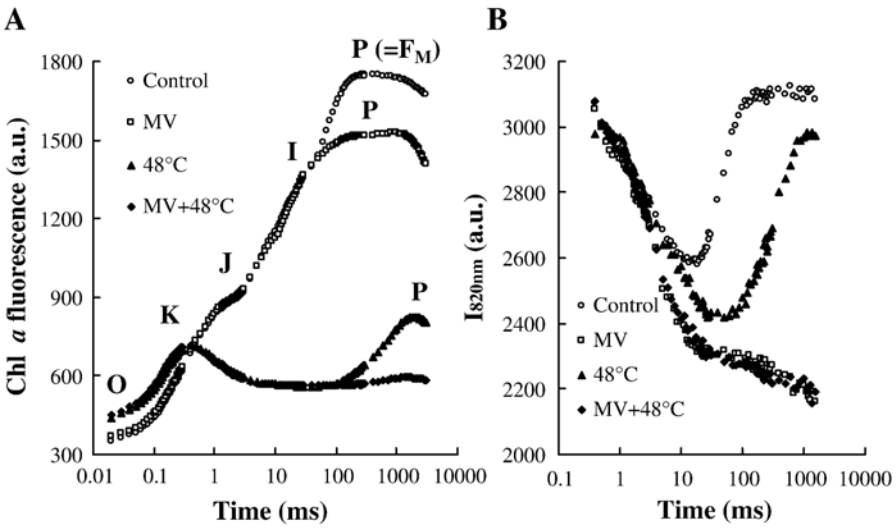

Fig. 5. Chl *a* fluorescence (A) and simultaneously measured 820 nm transmission kinetics (B) of heat-treated, MV-treated, MV+heat-treated and untreated barley leaves. The excitation light was produced by 650 nm LEDs and chl *a* fluorescence emitted at wavelengths >700 nm was measured.

the $t_{1/2}$ of the electron donation to PSII is slow compared to the control (cf. Fig. 2). As shown in Fig. 4B, the re-reduction of $P700^+$ and PC^+ is DCMU-sensitive both in the control and in the heat-treated samples. This finding substantiates our conclusion on the existence of an electron donor pool and the functioning of an electron transport through PSII in the absence of active OECs.

In unreported experiments we also confirmed that the oxidation of cytochrome *f* in heat-treated samples occurred as in control samples but the re-reduction of cytochrome *f* was somewhat slower in heat-treated leaves. (Flash-induced absorbance transients (see e.g. [40]) were recorded between 505 and 572 nm. Redox transients of the cytochrome *b*/*f* complex were identified based on the transient spectra, or for cytochrome *f*, as $\Delta A_{554} - \Delta A_{545}$, after correction for ΔA_{515}).

It is also interesting to note that heat treatment speeds up the fluorescence and transmission decrease at around 1 s (Fig. 4, see also Fig. 1B, P peak). We have shown earlier that the IP phase of the OJIP transient depends on PSI activity and it represents the reduction of the Fd pool in the presence of inactive FNR [15,27]. In the following section the possible role of the acceptor side of PSI on the chl *a* fluorescence transient was studied in heat-treated leaves.

3.5. Methylviologen (MV) treatments

In light-adapted leaves the PSI acceptor side is active (FNR is activated in 1–2 s; reviewed in [67]) and as a consequence, the IP phase is missing [27]. The IP phase is not present in leaves treated with methylviologen (MV), a compound that is known to accept electrons from the FeS-clusters of PSI; therefore it allows electrons to bypass the block that is transiently imposed by the inactive FNR [15].

Methlyviologen treatment of intact barley leaves made the IP phase disappear (Fig. 5A) and the re-reduction of PC^+ and $P700^+$ did not occur during the red light pulse (Fig. 5B), just like in pea leaves; [15]). In MV+heat-treated leaves the fluorescence peak in the 0.2–2 s time range was not observed (Fig. 5A) and the re-reduction of PC^+ and $P700^+$ did not occur (Fig. 5B). These data show that similar to the non-heat-treated leaves, the fluorescence rise to P depends on PSI activity [15].

3.6. Double-pulse experiments

In order to obtain further information about the photosynthetic electron transport processes in heat-treated leaves, double pulse experiments were carried out to study the regeneration of several fluorescence parameters. The regeneration kinetics of the O ($F_{20\ \mu s}$) and J ($F_{3\ ms}$) steps in the dark depend on the re-oxidation of Q_A^- and the PQ-pool, respectively [15,38]. In untreated leaves, the $F_{20\ \mu s}$, $F_{400\ \mu s}$ and $F_{3\ ms}$ values gradually decreased upon increasing the time intervals between the two light pulses (Fig. 6A). However, in the case of heat-treated leaves, the $F_{20\ \mu s}$, $F_{400\ \mu s}$ and $F_{3\ ms}$ values initially increased upon increasing the time interval between the two light pulses, and they reached their maxima 0.5 s after the first pulse (Figs. 6B and D). This initial $F_{20\ \mu s}$, $F_{400\ \mu s}$ and $F_{3\ ms}$ increase may indicate an ongoing reduction of the PQ-pool and Q_A in darkness that lasts for a short period of time after switching off the light. This may be explained by a stimulated dark-reduction of the PQ-pool [1,2] and a heat-induced shift in the redox equilibrium between Q_B and Q_A towards Q_A [5,6,8,9]. We note that FR pre-illumination of dark-adapted heat-treated leaves only slightly changed the chl *a* fluorescence transients indicating that in dark-adapted samples the PQ-pool was mainly in the oxidized state (data not shown).

In untreated leaves, re-oxidation of Q_A^- may occur via two pathways [41]: (i) recombination reactions between Q_A^- and the S_2/S_3 states of the OEC ($t_{1/2}$ of less than a second), and (ii) forward electron transport in those centers that are in the S_0 or S_1 states and that depends on the availability of oxidized PQ-molecules. The $F_{3\ ms}$ value can be used as an indicator for the PQ-pool redox state [12,38] and its dark-recovery is considerably slower than the dark-recovery of the $F_{20\ \mu s}$-value that

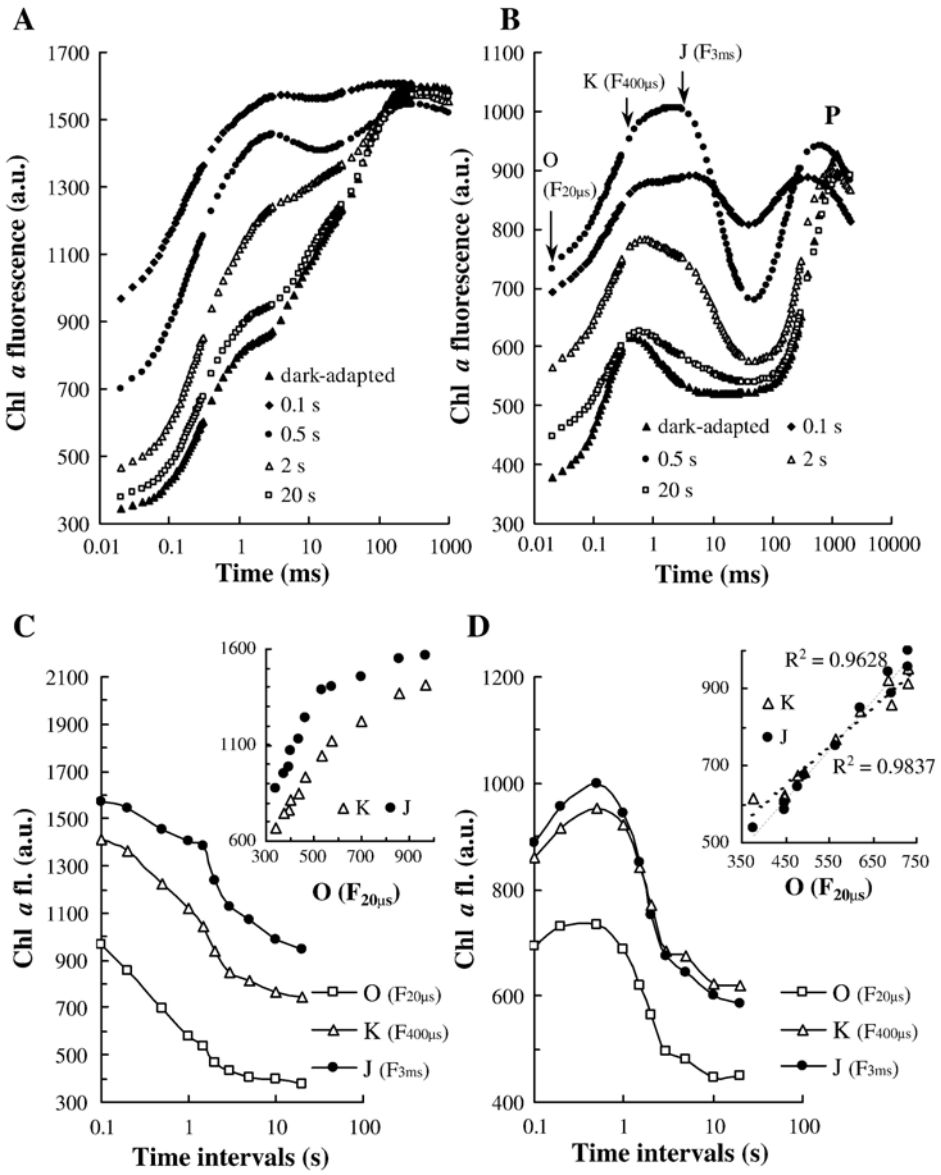

Fig. 6. Chl *a* fluorescence transients of a double-pulse experiment carried out on untreated (A) and heat-treated barley leaves (B). Dark adaptation kinetics of $F_{20\ \mu s}$, $F_{400\ \mu s}$ and $F_{3\ ms}$ values of untreated (C) and heat-treated barley leaves (D), derived from chl *a* fluorescence transients like the ones presented in panels A and B. The excitation light was produced by 650 nm LEDs and chl *a* fluorescence emitted at wavelengths > 700 nm was measured.

reflects the re-oxidation of Q_A^- [15]. In heat-treated leaves, the dark-recovery of $F_{20\ \mu s}$, $F_{400\ \mu s}$ and $F_{3\ ms}$ occurred in parallel (Fig. 6D) and linear relationships were found between $F_{20\ \mu s}$ and $F_{400\ \mu s}$ ($R^2 = 0.96$) and between $F_{20\ \mu s}$ and $F_{3\ ms}$ ($R^2 = 0.98$; inset of Fig. 6D). No such correlation can be found in control leaves (inset of Fig. 6C). This suggests that in heat-treated leaves, re-oxidation of Q_A^- occurred only via forward electron transfer and there were no recombination reactions between the donor side and Q_A^- (recombination reaction between Q_A^- and Tyr_Z^+ occurs with $t_{1/2} \sim 120$ ms [42]). This further confirms that the OEC was completely inhibited by the heat pulse and that Tyr_Z^+ had been re-reduced by an alternative electron donor before recombination could take place.

An interesting fluorescence feature can be observed in the case of heat-treated leaves (Fig. 6B): 0.1 s after the first light pulse, both the K and the J steps were present in the fluorescence transients. In the case of the 0.5 s time interval the J step became more prominent. The discussion of this phenomenon is beyond the scope of the present study, but see [38,43].

3.7. Re-reduction of $P700^+$ and PC^+ in darkness

In heat-treated samples the re-reduction of $P700^+$ and PC^+ in darkness following a FR illumination is stimulated, which may be caused by a stronger electron donation by stromal reductants like Fd and NADPH to the PQ-pool [1,2]. Immediately after the heat-treatment $P700^+$ and PC^+ re-reduction is ~10 times faster than in untreated samples but this effect does not persist for long [2]. Our measurements were carried out 30–60 min after the heat pulse. The $t_{1/2}$ of the re-reduction of $P700^+$ and PC^+ was

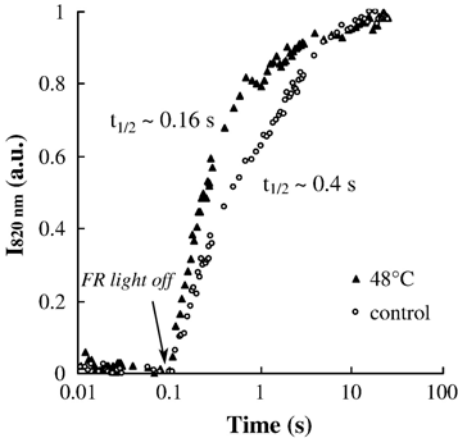

Fig. 7. Dark-recovery of the 820 nm transmission signal of heat-treated and untreated barley leaves following 15 s of FR pre-illumination. The FR-LED had a peak emission at 718 nm.

~0.4 s for the control and ~0.16 s for the heat-treated samples, which means that the stimulation of $P700^+$ and PC^+ re-reduction was still significant (Fig. 7). This indicates that dark-reduction of the PQ-pool is stimulated and this may represent a possible explanation for the increase of the $F_{20\ \mu s}$, $F_{400\ \mu s}$ and $F_{3\ ms}$ values during the first 0.5 s of darkness after a red light pulse (Figs. 6B and D).

4. Discussion

4.1. The initial fluorescence rise in heat-treated leaves

The heat pulse exerted little effect on the very beginning of the fluorescence transient: the rising parts of the K peak and the J step overlap completely during the first 70–150 μs depending on the light intensity (Fig. 1C). This implies that the heat-treatment had little effect on the effective cross-section of PSII (σPSII; [44]). This means that heat-induced antenna dissociation (e.g. [45] plays no or a very limited role under our experimental conditions. The initial overlap of the K and J steps also implies that the probability of charge recombination is not higher and there is no stronger accumulation of $P680^+$ in heat-stressed leaves than in control samples at the beginning of illumination. Also, 820 nm transmission measurements (Fig. 4B) show that $P680^+$ accumulation is unlikely during the first 10 ms of illumination, because the build-up of $P680^+$ should have led to a faster decrease of the 820 nm transmission signal ($P680^+$ absorbs at 820 nm just like $P700^+$, e.g. [30]).

Photosystem II contains several other co-factors (Cyt b_{559}, Tyr_D, Chl_Z, β-carotene) that could act as potential electron donors (reviewed in [46]). It has been estimated that the excitation rate of PSII for pea leaves is once every 50 μs at 12 000 μmol photons m^{-2} s^{-1} light intensity [47]. This would suggest that the overlapping parts of the K and J steps represent approximately one charge separation with Tyr_Z being the electron donor [20]. If any of the above-mentioned additional co-factors were able to stabilize an additional charge separation, it would have prolonged the fluorescence rise beyond the K peak. These co-factors are not likely to donate electrons to $P680^+$ for two other reasons: (i) the pool of available electrons is too small to explain the observed electron transport activity (Fig. 4B), and (ii) there is little sign of heterogeneity in the regeneration kinetics of the K-peak (Fig. 2C).

The fluorescence transients normalized to $F_{20\ \mu s}$ in Fig. 1B demonstrate that the K peak is highly light-intensity dependent but the fluorescence rise in the 0.2–2 s range is not. This indicates that the fluorescence rise in the 0.2–2 s range is limited by electron transport reactions, which is confirmed by 820 nm transmission measurements (Fig. 4B).

4.2. Electron donation to PSII by alternative sources

The results of this in vivo study indicate that if OECs are inactivated, PSII reaction centers have access to a large pool of alternative electron donors making partial reduction of Q_A and the electron transport chain possible. The $t_{1/2}$ for this electron donation process is ~30 ms (Fig. 2C). This electron donation pathway is rather robust since after a heat pulse of 54 °C the alternative electron donors were still capable of donating electrons to PSII (Fig. 3).

Several compounds are known to be able to donate electrons to the donor side of PSII in vitro in the absence of the manganese cluster: ascorbate (Asc), Mn^{2+}, NH_2OH, TMPD (e.g. [31,48,49]). Alternative electron donors have been suggested to play an important role also under in vivo conditions (e.g. [50,51]) but their existence has never been experimentally proven. In vivo, in the absence of active OEC, Asc is probably the only alternative electron donor that can supply electrons in sufficiently high amounts to PSII. The concentration of Asc in chloroplasts is rather high; ~25–50 mM in C_3 plants [52,53]. In the lumen, where violaxanthin de-epoxidase uses Asc as co-substrate [54], the concentration of Asc was estimated to be 3.8 mM [55]. In isolated thylakoids, Katoh and San Pietro [48] and Mano et al. [51] found that the K_m value for Asc was ~2.5 mM and they estimated that the Asc-dependent electron flux was ~50 μmol NADPH mg Chl^{-1} h^{-1}. The donation time that can be derived from these values is of the same order of magnitude as the ~30 ms electron donation halftime that we obtained in heat-treated leaves.

It is worth mentioning that Asc is not a good electron donor to PSII reaction centers with an active OEC [51]. It has also been suggested that Asc can donate electrons to PSI, but this electron donation is significant only at relatively high (>10–20 mM) Asc concentrations [51,56] that are unlikely to occur in the lumen. Indeed, we did not find evidence for electron donation by alternative electron donors to $P700^+$. Fig. 4 shows that if all electron flow from PSII is inhibited by DCMU, $P700^+$ and PC^+ were oxidized within 1–2 s of red light illumination in the heat-treated samples, just like in control samples.

The lack of electron donors in manganese-depleted samples has been suggested to be responsible for low-light photoinhibition (e.g. [57]). The absence of electron donors would lead to the accumulation of $P680^+$ leading in turn to photo-oxidative damage. The 820 nm transmission measurements presented in

Fig. 4B demonstrate that alternative donors can maintain a limited electron flow through PSII. In low light, this might be enough to protect PSI and PSII reaction centers from further damage even in the total absence of active OECs. This protection can be physiologically relevant given the fact that under natural stress conditions the inactivation of OECs occurs stochastically. Indeed, it has been observed that there is no difference between the photosynthetic activities of plants kept in darkness or in relatively low light (100 μmol photons m^{-2} s^{-1}) for 30 min following a heat pulse [23]. However, a few hours of light treatment following a heat pulse makes the K peak disappear indicating a further damage (or degradation) of the PSII reaction centers [23].

Photosystem I itself is heat-tolerant but exposure of isolated chloroplasts to heat in the light causes a selective destruction of PSI pigments. It has been shown that it is a direct consequence of the interruption of the electron flow originating from PSII [58]. Therefore, it is conceivable that the alternative electron donors of PSII may have some protective role also for PSI.

A pathway for the maintenance of the Asc-concentration in the lumen has been proposed by Mano et al. [51,59]. Upon donating electrons Asc is oxidized to monodehydroascorbate (MDA). The MDA radicals in the lumen are then disproportionated to Asc and dehydroascorbate (DHA). DHA is able to diffuse to the stroma where DHA reductase catalyses the reduction of DHA by glutathione. Asc^- is then thought to be transferred to the lumen by a postulated transporter [51,59] or by diffusion [55].

4.3. Dark-reduction of the PQ-pool

The heat-induced stimulation of electron transport around PSI has been in the focus of research on heat-stress effects on the thylakoid membrane over the last 10 years. Re-reduction of $P700^+$ and PC^+ after a far-red pulse was shown to be stimulated up to 10-fold after a heat treatment [2]. However, it was also shown that this stimulatory effect disappears quite quickly after the heat-treatment [2]. The only two-fold stimulation that is observed here (Fig. 7) could very well be due to the fact that our measurements were carried out ~30 min after the treatment. A stimulated non-photochemical reduction of the PQ-pool can be due to an increased cyclic electron transport around PSI [60–63] or to increased chlororespiration [64,65]. However, FR-preillumination did not decrease the F_0 value of dark-adapted, heat-treated leaves (data not shown). Therefore it is likely that the PQ-pool and Q_A were mainly in the oxidized state and the stimulation of chlororespiration was not significant.

Two cyclic PSI electron transport pathways have been described in higher plants: (i) a NADPH-dehydrogenase (NDH) dependent pathway [66], and (ii) a ferredoxin-PQ-reductase (FQR) dependent pathway [60]. Of these, the NDH pathway does not seem to be relevant to our study, because the data indicate (Fig. 4) that in heat-treated barley leaves FNR is inactive in darkness, as expected (e.g. [67]). Havaux et al. [68] have shown that the relative contribution of the two cyclic pathways varies from one plant species to another. In barley protoplasts, it has been shown that the slow component of the far-red light stimulated luminescence (related to PSI cyclic electron transfer) is sensitive to antimycin A [69]. Therefore it is likely that in non-stressed barley plants the FQR pathway is active. In other words, a heat-stimulation of the FQR-pathway could explain the two-fold increase in $P700^+$ and PC^+ reduction (Fig. 7) and the increase of $F_{20\ \mu s}$, $F_{400\ \mu s}$ and $F_{3\ ms}$ that lasts for ~0.5 s after switching off the light (Fig. 6). However, the 820 nm transmission measurements (Fig. 4B) demonstrate that in the light the electron transport activity of heat-treated leaves was very sensitive to DCMU. This means that cyclic PSI electron transport contributed very little and the photosynthetic electron transport was dominated by PSII-mediated electron transport that originates from the alternative electron donors of PSII.

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