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Regulation of antenna structure and electron transport in Photosystem II of *Pisum sativum* under elevated temperature probed by the fast polyphasic chlorophyll *a* fluorescence transient: OKJIP

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Abstract

Chlorophyll *a* (Chl *a*) florescence induction kinetics from the minimum yield F_0 to the maximum yield F_m provide information on the filling up of the plastoquinone pool with reducing equivalents. In this paper, we have examined the effect of high temperature (above 40°C) on Chl *a* fluorescence rise kinetics starting from 40 μs (to 1 s) in pea leaves (*Pisum sativum*). The variable Chl *a* fluorescence is strongly quenched after heat treatment. With increasing temperature or the duration of heat treatment a typical O-J-I-P transient (Strasser et al. (1995) Photochem. Photobiol., 61, 32–42) is transformed into an O-K-J-I-P transient, with an additional rapid step called K detected in the 200–300 μs range. After prolonged heat treatment, the K-step becomes a dominant peak in the Chl *a* fluorescence transient followed by a large dip. We have investigated the origin and the possible interpretation of these changes by using NH_2OH which acts as an electron donor to PS II, and DCMU which is known to block the PS II electron transport chain by displacing Q_B. From the present data we propose that the appearance of this K-step is due to two effects: (1) inhibition of the water splitting system that leads to a much slowed turn over of the reduction of Q_A; (2) changes in the architecture of the antenna of PS II which affect the energy migration properties within the photosynthetic unit. The K-step can thus be used as an indicator of the heterogeneity of photosynthetic units and as an indicator for the physiological state of the photosynthetic sample.

Keywords: Chlorophyll *a* fluorescence; Heat stress; OKJIP transient; Photosystem II reaction center

1. Introduction

Many environmental factors such as light, water, CO_2 and temperature affect the growth of the plants. Lack or excess of any of these factors causes stress [1,2]. Among all cell functions, the photosynthetic activity of chloroplasts is believed to be one of the most heat sensitive (for reviews, see [3–5]). When leaves or algae are incubated at higher temperature, their O_2 evolution, CO_2 fixation and photo-phosphorylation capabilities are dramatically inhibited.

Abbreviations: Chl *a*, chlorophyll *a*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; K, J, I, intermediate steps of Chl *a* fluorescence rise between F_0 and P; F_0, F_m, initial and maximum Chl *a* fluorescence; F_p, Chl *a* fluorescence peak appearing later than the intermediate steps F_K, F_J and F_I; NH_2OH, hydroxylamine; PQ, plastoquinone; PS II, Photosystem II; Q_A, primary bound plastoquinone; Q_B, secondary bound plastoquinone; RC, reaction center.

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The effect of high temperature on photosynthetic membranes results in the loss of grana stacking, due to the dissociation of the peripheral antenna complex of Photosystem II (PS II) from its core complex [6,7]. Heat inactivation of chloroplasts has also been shown to be correlated with the release of Mn from the oxygen evolving complex [8,9], while other studies suggest an alteration in Q_A to Q_B electron transfer at the acceptor site [10]. Chl *a* fluorescence has been extensively used to evaluate the extent of thermal damage of the photosynthetic apparatus in intact leaves [10–15]. Here, we have used the technique to measure the fast Chl *a* fluorescence transient up to 1 s, which offers additional possibilities to probe the nature of damage by thermal and other stresses [16–20]. At room temperature, the Chl *a* fluorescence transient follows a polyphasic pattern of O-J-I-P from the initial fluorescence level (F_0), to the maximum fluorescence level (F_p or F_m) [21–23].

It has been reported earlier [16–18] that the O-J-I-P Chl *a* fluorescence transient exhibits two major changes after heat treatment of the leaf discs: (1) quenching of the variable Chl *a* fluorescence (F_p-F_0) due to a large decrease in the maximum fluorescence F_p and a small increase in F_0, and (2) the transformation of the polyphasic O-J-I-P Chl *a* fluorescence rise into an O-K-J-I-P fluorescence transient with a new rapid step K detected at about 200–300 μs. The K-step can even represent the maximum fluorescence intensity in the Chl *a* fluorescence induction transient before the fluorescence declines again. In this paper, we report the phenomenological appearance of the K-step in heat stressed leaves of pea, as well as in algae and in several plants growing in hot climates in the field.

2. Materials and methods

Most of the experiments were done with 6 mm leaf discs from dark adapted fully matured leaves of pea (*Pisum sativum* L.) plants grown in a greenhouse at 22/18°C (day/night) under natural sun light. Cells of *Haematococcus lacustris* (Girod) Rostafinski (Volvocales) were grown autotrophically at 21°C (±2°C) in Erlenmeyer flasks under white light (day light fluorescence lamp, 12.5 $W \cdot m^{-2}$) in a medium with the addition of 0.3 μM of thiamin as described by Hedlich [24].

For heat stress treatment, pea leaf discs were immersed in water in a small beaker. The beaker was directly plunged in a circulating water bath (Colora Messtechnik, Lorch, Baden-Württemberg, Germany) at different temperatures ranging from 25°C to 48°C for different times. For the NH_2OH or DCMU treatment, leaf discs were incubated in 50 mM NH_2OH and/or 250 μM DCMU for 2 h in the incubation medium (100 mM sorbitol, 50 mM Hepes, 0.5 mM $CaCl_2$ and 0.5 mM $MnSO_4$, pH 7.5) in complete darkness.

Chl *a* fluorescence was measured at room temperature using a Plant Efficiency Analyzer (PEA, Hansatech, King's Lynn, Norfolk, England) as described before [20]. Excitation light of 600 $W \cdot m^{-2}$, from an array of six light-emitting diodes (peak wavelength, 650 nm), was focused on the surface of the leaf disc to provide a homogeneous illumination light spot of about 4 mm in diameter. Chl *a* fluorescence signals were detected using a PIN photocell after passing through a long pass filter (50% transmission at 720 nm).

The fast fluorescence transients were recorded and digitized on line with 12-bit resolution from 10 μs to 1 s, with a time resolution of 10 μs for the first 200 data points. The fluorescence signal at 40 μs (the fourth digitized data point) was considered as a reliable value for the initial fluorescence intensity, F_0. This was established in control measurements with a fast digital oscilloscope.

3. Results and discussions

3.1. Effect of heat stress on the Chl a fluorescence induction curve

Changes in the shape of Chl *a* fluorescence transients of pea leaves, incubated in water at different temperatures for 5 min, are shown in Fig. 1. A leaf disc incubated at 25°C exhibits a polyphasic rise called O-J-I-P Chl *a* fluorescence transient; the O- to J-phase (ends at about 2 ms), the J- to I-phase (ends at about 30 ms) and the I- to P-phase (ends about at 500 ms). The O- to J-phase is due to the net photo-

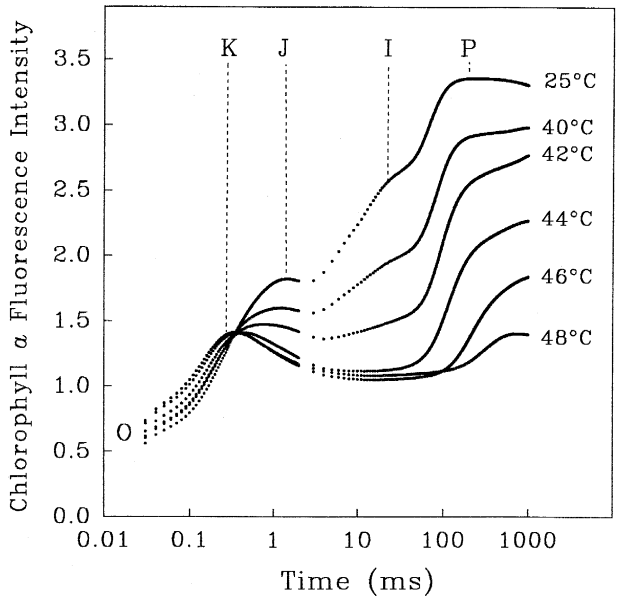

Fig. 1. Temperature dependence of the appearance of the K-step. Pea leaf discs were heated at the indicated temperatures for 5 min and readapted at room temperature for 10 s before the measurements. These are the original data, as measured, without any normalization.

chemical reduction of Q_A to Q_A^-. This phase is also influenced by the S-states of the donor side of PS II [21,25,26] and the secondary reaction $Q_A^- Q_B$ to $Q_A Q_B^-$. The intermediate step I and the final step P have been proposed to be due to the existence of fast and slow reducing plastoquinol (PQ) pool-centers, as well as due to the different redox states of the RC of PS II which reduces the PQ pool [22]. After the heat stress, the major effect is the decrease of the 'P' level. This decrease of the fluorescence intensity can be restored by the addition of DCMU (see Table 1). Therefore, it is due to a blockage of electron donation from the PS II donor side. Details are discussed below.

3.1.1. Quantum yield of excitation energy trapping of PS II

There have been a large number of investigations carried out on heat stressed plants showing a decrease in the rate of O_2 evolution, electron transport and the ratio F_v/F_m (see [12],[27–29]). The expression F_v/F_m is an excellent measure of the quantum yield of primary photochemistry of PS II [30]. The measurements shown in Fig. 1 indicate, as mentioned earlier [14–17], that by increasing the leaf temperature, the immediate effect seen was the decrease of the fluorescence intensity at F_p and a very slight increase of F_0 resulting in reduced variable fluorescence and decreased F_v/F_m ratio. Similar results have been published by several investigators (see, e.g., [11],[31,32]). F_0 is the level of fluorescence emission when all the primary quinone acceptors (Q_A) are in the oxidized state (see for reviews [33,34]). An increase in F_0 has been attributed to the physical separation of the PS II reaction centers from their associated pigment antennae resulting in blocked energy transfer to the PS II traps [6,35,36], although a part of this phenomenon could possibly reflect the accumulation of the reduced form of Q_A^- [10]. Recently Briantais et al [37] suggested that heat stress

Table 1
Effect of temperature on different parameters [56] of fast fluorescence transients

T(°C)	DCMU	F_0 $F_{50\ \mu s}$	F_1 $F_{150\ \mu s}$	F_2 $F_{300\ \mu s}$	F_3 $F_{2\ ms}$	F_m	F_v	F_v/F_m
25	−	557	721	906	1274	2646	2086	0.789
46 *	−	825	1047	1151	1001	1507	682	0.453
25	+	751	1138	1552	2127	2263	1512	0.668
46	+	796	1135	1438	1875	2188	1392	0.636
T(°C)	DCMU	F_v/F_0	$F_1 - F_0$	$V_{150\ \mu s}$	$V_{300\ \mu s}$	V_J	$V_{150\ \mu s}/V_J$	$V_{300\ \mu s}/V_J$
25	−	3.750	164	0.079	0.167	0.343	0.229	0.487
46 *	−	0.827	222	0.326	0.478	0.258	1.261	1.852
25	+	2.013	387	0.256	0.530	0.910	0.281	0.582
46	+	1.749	339	0.244	0.461	0.775	0.314	0.595

Leaves were heated for 5 min at indicated temperatures and then kept in the incubation medium without or with DCMU (250 μM) in the dark for 2 h. These are the average data from 15 different experiments performed during a year. $V_x = (F_x - F_{50\ \mu s})/(F_m - F_{50\ \mu s})$.
* Numbers belong to a state when not all RC are closed.

decreases the quantum efficiency of PS II photochemistry due to several effects: (1) a decrease of the rate of primary charge separation; (2) a reduction in the stabilization of charge separation; (3) an increase in the recombination rate constant of the radical pair of the RC; as well as (4) the disconnection of some minor antenna from the reaction center II.

3.1.2. Appearance of the K-step

After the heat stress, in addition to the major effects on initial and maximum fluorescence intensity, the polyphasic O-J-I-P Chl *a* fluorescence transient shows an additional step called K-step (in alphabetical order, I, J, K), which appears between the F_0 and the J-step (Fig. 1, see also [16], [17], [18], [19]). The K-step starts to emerge after a treatment of about 42°C for 5 min, and becomes the dominant peak when the leaf discs were treated at 48°C (Fig. 1). This step levels off at about 200–300 μs and is clearly visible when the graphs are plotted on a logarithmic time scale, as shown in Fig. 1. The transformation of a typical O-J-I-P Chl *a* fluorescence transient into an O-K-I-P transient, via an intermediate mixed O-K-J-I-P transient, is dependent on the temperature (Fig. 1) and on the duration of the high temperature treatment [16].

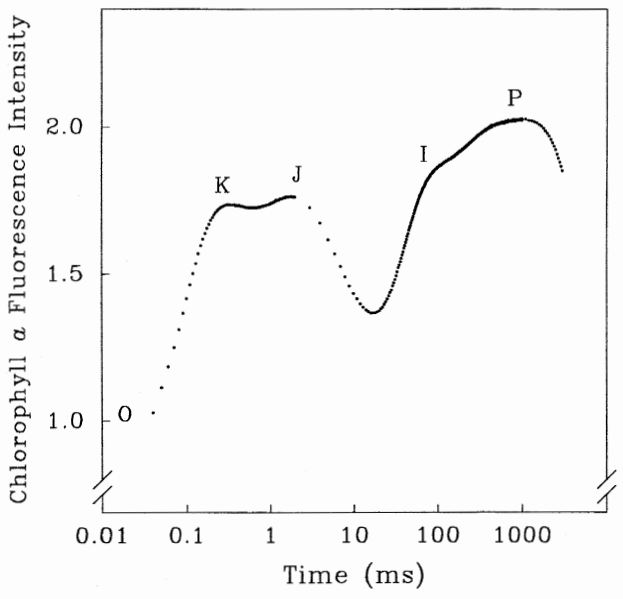

Fig. 2. Chlorophyll *a* fluorescence transients of a pea leaf disc after heat treatment (13 min at 44°C) and dark adaptation at room temperature for 10 s. The graph plotted on logarithmic time scale distinctly shows the K, J, I steps in between O and P.

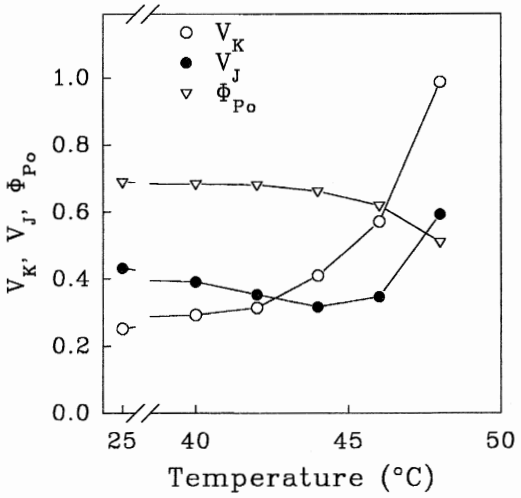

Fig. 3. Effect of temperature on the maximum quantum yield of excitation energy trapping (Φ_{P0}) the relative variable fluorescence at the J-step (V_J), and the K-step (V_K) of pea leaves. Φ_{P0} was measured in presence of DCMU.

Since it has been shown earlier [22] that, the O-, J-, I- and P-steps can be shifted on the time scale depending on the light intensity used, it was important to find the optimal temperature and the optimal time of treatment to show, in a non ambiguous way, that the K-step is not due simply to a shift of the J-step to shorter time. Although these four peaks are not always very well separated, Fig. 2 shows all the steps of the Chl *a* fluorescence transient, K, J, I and P, thus confirming that the K-step and the J-step are two different phenomenon. Some times OJIP transient is converted to OKIP due to intermediate mixed state.

The existence of the K-step, independent of the J-step, can also be seen in Fig. 3. With increasing leaf temperature, the relative variable fluorescence at the K-level measured at 200 μs ($V_K = (F_{200\ \mu s} - F_{50\ \mu s})/(F_m - F_{50\ \mu s})$) became significantly higher at 44°C and it increased further with increasing leaf temperature. On the other hand, a slight decrease in the relative variable fluorescence at 2 ms ($V_J = (F_{2\ ms} - F_{50\ \mu s})/(F_m - F_{50\ \mu s})$) was observed up to 46°C. These data further confirm that K and J are two different steps of the Chl *a* fluorescence induction kinetics. The maximum quantum yield of excitation energy trapping ($\Phi_{P0} = 1 - F_0/F_m$) of the leaf, measured in presence of DCMU, decreased steadily with increasing leaf temperature (Fig. 3).

3.2. Does the K-peak disappear after transferring the heated leaf disc to room temperature?

If after heating the leaf disc for 5 min up to 40°C, they are changed back at room temperature, the decreased Φ_{P0} recovers and the K-step disappears (data not shown). At higher temperatures (> 40°C) when the effects on Φ_{P0} become irreversible, the K-step is also irreversible. For example, the Chl *a* fluorescence transients in Fig. 4 were obtained from pea leaf discs which were kept in the dark for 2 h at room temperature after being for 5 min at 25°C (curve a) and 46°C (curve d), respectively. These results indicate that a heat treatment at 46°C for 5 min produces irreversible injury.

3.3. Effect of hydroxylamine on the K-phase

Leaf discs treated at higher temperatures show a large dip after the K-step followed by an increase of Chl *a* fluorescence intensity (Fig. 1, Fig. 2 and Fig. 4). There are two possible explanations for the appearance of this large dip after the K-step; (1) the reopening of the centers by electron transfer from Q_A to Q_B; and/or (2) the accumulation of centers with P-680$^+$ which is known to have a low fluorescence yield. It has been reported earlier that the major injury due to higher temperature is on the water oxidizing side [3,4]. If we assume that the decrease in the variable fluorescence, and the dip after the K-step are due to a lack of electron donation from the water splitting system, then supplying the electrons with an artificial electron donor should restore the fluorescence transient to some extent. To verify this assumption, in this experiment, we have used NH_2OH. Although NH_2OH disconnects the water splitting system from the PS II reaction center, it also acts as an artificial electron donor for PS II [38–40]. NH_2OH treated leaf discs showed still a lower level for the J-step in comparison to the non-heated leaf (Fig. 4b). On the other hand, there was no suppression of the J-step to I and P part of the induction curve. When the heat stressed leaf discs were incubated in the suspension medium containing NH_2OH, the dip after K was abolished and the variable fluorescence was also partially restored (Fig. 4c). This confirms that NH_2OH restores the lack of electron donation which arises through the inhibition of the water splitting system after heat treatment. This also verifies that the dip after K is due to the emptying of an electron pool on the donor side which can be refilled by NH_2OH.

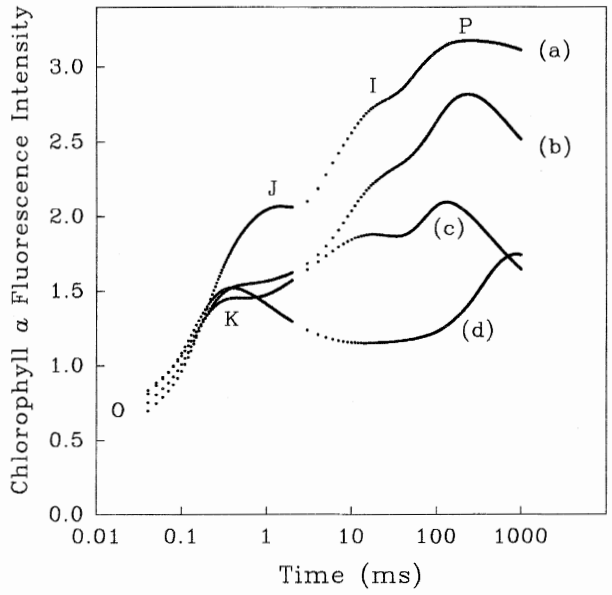

Fig. 4. Effect of NH_2OH on Chl *a* fluorescence transients of heat stressed pea leaf discs. a: control and (d) heat stress (5 min at 46°C) leaf discs kept in incubation medium for 2 h at room temperature; b: control and (c) heat stress (5 min at 46°C) leaf discs kept in incubation medium with 50 mM NH_2OH for 2 h at room temperature.

3.4. Effect of DCMU on the K-step

Although a large fraction of the dip after the K was recovered after NH_2OH treatment, the quenching in the variable Chl *a* fluorescence (F_p-F_0) was not fully eliminated (Fig. 4c). The fluorescence intensity remained lower at the F_p-level in comparison to the non-heated leaf. The F_p is the fluorescence of PS II with only partially closed RCs. It appears that the light intensity used is not enough to reach the true F_{max} in heated samples treated with NH_2OH. We explain this phenomenon in the following way: (1) the heat treatment slows down or totally damages the water splitting system; (2) NH_2OH restores electron donation to PS II, however with quite low efficiency [39]; (3) heat stress inhibits PS II activity while at the same time stimulates PS I activity [12]. Thus, after heat stress, higher PS I activity drained the electrons

much faster from the PS II acceptor side and the light intensity used was not enough to reach the true F_m.

Therefore, we measured the effects of heat in the presence of DCMU which blocks electron transport beyond Q_A^- by displacing Q_B [41,42]. For this experiment, leaf discs were heat treated at different temperatures for 5 min, and then kept at room temperature for 2 h in 250 μM DCMU to allow complete penetration. As reported earlier ([22], also see Fig. 5), the DCMU-treated leaf discs displayed maximum fluorescence intensity (at about 2 ms) where the J-step appears in the untreated control leaf. The heat treated sample, however, showed a pronounced K-peak with DCMU which appeared earlier (in between 200–300 μs), depending on the heat treatment, than in the unheated control. Addition of DCMU, in absence of any artificial electron donor, restored almost all the Chl *a* fluorescence in heat treated leaves. It is possible that (1) some endogenous electron donors may still be present with a limited donation capacity and their action is visible only in presence of DCMU; (2) DCMU prevents the accumulation of P+, a Chl *a* fluorescence quencher, by not allowing more than one turn over per center.

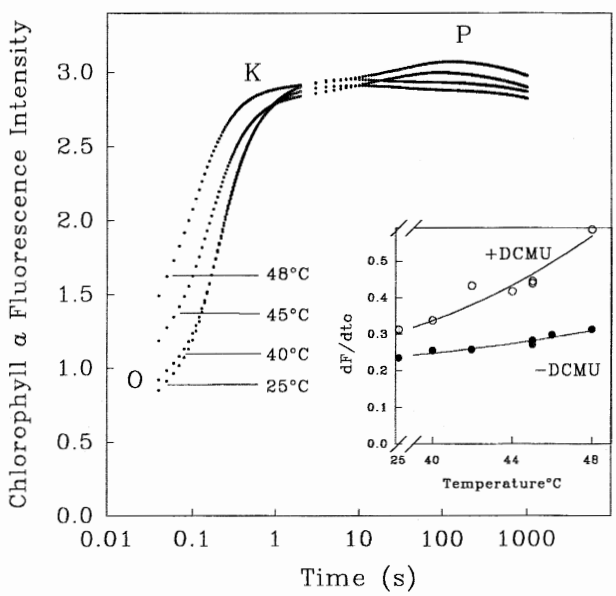

Fig. 5. Effect of DCMU (250 μM) and heat treatment on Chl *a* fluorescence transients of pea leaf discs. After the heat stress at different temperatures for 5 min, the leaf discs were kept in incubation medium with 250 μM DCMU for 2 h in complete darkness at room temperature. Insert shows the changes in the Chl *a* fluorescence intensity from 40 μs up to 100 μs in the presence or absence of DCMU at different temperatures. The addition of NH_2OH to the heat treated sample in the presence of DCMU did not change the shape of the curves (not shown in this figure).

The Chl *a* fluorescence rise in heated samples is much faster in the DCMU treated leaves than in the control leaves (insert in Fig. 5). Fluorescence kinetics of the heated leaf discs were also measured after incubating them in DCMU + NH_2OH or DCMU alone but they did not show any significant difference among themselves (data not shown).

The slope at the origin of the relative variable fluorescence (defined as $dV/dt_0 = (F_{150\ \mu s} - F_{50\ \mu s})/(F_m - F_{50\ \mu s})$) is a measure of the rate of primary photochemistry $((dQ_A^-/Q_{A(total)})/dt_0)$. Fig. 6 displays the fast fluorescence rise up to 500 ms after heat treatment at different temperatures. Plots in panels a–d of Fig. 6 are different presentations of the same data to illustrate particular points. Leaf discs kept at 25°C show an almost linear increase in the rate of fluorescence rise up to 250 μs and then the rate starts to fall. In contrast, the leaf discs kept at 48°C show a faster increase in the fluorescence rise up to 100 μs. However, if the curves are normalized with respect to their F_0 values, no significant differences were observed in the fluorescence rise kinetics up to 120 μs (Fig. 7b). The relative variable fluorescence (V_t) and variable fluorescence normalized by the variable fluorescence at 2 ms (V_t/V_J) are presented in panels c (Fig. 6c) and d (Fig. 6d) respectively. From all these presentations, one can see that the sigmoidal shape of the fluorescence rise in the leaves incubated at 25°C disappears in favor of an exponential rise after the heat treatment, indicating changes in the energetic cooperativity (grouping) between the antenna of several photosynthetic units.

3.5. Appearance of the K-step in plants grown in a natural environment

We believe that the process occurring in the leaves of higher plants which is responsible for eliciting the rapid K-step in the fluorescence rise, is a natural phenomenon. It is always present, but for dynamic reasons it does not appear clearly in an unstressed system. However, the K-step becomes dominant after heat treatment as reported here. All plants we have

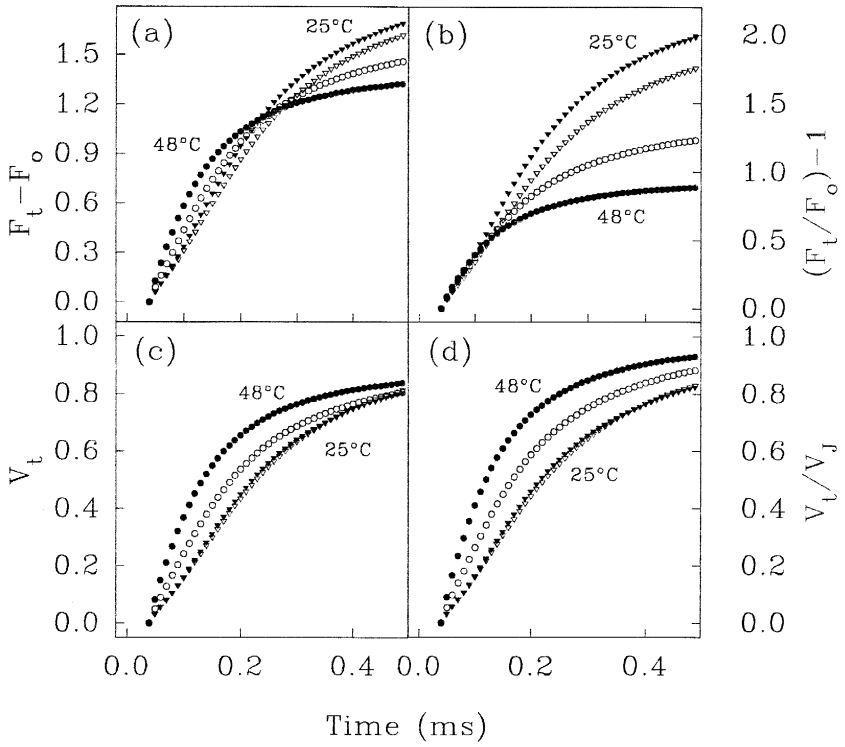

Fig. 6. a: the fast rise of the variable fluorescence $(F_t - F_0)$ without any normalization during the first 0.5 ms after heat treatment for 5 min at 25 (△), 40 (▲), 46 (○) and 48 (●) °C and incubation for 2 h in DCMU at room temperature. b: same data as in (a) but normalized to the initial fluorescence intensity, F_0. c: the relative variable fluorescence, $V_t = (F_t - F_0)/(F_m - F_0)$. d: the variable fluorescence $(F_t - F_0)$ normalized by the variable fluorescence at 2 ms, $V_t/V_J = F_t/(F_{2\ ms} - F_0)$. The slope at the origin in the presence of DCMU is a measure of the maximum fractional rate of primary photochemistry, $(dQ_A^-/Q_{A\ (total)})/dt_0$, according to Eq. (7). The expression V_t/V_J without DCMU substitutes the expression V_t in presence of DCMU [56,57].

investigated so far showed this K-step in the polyphasic fluorescence transient after heat treatment, although the sensitivity of each plant to temperature is different. We have also observed the K-step in different higher plants growing naturally in ecosystems associated with a dry and hot environments. The Chl *a* fluorescence transients obtained from leaves of *Cycus revoluta* (curve a), *Permelia sp*. (curve b) and green fruits of *Juniperus sp*. (curve c) growing in fields of hot climate, shown in Fig. 7, clearly indicate the existence of the K-step.

Another example of the presence of a K-step was observed in an algal culture of *Haematococcus lacustris*. The main feature of this flagellate is that it accumulates secondary carotenoids in the cells or in their spores at the cessation of cell division. The secondary carotenoids can also be accumulated in the flagellates by manipulation of the growth medium and the light conditions [43]. When the Chl *a* fluorescence induction kinetics were measured in the green flagellates of *H. lacustris*, the K-step was totally absent, but this step was clearly present in the red flagellates (Fig. 8). In the same way, the K-step was not obvious in green spores of *H. lacustris* but it was present in the red spores (Fig. 8). After heat treatment of the green cells of *H. lacustris*, the K-peak appeared (data not shown) as observed in green pea leaves suggesting that the appearance of the K-step of red flagellates and red spores of *H. lacustris* reflect the same changes in the photosynthetic apparatus as those induced by heat stress. Whenever the water splitting system is partially inhibited a K-step starts to appear, eg., under a stress environement, heat treatment in the laboratory or during specific phases of the life cycle such as red spores.

On the basis of these observations we conclude that the steps O-K-J-I-P of the fluorescence transient are always present in oxygenic plants. Each step of

the O-K-J-I-P transient is related to various electron transport reactions, and the kinetics are affected by the heterogeneity of the photosynthetic system.

3.6. Quantitative criteria of the K-step

In the control leaf discs the F_v/F_m ratio was 0.79, and was reduced to 0.45 after the heat treatment at 46°C for 5 min (Table 1). But in the presence of DCMU the F_v/F_m ratio was 0.67 in control, and 0.64 in heat treated samples (Table 1, averaged data collected from 15 different experiments during 1 year). This indicates that in the absence of DCMU, in heat treated samples, the measured fluorescence F_m does not correspond to the state where all reaction centers are closed. At this temperature range (25–46°C) the average rate of primary photochemistry measured between 50 and 150 μs in presence of DCMU ($(dQ_A/Q_{A(total)})/dt_0 \approx (F_{150\,\mu s} - F_{50\,\mu s})/(F_{2\,ms} - F_{50\,\mu s})$) was not affected by the heat treatment (Table 1). However, the slight sigmoidal shape of the fluorescence rise in the DCMU treated sample of the control leaf discs disappeared in favor of an exponential rise after heat treatment (Fig. 6). This can be seen as a small increase in the rise of the relative variable fluorescence at the origin normalized by V_J (measured as $(V_{150\,\mu s} - V_{50\,\mu s})/V_{2\,ms}$) from 0.281 to 0.314 after heat treatment (Table 1; Fig. 6).

In another type of experiment where the fluorescence intensity was measured as a function of sample temperature, the critical temperature (T_C) was defined in a very narrow range (within 2–3°C) for a given plant material [15]. T_C matches the behavior of the quantum yield of excitation energy trapping (Φ_{P0}) in our experiment. When the heat treatment is above 46°C Φ_{P0} decreases appreciably. Above 46°C the initial fluorescence rise dF/dto, as well as the initial

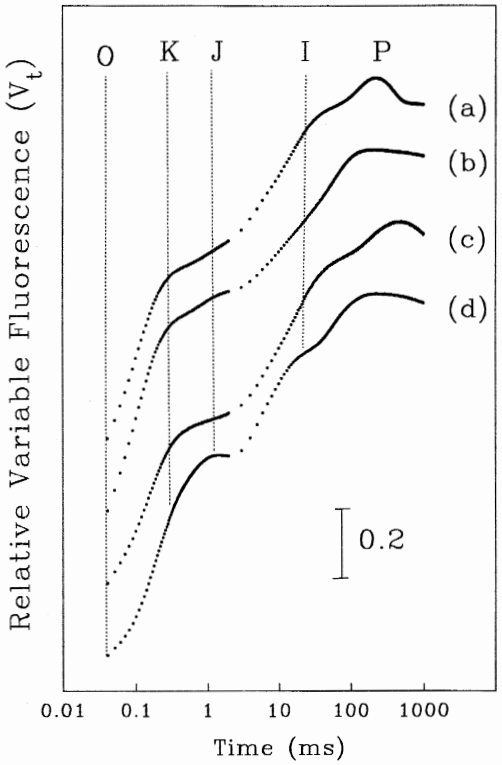

Fig. 7. Relative variable Chl *a* fluorescence ($V_t = (F_t - F_0)/(F_m - F_0)$) of leaves of *Permelia sp.* (a), *Cycus revoluta* (b) and the fruits of *Juniperus sp.* (c). Measurements were done directly on the sample in the field after 1 h of dark adaptation. All the traces show the existence of a K-step. For comparison, curve (d) has been plotted which has been measured from a pea leaf, grown in the green house, displays only O-J-I-P steps of the Chl *a* fluorescence transient.

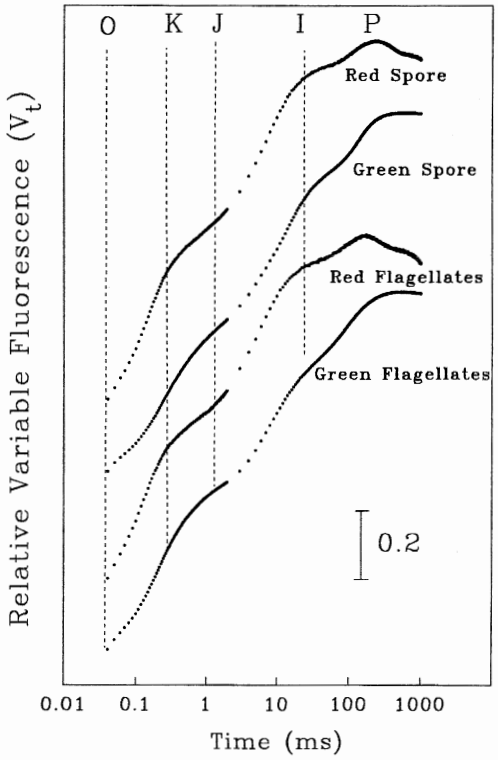

Fig. 8. Relative variable Chl *a* fluorescence ($V_t = (F_t - F_0)/(F_m - F_0)$) of dark adapted green or red flagellates of *Haematococcus lacustris* and its red or green spores. A clear K-step is visible in red spores and red flagellates.

rate of primary photochemistry, $((dQ_A^-/Q_{A(total)})/dt_0)$ increase (insert in Fig. 5). Under such heat treatment, and under the given light intensity, fluorescence transient in the presence of DCMU levels off much faster (about 300 μs) than in the control samples (2 ms), as reported earlier [16].

3.7. Mechanistic effect of heat treatment and the possible explanation for the appearance of the K-step

The architecture of a control sample has to be considered as a typical tripartite unit with a partial energetic connectivity between the photosynthetic units. As shown in the scheme in Fig. 9, we consider three categories of pigment protein complexes: (1) the reaction center (RC); (2) the core antenna (Core) which is firmly associated with the RC; and (3) additional light harvesting complexes (LHC) of different polypeptide composition. The energy migration (as an energy cycling back and forth) between the core antenna and the reaction center, is characterized by the expression T, which is the product of the probabilities for energy migration from the core antenna to the RC and back to the core antenna (according to the terminology of Butler [44] Φ_T and Φ_t, respectively). Therefore, the trapping probability $T = \Phi_{T\ *} \cdot \Phi_t$. Analogous to the T, the energy cycling between the LHCs and the core antenna is given by the expression C (where according to Butler $C = \Psi_{TII} * \Psi_{tII}$ [45]). In the same way energy cooperativity between LHCs or/and core antenna of several photosynthetic units is given by the expression according to Strasser [46,47] defined as the overall grouping probability G. The expressions T, C, and G are probabilities for energy migration and therefore, they can be expressed entirely by deexcitation rate constants. The fluorescence transient follows the empirical equation:

$$F_t = F_0 + F_v * V_{(t)}, \text{ where } F_v = F_m - F_0 \text{ and } V_t = (F_t - F_0)/(F_m - F_0) \quad (1)$$

After rearrangement of Eq. (1), the quantum yield for the primary photochemistry of PS II can be derived according to Paillotin [30] and Havaux et al. [48]:

$$\Phi_{Pt} = 1 - \frac{F_t}{F_m} = \frac{F_v}{F_m}(1 - V_t) = \Phi_{P0} * (1 - V_t) \quad (2)$$

For a dark adapted sample, assuming that all RCs are open ($V_t = 0$), the ratio of the maximal exciton trapping and the absorption ($\Phi_{P0} = TR_0/ABS$) can be calculated with the experimental measurements F_0 and F_m only, as predicted by Paillotin [30], and tested by Genty et al. [49]:

$$\Phi_{P0} = \frac{TR_0}{ABS} = 1 - \frac{F_0}{F_m} = \frac{F_v}{F_m} \quad (3)$$

Three biophysical equations can be independently derived which describe the dynamics of such a PS II unit.

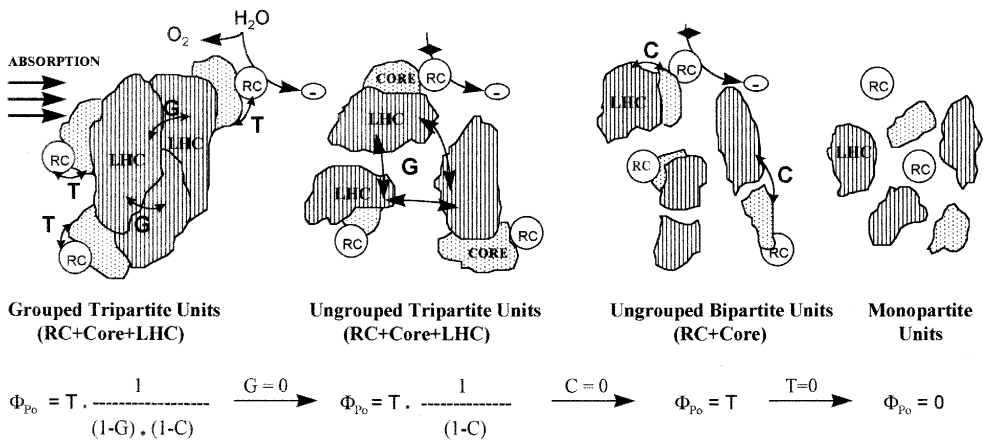

Fig. 9. Alteration in the architecture of PSII units after heat treatment and their effect on the maximum quantum yield for primary photochemistry, Φ_{P0}. Monopartite units correspond to pigment protein complexes which are totally disconnected from the RC. ♦ indicates the block in electron donation from the oxygen evolving complex. Core antenna complexes are shaded with dots and LHC complexes are shaded with vertical lines.

(1) The energy flux theory of Strasser [46,50,51] using the values of F_0 and F_m only, independent of the complexity of the model [47], leads to the following equation which correlates the maximum yield of primary photochemistry of PS II to the probabilities of energy cycling between antenna and RC (expression, T), the average probability of energy cycling between the LHCs and core antenna (expression, C), and the average probability of energy cycling between connected antenna of PS II units (expression, G). Therefore Φ_{P0} can be expressed in terms of these deexcitation probabilities which depend only on the deexcitation rate constants of the pigment protein complexes [47]:

$$\Phi_{P0} = \frac{TR_0}{ABS} = 1 - \frac{F_0}{F_m} = \frac{F_v}{F_m} = \frac{T}{(1-C)*(1-G)} \quad (4)$$

(2) The relative variable Chl a fluorescence in presence of DCMU, V_t is correlated with the fraction of closed reaction center $Q_A^-/Q_{A(total)}$ as described first by Joliot and Joliot [52], according to Eq. (5):

$$V_t = \frac{F_t - F_0}{F_m - F_0} = \frac{Q_A^-/Q_{A(total)}}{1 + k\left[1 - \left(Q_A^-/Q_{A(total)}\right)\right]}, \quad (5)$$

where k stands for the curvature parameter of the hyperbola, V_t vs. $Q_A^-/Q_{A(total)}$. According to Joliot and Joliot [52] $k = p/(1-p)$, where p is the probability that an exciton of a closed unit can be transferred to a neighbour unit. The biophysical meaning of the curvature constant k of the hyperbola in the Eq. (5) has been expressed in a different way by Strasser [46,47] as:

$$k = G * \frac{\Phi_{P_0}}{1 - \Phi_{P_0}} = G * \frac{F_v}{F_0} \quad (6)$$

The same description of k has been reported recently by Trissl and Lavergne [53], Lavergne and Trissl [54] and Trissl [55]. These authors derived the curvature parameter, k, of the hyperbola, V_t vs. $Q_A^-/Q_{A(total)}$, using the connectivity concept of Paillotin [30], where G corresponds to the probability for energy migration between open photosynthetic units in a so called 'embedded' model of PS II.

(3) The original slope of the fluorescence transient normalized to the maximum variable fluorescence dV/dto can be correlated with the differentiated Eq. (5). For the time approaching zero, we can write:

$$\frac{dV}{dt_0} = \frac{dQ_A^-/Q_{A(total)}}{dt_0} * \frac{1}{1+k} \quad (7)$$

Based on the results stated here, presumably the effects of mild heat stress are reversible, but the effects of more severe heat stress are irreversible. Our hypothesis is: mild heat stress (between about 30–40°C) provokes, among other effects, a decrease of the energetic cooperativity between the PS II units, which is seen as a decrease in sigmoidicity of the fluorescence transient. Therefore, the overall grouping probability G decreases until it reaches a value close to zero (indicating no or very low cooperativity between the PS II units). As a consequence, the curvature constant k for the hyperbola becomes zero as well. Thus, the fluorescence transient close to the origin, in the presence of DCMU, becomes exponential, and the fluorescence rise at the origin (dV/dt_0) increases according to Eq. (7).

Due to a break down of the oxygen evolving system in the heat-treated plants, the continuous supply of electrons to the reaction center is dramatically decreased. After the initial fast fluorescence rise (which is due to the reduction of Q_A), the reoxidation of Q_A^- to Q_A by transferring the electrons from Q_A to Q_B continues, but due to the lack of electrons from the donor side, the fluorescence intensity decreases again, forming a peak called the K-step.

If the heat treatment is more severe, a strong decrease in the Φ_{P0} is observed due to the loosening of one or several LHCs from the core antenna. In biophysical terms, the average coupling probability C decreases and according to Eq. (4) Φ_{P0} decreases.

The remaining, so called, small units are excited mainly by the core antenna chlorophylls and they show an increase of F_0 and a fast rise to the K-step or to the high fluorescence level in presence of DCMU. If the heat treatment is more severe, the RC gets disconnected from the core antenna complexes. This would result in the further increase in F_0, while the expression T decreases (Fig. 9). Also, the quantum yield for primary photochemistry Φ_{P0} tends towards zero.

4. Conclusion

Our results indicate that the responses to heat treatment by a leaf follows a mechanism which first leads to a downregulation of the donation of electrons by the oxygen evolving system, and then to the reorganization of the architecture of the light harvesting and antenna complexes. This phenomenon can be seen by the appearance of a K-step (at about 200–300 μs) in the fluorescence rise from F_0 to F_p which reflects two major events: (1) a limitation of the electron donation by the oxygen evolving system; and (2) changes in the architecture of the antenna of PS II giving rise to an altered energy distribution between the pigment protein complexes and the whole photosynthetic units. After heat treatment, the original fluorescence rise loses its sigmoidal character when the connectivity probability between the PS II antenna (G) approaches zero. The coupling probability (C) between the LHCs and the core antenna decreases (see model Fig. 9 for detailed description). The maximum quantum yield for photochemistry Φ_{P0} also decreases. Therefore, the K-step in the fluorescence transient indicates the state of PS II RC of a sample. The K-step can be taken as an index of the balance between electron donation and electron transport through the RC of PS II. The molecular processes involved in the effect of heat on electron transport through PS II, and on the association of the pigment protein complexes of PS II, remain to be further elucidated.

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