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POLYPHASIC CHLOROPHYLL *a* FLUORESCENCE TRANSIENT IN PLANTS AND CYANOBACTERIA*

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Abstract—The variable chlorophyll (Chl) *a* fluorescence yield is known to be related to the photochemical activity of photosystem II (PSII) of oxygen-evolving organisms. The kinetics of the fluorescence rise from the minimum yield, F_0, to the maximum yield, F_m, is a monitor of the accumulation of net reduced primary bound plastoquinone (Q_A) with time in all the PSII centers. Using a shutter-less system (Plant Efficiency Analyzer, Hansatech, UK), which allows data accumulation over several orders of magnitude of time (40 μs to 120 s), we have measured on a logarithmic time scale, for the first time, the complete polyphasic fluorescence rise for a variety of oxygenic plants and cyanobacteria at different light intensities. With increasing light intensity, the fluorescence rise is changed from a typical O–I–P characteristic to curves with two intermediate levels J and I, both of which show saturation at high light intensity but different intensity dependence. Under physiological conditions, Chl *a* fluorescence transients of all the organisms examined follow the sequence of O–J–I–P. The characteristics of the kinetics with respect to light intensity and temperature suggest that the O–J phase is the photochemical phase, leading to the reduction of Q_A to Q_A^-. The intermediate level I is suggested to be related to a heterogeneity in the filling up of the plastoquinone pool. The P is reached when all the plastoquinone (PQ) molecules are reduced to PQH_2. The addition of 3-(3-4-dichlorophenyl)-1,1-dimethylurea leads to a transformation of the O–J–I–P rise into an O–J rise. The kinetics of O–J–I–P observed here was found to be similar to that of O–I_1–I_2–P, reported by Neubauer and Schreiber (*Z. Naturforsch.* **42c**, 1246–1254, 1987). The biochemical significance of the fluorescence steps O–J–I–P with respect to the filling up of the plastoquinone pool by PSII reactions is discussed.

INTRODUCTION

The time course of the variable chlorophyll (Chl)‡ *a* fluorescence of plants was related to the time course of photosynthesis by Kautsky and Hirsch.[1] In all dark-adapted oxygen-evolving systems, the intensity of Chl *a* fluorescence emission shows a characteristic variation in time, known as fluorescence transient or induction.[2,3] This fluorescence transient can be divided into two segments: (1) a fast rise in fluorescence intensity from an initial low level called F_0 to a maximum intensity level called F_P and (2) after this maximum, the fluorescence intensity decreases, within minutes, to a lower level F_S or F_T, which, for samples in good physiological condition, is equal or very close to the values of F_0. During the fast fluorescence rise (up to ~1 s, with saturating light) and the slow decrease to a steady-state fluorescence, the light absorption of the sample can be considered constant; thus, the fluorescence intensity changes are changes in the fluorescence yield. Since its discovery, this variable Chl *a* fluorescence has been the subject of many reviews and debate in photosynthetic research.[2–5] The easier it became to measure this signal due to the availability of small, inexpensive and portable instruments, the more it became important not only in basic research, but in biotechnology, industrial screening and environmental research. From measurements in the laboratory, on intact leaves on the plant in the field, or remote sensing from the satellite, the variable Chl *a* fluorescence phenomenon has become one of the most important tools in plant physiology research.[6] The variable Chl *a* fluorescence originates in photosystem II (PSII) (see reviews in Govindjee *et al.*[4]). However, a full understanding in terms of the biochemistry and biophysics of this noninvasive and highly sensitive fluorescence signal, reflecting various photosynthetic reactions, is still unavailable.

Duysens and Sweers[7] proposed the concept that a quencher (Q) is involved in the primary photochemistry of the PSII reaction center (RC) complex. Photosystem II of oxygenic organisms reduces Q to a nonquenching form Q^-, whereas photosystem I (PS) reoxidizes Q^- back to Q. With this concept, the fast fluorescence rise from the initial fluorescence F_0 to the maximum fluorescence intensity F_P corresponds to the conversion of the quenched to the unquenched state of

*This paper is dedicated to Warren L. Butler on the occasion of his 70th birthday, which would have been on 28 January 1995.

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‡*Abbreviations*: Chl *a*, chlorophyll *a*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_J, F_I, intermediate steps of Chl *a* fluorescence rise between F_0 and F_P; F_0, F_m, initial and maximum Chl *a* fluorescence; F_P, Chl a fluorescence peak appearing later than the intermediate steps F_J and F_I; F_S, steady-state level of Chl *a* fluorescence; F_t, Chl *a* fluorescence at time t; F_v, variable fluorescence at time t; LED, light-emitting diodes; P680, primary electron donor of reaction center II; PEA, Plant Efficiency Analyzer; Pheo (or Ph), pheophytin; PQ, oxidized plastoquinone; PQH_2, reduced plastoquinone; PSI, photosystem I; PSII, photosystem II; PSU, photosynthetic unit; Q, quencher; Q_A, primary bound plastoquinone; Q_B, secondary bound plastoquinone; RC, reaction center.

the RC of PSII. The quenched form of the RC of PSII has been called "open" and it is able to perform primary photochemistry with the highest efficiency as soon as it is excited. On the contrary, it has been called "closed" in the unquenched state where it is not able to perform primary photochemistry with any significant efficiency. Further, Warren Butler[8] suggested that, in a different time domain, the oxidized form of the RC of PSII, $P680^+$, also quenches Chl *a* fluorescence. This explained the Chl *a* fluorescence yield changes observed, after a nanosecond flash, by Mauzerall.[9]

It was Butler[10,11] who first developed a dynamic description of the photosynthetic apparatus using the quenching concept of Duysens and Sweers[7] and the concept of open and closed systems linked to low and high Chl *a* fluorescence yield. For this purpose, three types of conditions were used to measure fluorescence transients: (1) Measurements at low temperature (77 K) where only the primary reactions of photochemistry occur (*e.g.* PPh to P^+Ph^-) secondary processes are slowed down (*e.g.* $Q_A^-Q_B$ to $Q_AQ_B^-$), and diffusion processes are blocked; (2) measurements at room temperature in the presence of the herbicide diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea; DCMU) that blocks the electron transport in the RC II complex from the primary bound plastoquinone Q_A^- to the secondary band plastoquinone Q_B and (3) *in vivo* measurements under physiological conditions. Based on results of these experiments, the energy fluxes and their rates in photosynthesis were formulated by Butler and coworkers[12–14] employing the first-order rate constants and probabilities of individual reactions, as generally accepted for the kinetic description of first-order reactions. Using this approach, the variable Chl *a* fluorescence attained a biophysical meaning and a mathematical description. However, one additional concept was necessary for the biophysical description of variable Chl *a* fluorescence: the concept of "energy-cycling," which describes the energy transfer back and forth between the RC and the antenna.[15] Joliot and Joliot[16] introduced a probability term for energy transfer from one antenna pool of PSII unit to another antenna pool of a neighboring PSII unit (PSII connectivity).

Formulated without explicit knowledge of the biochemical composition of the RC, the concept of Butler[11] advanced our understanding of the energy distribution events in the photosynthetic apparatus. Among other aspects, Butler considered energy distribution between the antenna and the RC of the PSII, the energy transfer from PSII to PSI, and the energetic connectivity between different photosynthetic units. One feature of the variable Chl *a* fluorescence is that it is proportional to the fraction of closed PSII RC as long as there is no energy transfer between the independent PSII units. In contrast, the variable Chl *a* fluorescence is a hyperbolic function of the fraction of closed PSII RC when connectivity (grouping) between the photosynthetic units exists (for references to different formulations of this phenomenon, see Strasser *et al.*[17]). Recently Lavergne and Trissl[18] have presented a detailed analysis of fluorescence transient in the presence of DCMU. Their results support the model of Paillotin[19] for the connectivity and energy transfer among few PSII units: excitons visit only 3–4 PSII units.

The advances in the understanding of the biophysics and biochemistry of PSII allows one today to formulate the very early electron transport events in its RC.[20–22] The excited RC Chl *a* of PSII ($P680^*$, P^*) donates an electron to a pheophytin (Ph) molecule within 3 ps, which in turn, reduces the plastoquinone Q_A to Q_A^- within 200 ps. The Q_A^- reduces the next electron carrier Q_B to Q_B^- within 100–200 μs (see Fig. 1). The Q_B acts as a "two-electron gate" and it undergoes two reduction steps to become Q_B^{2-}. After protonation events, Q_B^{2-} dissociates from the RC complex as a fully reduced plastoquinone, the plastoquinol (PQH_2). The reaction mechanism and the dynamics of the early electron transfer events until the reduction of Q_A to Q_A^- may be described by the exciton–radical pair model proposed by Holzwarth and coworkers (see a review by Holzwarth[23]). This description does not contradict the energy distribution concepts of Butler but may be considered as a modification and an extension of that model. Here, the concept of the shallow trap nature of P680 and the recombination probability of the radical pair (P^+, Ph^-) to produce an exciton are included. A thorough discussion is presented by Dau.[5]

The current explicit knowledge of the reactions of the electron carriers P680, Pheo, Q_A and Q_B, and the scheme of the exciton–radical pair model tell us that the earlier concepts of the open and closed RC may have to be modified. All forms of Q_AQ_B complex that contain the reduced form of Q_A^- are considered as closed and the others as open.[21,24] Therefore a photosynthetic system under continuous illumination determines its Chl *a* fluorescence behavior by the mixture of the different species of open (Q_AQ_B, $Q_A\square$, $Q_AQ_B^-$, $Q_AQ_B^{2-}$, $Q_AQ_BH_2$) or closed ($Q_A^-Q_B$, $Q_A^-\square$, $Q_A\,Q_B$, $Q_A^-Q_B^{2-}$, $Q_A^-Q_BH_2$) RC.

Independent of the state of Q_B, each electron that is transferred from the PSII RC to Q_A transforms the state of the RC from an open into a closed state. However, we do not exclude the influence of the charge on Q_B^- on this transfer step. The Chl *a* fluorescence yield of a single PSII unit rises from a low to a high value as Q_A is reduced to Q_A^-. On the macroscopic level, the mixture of all species of open and closed RC determines the Chl *a* fluorescence behavior of the sample. The quenching nature of $P680^+$ must be taken into consideration because its concentration may vary depending upon the equilibration on the donor side (S-state) of PSII (see *e.g.* Shinkarev and Govindjee[25]).

The polyphasic rise of Chl *a* fluorescence in continuous light and under physiological conditions has been reviewed by several authors.[2–4] In addition to the effects related to Q_A, the Chl *a* fluorescence rise also includes the influence of the S-states,[26–28] the differential influence of the so called "inactive" PSII[30,31] and of the electron acceptor side of PSI.[32] The general trend of the fast fluorescence rise had been designated as the O–I–P rise, where I is the intermediate step in the fluorescence curve marked by an inflection.[2,33,34] In some cases the fluorescence intensity even decreases after the step I, creating a so-called dip D.[35] Neubauer and Schreiber[36] reported two intermediate steps between O and P, called I_1 and I_2. The I_1 of Neubauer and Schreiber[36] develops in high light intensities and it is equivalent to the inflection "J" in this paper, or the peak that had been observed earlier by Delosme[37] and later by Genty *et al.*,[38] after exposure to high light intensities and with fast (microsecond) time resolution.

In this paper we show that there are several different dynamic steps in the fast Chl *a* fluorescence rise curve, appearing and disappearing depending upon the experimental

conditions. We not only confirm the data reported by Neubauer and Schreiber[36] but show, for the first time, the polyphasic fluorescence rise over a wide time range (40 μs to 120 s) for a variety of oxygenic plants and cyanobacteria. Our ability to show this transient rise as O–J–I–P is primarily due to the use of log scale for time in our presentation and the use of light-emitting diodes (LED). Sometimes in the past, the same events have been labeled with different labels, or the same labeling has been used for different events. We have chosen O–J–I–P as the symbols for the sequence of events, used in earlier papers at two conference proceedings.[39,40] We show in this paper that all fluorescence transients of plants under physiological conditions follow the sequence of dynamic events we have called O–J–I–P. Depending on the experimental condition, a fluorescence decrease (or a dip) can be observed after each step. The description of this overall phenomenon provides the experimentalist with several independent phenomenological parameters that are of importance for the screening of cultivars and mutants in biotechnology or functional analysis of plants under stress. We will also discuss in this paper the biochemical significance of each step observed in the O–J–I–P fluorescence transient.

MATERIALS AND METHODS

Plant materials The experiments were done with fully mature intact leaves of 3–4 week old pea (*Pisum sativum*) and several year old camellia (*Camellia japonica*) plants. Plants were grown in the greenhouse, at 22°C 18°C (day night) under natural sunlight, in small pots in a soil mixture Optima (Optima-Werke H. Gilgen, Munchenstein, Switzerland) with alternate day watering.

Different strains of cyanobacteria (*Anabaena PY, Planktothrix rubescens* and *Limnothrix redekei*) were cultivated in 300 mL culture tubes in a defined nutrient medium at 27°C, bubbled with 0.27% (vol vol) CO_2 air mixture from the bottom of the flask and illuminated with one fluorescent tube (1000 lux), as described earlier.[4] The nutrient medium contained[41] 0.6 m*M* $CaCl_2$, 8 m*M* $NaNO_3$, 0.4 m*M* K_2HPO_4, 0.4 m*M* $MgSO_4$, 10 μ*M* NaFeEDTA, 10 μ*M* H_3BO_3, 10 μ*M* $MnCl_2$, 2 μ*M* Na_2MoO_4, 0.2 μ*M* $ZnSO_4$, 0.2 μ*M* $CuSO_4$ and 0.2 μ*M* $CoSO_4$ in distilled water.

Chl a fluorescence measurements Chlorophyll *a* fluorescence transients were measured at room temperature by a Plant Efficiency Analyzer (PEA, built by Hansatech Ltd, King's Lynn, Norfolk, PE30 4NE, England) with different excitation light intensities. One hundred percent light intensity corresponds to about 600 W m^{-2}. Illumination was provided by an array of six LED (peak at 650 nm) focused onto the sample surface to provide homogeneous illumination over the exposed area of the sample (4 mm diameter). The fluorescence signals were detected using a PIN-photodiode after passing through a long-pass filter (50% transmission at 720 nm). All the fluorescence transients were recorded in a time span from 40 μs to 120 s with a data acquisition rate of 10 μs for the first 2 ms and 12 bit resolution. After 2 ms and after 1 s the instrument switches automatically to slower digitization rates. The fluorescence signal at 40 μs was considered as F_o. The instrument uses a built-in routine to extrapolate the value of F_o. Because we did not find any significant difference (within 10%) between the F_o at 40 μs and F_o value given by the instrument under the light intensities used, we prefer to use the fluorescence yield detected at 40 μs after the onset of light as F_o.

In this paper, we show fluorescence transients both on logarithmic and on linear time scales (40 μs to 120 s). This allows a visualization of the complete transient.

RESULTS

Chl a fluorescence transients in pea and camellia leaves

For a clear understanding of the results, we describe briefly PSII reactions, as all of the variable Chl *a* fluorescence at

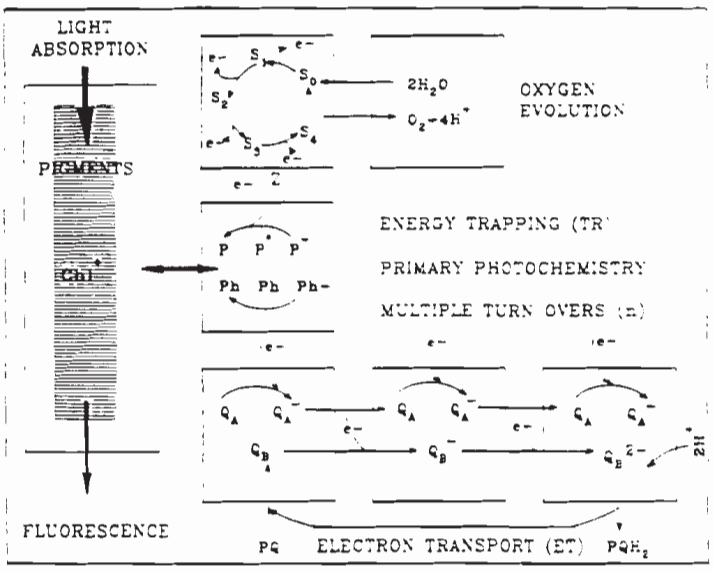

Figure 1. Diagrammatic sketch of PSII. The long hatched box on the left represents the PSII antenna system, where most of the absorption of light and fluorescence originates. In primary photochemistry, charge separation occurs: $P680^+$ (P^+) and $Pheo^-$ (Ph^-) are created. The re-reduction of the P^+ is achieved by electron donation *via* an intermediate Z from the S-cycle, which splits water into molecular O_2, electrons and protons. P* donates an electron to pheophytin, Ph, which in turn reduces the plastoquinone Q_A to Q_A^-. The Q_A^- reduces the next electron carrier Q_B to Q_B^-. Then Q_B^- undergoes a second reduction step to become Q_B^{2-}. After protonation events, Q_B^{2-} dissociates from the RC as plastoquinol (PQH_2).

room temperature arises from the PSII antenna system (Fig. 1). In addition to the major use of the absorbed energy for photochemistry, the de-excitation pathways include loss of energy as heat, and fluorescence. In primary photochemistry, charge separation occurs: $P680^+$ (P^+) and $Pheo^-$ (Ph^-) are created, and there is the possibility of an exciton formation by recombination of this charged pair; this exciton can return to the antenna system (see Fig. 1, middle box). The re-reduction of the oxidized RC Chl *a*, P^+, is achieved by electron donation, *via* an intermediate Z, from an Mn complex, which is able to split water into molecular oxygen, electrons and protons. The redox states of the Mn complex are represented by the so-called S-states.[42] On the electron acceptor side, Q_A is reduced, reoxidizing the Ph^-. Then Q_B, the next electron acceptor, is reduced by Q_A^- twice; it leaves the RC complex as PQH_2. Then PQH_2 is reoxidized by cytochrome b_6/f complex. Oxidized plastoquinone (PQ) enters the intact RC complex on the electron acceptor side reforming Q_B. The Chl *a* fluorescence transient, which exhibits several intermediate steps, is correlated with the reduction of the PQ pool. The relationship of these events to the fluorescence transient O, J, I and P is suggested to be as follows: O, minimal Chl *a* fluorescence yield (highest yield of photochemistry); O to J, reduction of Q_A to Q_A^- (photochemical phase, light intensity dependent); J to I to P, nonphotochemical phase. However, it still represents a further accumulation of Q_A^-. We speculate that the phase generally labeled as I (or as PI for plateau or as I_1, see Table 1) is due to the heterogeneity in the PSII acceptor side: (1) a mixture of different redox states of Q_B or (2) several types of biochemically different PSII complexes, or both. Some reduce the PQ pool faster than the other. In

Table 1. Nomenclature used by different authors for the different steps in the Chl *a* fluorescence rise curve from the initial fluorescence 0 to the maximum fluorescence P*

Authors	Maximum fluorescence (below 1 s)	First step before maximum (below 100 ms)	Second step before maximum (below 2 ms)	Third step before maximum (below 300 μs)	Initial fluorescence (below (50 μs)
Lavorel[56]	P	—	—	—	0
Delosme[47]	—		I	—	0
Forbush and Kok[57]	Max	PI	—	—	0
Munday and Govindjee[35]	P	I	—	—	0
Neubauer and Schreiber[58]	P	I_2	I_1	—	0
Strasser and Govindjee[39,40]	P	I	J	—	0
Eggenberg *et al.*[51]	P	I	J	(K)†	0

*Steps shown below are arranged (left to right) from P to 0. After each step (*e.g.* after J or after I), a dip can be observed in some samples. The label D for dip was first used by Munday and Govindjee.[35]

†Recently B. Guissé, in our laboratory, found that after heat (≥42°C) treatment (K) becomes the dominant step in the Chl *a* fluorescence transient.

addition, the O–J phase includes contributions due to the donor side of PSII,[26–29] and the entire transient is affected by the electron transfer going through PSI.[32,43]

Figure 2A shows a typical Chl *a* fluorescence transient (40 μs to 120 s) of a 1 h dark-adapted pea leaf exposed to 600, 60 and 6 W m^{-2} 650 nm light, plotted on a linear time scale. All the curves start from the same F_0 intensity and increase to a maximum peak (P, F_P). This is followed by a decay to the terminal steady-state T, which is very close to F_0. With 6 W m^{-2} light, the fluorescence decay proceeds *via* a transient labeled as SMT where S is for a quasi-steady-state and M for another maximum.[2] As has been shown earlier in a preliminary report,[39,40] the fluorescence induction kinetics plotted on logarithmic time scale reveals two intermediate steps between F_0 and F_P, labeled as J (F_J) and I (F_I) (see Fig. 2B, 100% curve). There is a rapid initial rise from the F_0-level to a first intermediate level J, which is followed by a dip; then, it further rises to a second intermediate level I, and after passing through a dip, the fluorescence intensity reaches the peak level P. Upon excitation of a healthy leaf with just saturating light, the first rise from 0 to J occurs within 2 ms. The second intermediate point I levels in about 20 ms and the final peak P in about 200 ms. The time for reaching each intermediate steps varies depending on the light intensity used. For example, with 600, 60 and 6 W m^{-2} of exposure, the P levels off in about 100 ms, 500 ms and more than 1 s, respectively.

The detailed behavior of the intermediate steps J, I and P as a function of light intensity in camellia leaves either dark or light adapted (1 h to 180 W m^{-2} light followed by a dark period of 30 s) is shown in Figs. 3 and 4, respectively. From top to bottom, the transients were obtained by using 96%, 48%, 24%, 12%, 6% and 3% of the maximum light intensity (600 W m^{-2}). Due to state 1 to state 2 transition[44] and photoinhibition,[45] the Chl *a* fluorescence yield of light-adapted leaves was lower than that of dark-adapted leaves (*cf.* Fig. 3 with Fig. 4). Although the O, I, P transients are visible on a linear time scale (Fig. 3A), the complete O, J, I and P steps were clearly seen only on the log time scale (Fig. 3B). The time of appearance of J and I steps was clearly intensity dependent. The I occurred at shorter times at higher light intensities, and the J was barely visible at the lower light intensities.

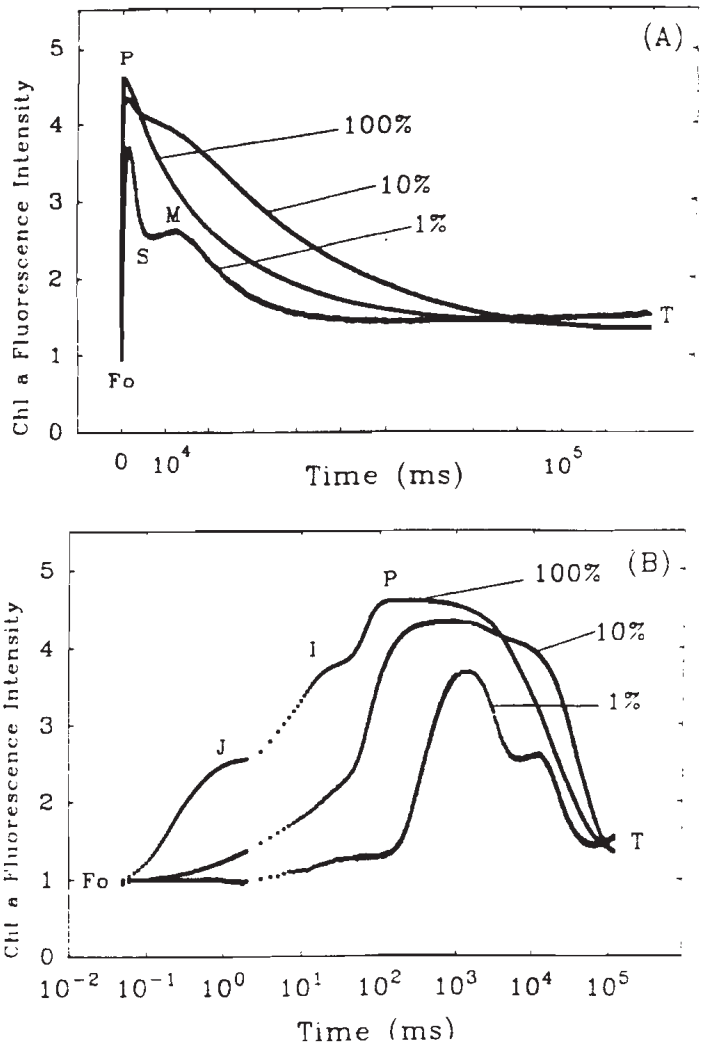

Figure 2. Chlorophyll *a* fluorescence transients of a dark-adapted pea leaf, excited with red (650 nm) LED giving an intensity of 600, 60 or 6 W m^{-2}, presented either on a linear (A) or on a logarithmic (B) time scale.

In dark-adapted leaves, the I level was visible at all the intensities used (Fig. 3). In contrast, light-adapted leaves did not show any distinction between the I and the P levels (Fig. 4). Thus we speculate that the PQ pool heterogeneity was abolished during the light adaptation. In light-adapted leaves, these J, and I = P levels shift to shorter times with increasing light intensity, as expected.

The inserts in Figs. 3A and 4A show the Chl *a* fluorescence induction curves during the first 2 ms of exposure to different light intensities. They demonstrate the intensity-dependent phenomenon. To quantify this result, the rate of the fluorescence increase between 50 to 300 μs is presented in Fig.

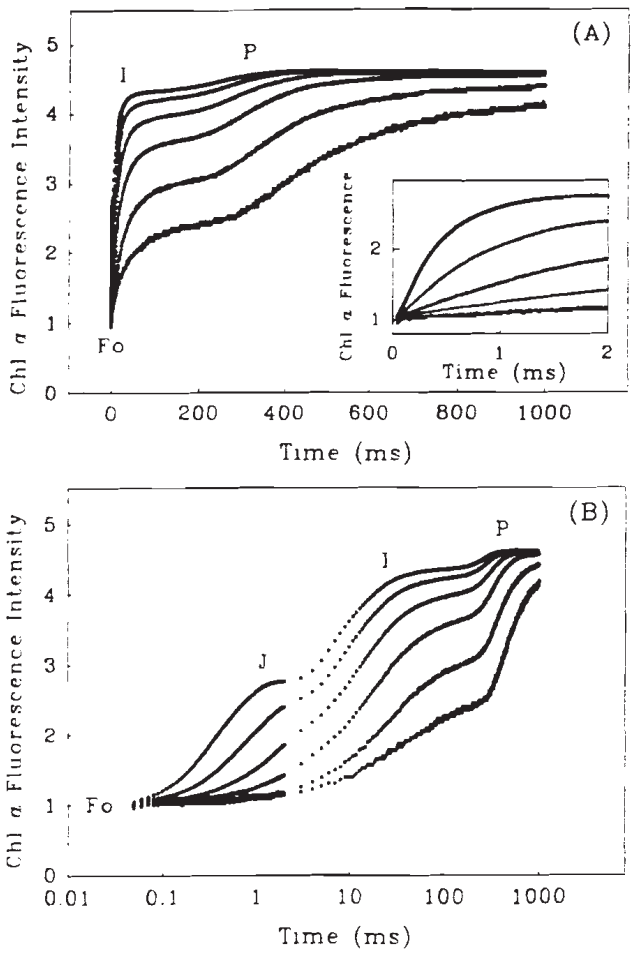

Figure 3. (A): Chlorophyll *a* fluorescence transient of a dark-adapted camellia leaf exposed to different light intensities. From top to bottom, the transients were obtained by using 576, 288, 144, 72, 36 or 18 W m^{-2} light intensity. All the measurements were done on one spot of the same leaf with a dark adaptation time of 10 min between each exposure with a different light intensity. The insert shows the fast rise kinetics during the first 2 ms exposed to 576, 288, 144, 72 or 36 W m^{-2} light intensity. (B): The same Chl *a* fluorescence transients as in (A) but plotted on a logarithmic time scale.

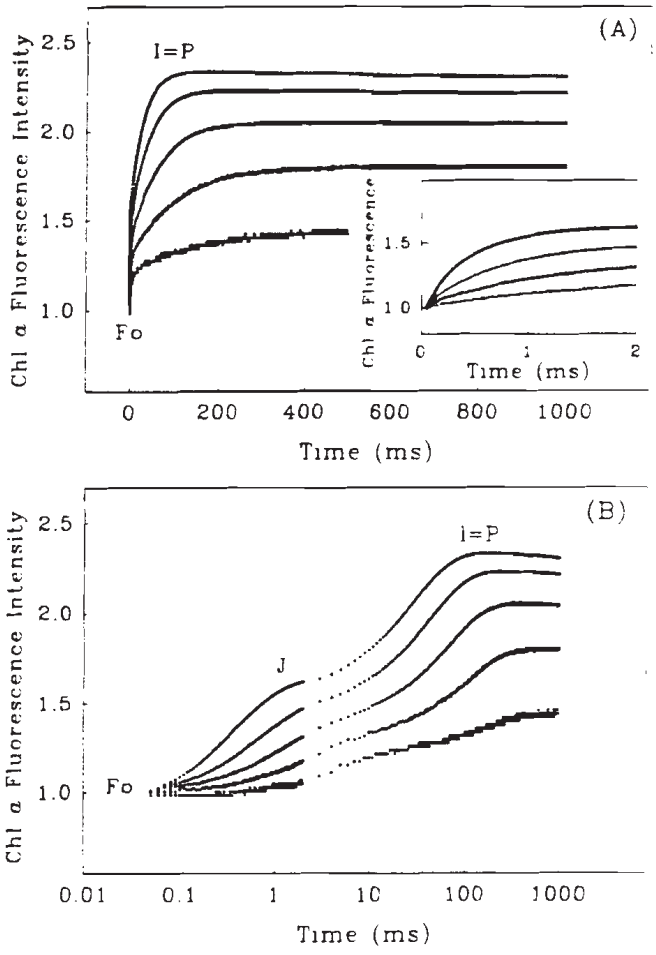

Figure 4. (A): Chlorophyll *a* fluorescence induction curves of a light-adapted camellia leaf exposed to different light intensities. The leaves were illuminated continuously for 1 h with 180 W m^{-2} light intensity and dark adapted for 30 s, followed by a 1 s pulse of 576 W m^{-2} light. The leaf was readapted to the same light intensity (180 W m^{-2}) for 10 min and exposed for 1 s to 288 W m^{-2} light after a dark adaptation for 30 s. The same procedure was repeated for other light intensities. From top to bottom, the transients were obtained by exposing the leaves to 576, 288, 144, 72 or 36 W m^{-2} of 650 nm light. The insert in Fig. 4A shows the fast Chl *a* fluorescence rise kinetics during the first 2 ms of a light-adapted leaf exposed to 576, 288, 144 or 72 W m^{-2} light intensity. (B): The same Chl *a* fluorescence transients as in (A) but plotted on a logarithmic time scale.

5. The slope of this phase in the dark- and light-adapted leaf is shown to be proportional to the light intensity. Further, this slope is highly insensitive to temperature up to 40°C (data not shown). The kinetics of the fluorescence induction of this fast phase were already studied by Delosme[37] with different light intensities; he had labeled this phase as I phase. The characteristics of this phase with respect to light and temperature suggest that the O–J phase is controlled by photochemical charge separation (the photochemical phase).

The relative variable fluorescence at any time t is defined as $V_t = (F_t - F_0)/(F_m - F_0)$. This experimental expression V_t can be taken as a measure of the fraction of the electron acceptor Q_A being in its reduced state. The variation of V with time at the beginning of the transient (theoretically at time zero) is labeled as dV/dt_0, a measure of the relative rate of photochemistry. This expression was calculated from the experimental values as:

$$dV/dt_0 = (F_{300\mu s} - F_{50\mu s})/(F_m - F_0).$$

The insert in Fig. 5 shows that the light dependency of the expression dV/dt_0 nearly coincides for both light and dark-adapted leaves. Thus, there is no fundamental difference in the primary photochemistry of PSII in the two cases. In spite of this, the maximum Chl *a* fluorescence yield of antenna is about five times higher in dark-adapted than in light-adapted leaves.

The amplitudes of the fluorescence steps J, I and P at fixed times, such as 2 ms, 30 ms, 500 ms, as a function of the light intensity are shown in Fig. 6. Both the intermediate levels J (2 ms) and I (30 ms) show a tendency toward saturation at high light intensities (600 W m^{-2}). The differences in the light intensity-dependent fluorescence yields at J and I levels are the same as that of I_1 and I_2 levels, as reported earlier by Neubauer and Schreiber.[36] The I saturates at lower intensities than J. Thus the equivalence of J with I_1, and I with I_2 is established. In spite of this equivalence, we prefer and recommend the use of I and J (J as the next letter in the alphabet after I) for the following reasons: (1) In the literature the label I was mostly used for the same event as for I in this paper;

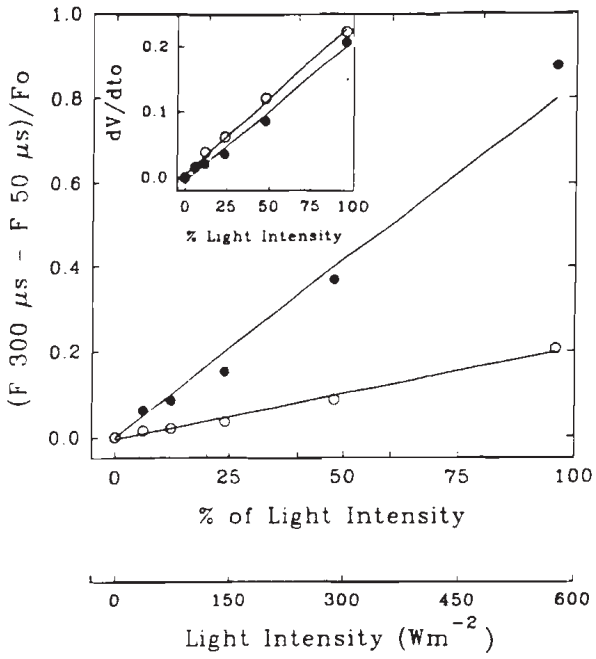

Figure 5. The effect of different light intensities on the rate of the fast fluorescence rise $[(F_{300\mu s} - F_{50\mu s})/F_0]$ of light- (○) and dark-adapted (●) camellia leaf. Data were obtained from Figs. 3 and 4. The insert shows the effect of the light intensity on the slope at the origin of the relative variable fluorescence. $dV/dt_0 = (F_{300\mu s} - F_{50\mu s})/(F_m - F_0)$ of light- (○) or dark- (●) adapted samples.

(2) the use of the subscripts is inconvenient and cumbersome; (3) the use of the same letter for steps that may belong to photochemical and nonphotochemical steps is not recommended and (4) the O–J–I–P nomenclature is now accepted and used in measurements with the PEA.[39,40]

To investigate further the behavior of the intermediate Chl *a* fluorescence levels, J and I, dark-adapted pea leaves were exposed for 1 s to 600 W m^{-2} 650 nm, further dark adapted

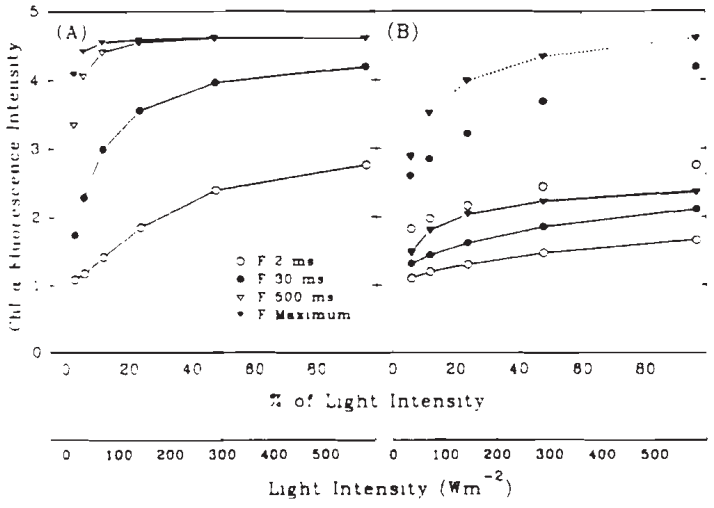

Figure 6. Chlorophyll *a* fluorescence intensities at 2 ms, 30 ms, 500 ms and F_{max} of the transient at different excitation light intensities. Dark- (A) and light-adapted (B) camellia leaf. Data were obtained from Figs. 2 and 3. For a better understanding of the shape of the curves obtained from a light-adapted leaf, the dotted curves have been normalized, so that their values at 576 W m^{-2} are the same as of dark-adapted samples. The factors used for normalization are: $F_{2ms} = 1.66$, $F_{30ms} = 1.99$, $F_{500ms} = 1.95$ and $F_{max} = 1.95$.

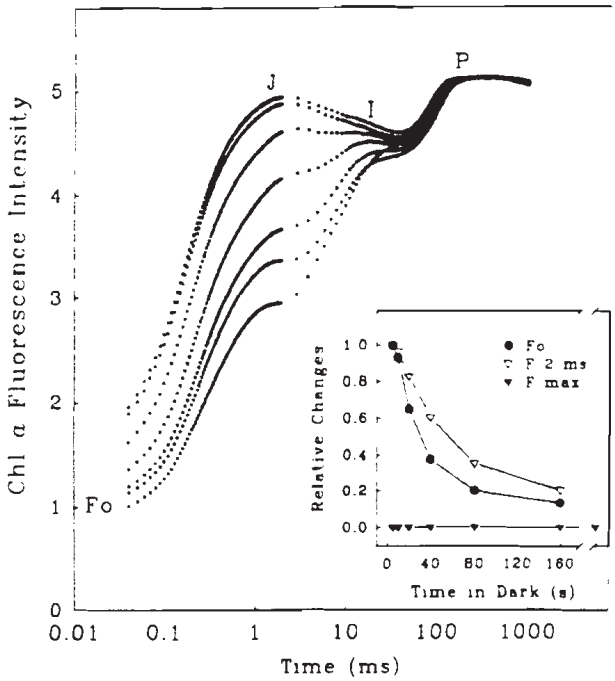

Figure 7. Changes in the fluorescence kinetics of a dark-adapted pea leaf exposed to 600 W m^{-2} light intensity after different times of dark adaptation. The leaf was exposed for 1 s to 600 W m^{-2} light intensity, dark adapted for 5 s and further exposed to the same light intensity (600 W m^{-2}) for 1 s. The same measurement was repeated after the dark adaptation of 10, 20, 40 or 160 s in between each exposure. From top to bottom, the transients were obtained after the dark adaptation of 5, 10, 20, 40, 80, 160 s or 1 h. The F_0 level of a 1 h dark-adapted leaf was adjusted to 1 and the same factor was used for the normalization of the other curves. The insert shows the relative values of the Chl *a* fluorescence at F_0, F_{2ms} and F_{max} after different times of dark adaptation.

for different times (5, 10, 20, 40, 80, 160 s) and exposed again to the same light intensity for 1 s (Fig. 7). All the curves were normalized at the P level. From these curves it is clear that once the sample was excited, it reached the closed state (F_m), but during dark adaptation, it started to reopen. If we expose the same sample again, the level of F_0 gives an idea of the closed state of the RC. If the measured F_0 is now higher than the first illumination, we conclude that Q_A^- has not been fully oxidized to Q_A during the dark period between the illuminations. It is also obvious from this experiment that the J level is very high after the second illumination in contrast to very small changes in the I level (Fig. 7).

The higher level of J during the second illumination shows that a higher proportion of closed RC are formed already within 2 ms. During the first illumination all RC are closed. In the dark, the closed RC reopen as a function of time. At the onset of the second illumination, the measured F_0 is higher than at the first illumination, indicating that not all RC have been opened in the dark between the two illuminations. Further, the shape of the variable fluorescence of the dark-adapted and the preilluminated sample are different. The open RC appear to close much faster in the second than in the first illumination. This difference must be related to the differences in the redox states and heterogeneity of the filling up of the PSII acceptor side (Q_AQ_B, PQ).

Figure 8 shows experimental results on a leaf that has been

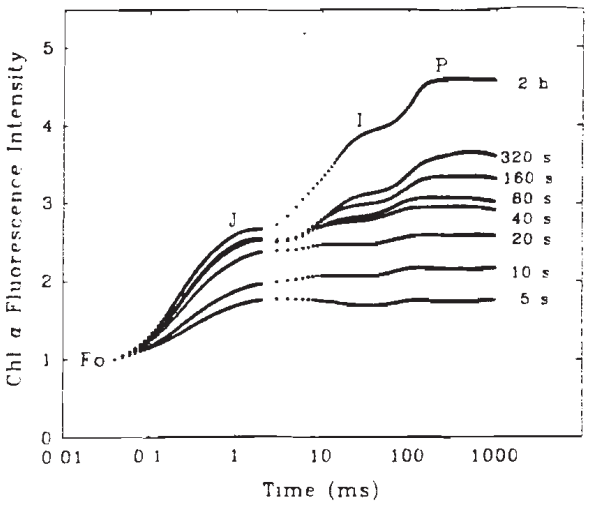

Figure 8. The effect of dark-adaptation time on the Chl *a* fluorescence induction kinetics. Pea leaves were illuminated with 600 W m^{-2} of red actinic light for 5 min, dark adapted for the different times as indicated in the figure and then illuminated further for 1 s with 600 W m^{-2} of red light.

light adapted for 5 min with light of 600 W m^{-2} and illuminated again after different times of dark adaptation. The regeneration of different intermediate steps (J, I, and P) has a different time dependence. Here, the J level recovers within seconds ($t_{1/2}$ about 20 s), whereas P and I levels require several minutes. This experiment establishes that this method of measuring changes in the yield of different levels can be used to evaluate the regeneration behavior of the electron acceptor side of PSII in plants under different environmental conditions. Interestingly, different behavior was observed in different plants. For example, the fluorescence transient in camellia regenerated nearly completely after the first exposure (Fig. 9A), while the regeneration kinetics in a pea leaf was relatively slow (Fig. 9B; also see Fig. 7). The shape of the induction curve after the second illumination depends basically on the given light intensity, plant material and the time of dark adaptation between the two exposures.

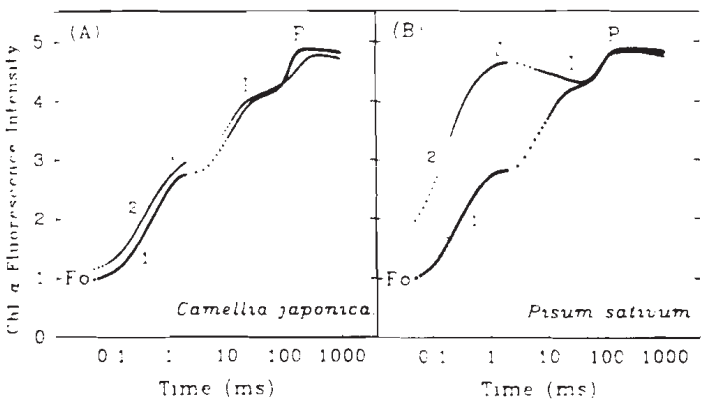

Figure 9. Chlorophyll *a* fluorescence transients of camellia (A) and pea leaf (B) excited with 600 W m^{-2} (1), followed by 9 s of dark adaptation and exposed further for 1 s to the same light intensity (2). The F_0 level of the transient obtained by the first exposure was adjusted to 1, and the transient obtained from the second exposure was adjusted with the same factor that was used for the first exposure.

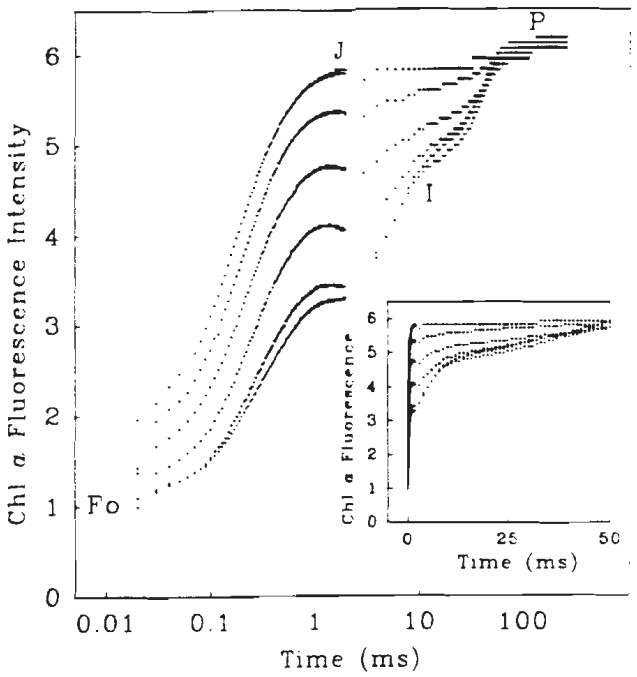

Figure 10. Effect of the time of DCMU penetration on the Chl *a* fluorescence rise from F_0 to F_{max} of an attached pea leaf. A drop of 100 μM DCMU was added on the abaxial side of the leaf and fluorescence transients were measured after different times on the same area. Different curves (from bottom to the top) correspond to the time after application of DCMU: 0, 0.25, 0.5, 1, 1.5 and 2 h. The insert shows the same results as in the main graph but plotted on a linear time scale up to 50 ms.

Chl a fluorescence transients in the presence of DCMU

The quantum yield of Chl *a* fluorescence at the F_0 level is independent of the light intensity, whereas at F_P it is first low, and then increases with the light intensity and finally it reaches a high and constant value.[2,3,35] This increase in the yield of Chl *a* fluorescence is related to the accumulation of Q_A^-. Thus, the addition of DCMU, which blocks electron flow beyond Q_A^-, must lead to a quick rise in Chl *a* fluorescence yield from F_0 to J (J = I = P). Experiments indeed show this result (see uppermost curve in Fig. 10). To test the penetration time of DCMU in a leaf, a drop of 100 μM DCMU was added on the abaxial surface of an intact pea leaf. Changes in the Chl *a* fluorescence transient were measured during the infiltration time of DCMU into the thylakoids of the leaf. A rise in the J level was observed with the penetration of DCMU (Fig. 10). In about 2 h, J became dominant and the induction curve was transformed into a well-defined monophasic form. The insert in Fig. 10 demonstrates the fast rise of the J phase on a linear time scale. The effect of DCMU on the O to J rise phase confirms its photochemical nature. Further, Fig. 10 demonstrates the usefulness of this method to measure the penetrability of herbicides that act at the Q_B site.

Chl a fluorescence transients in cyanobacteria

Chlorophyll *a* fluorescence transients of cyanobacteria have always been difficult to measure because of a very large F_0 due to a five times larger ratio of PSI to PSII fluorescence and a contribution of phycobilin fluorescence (see Note add-

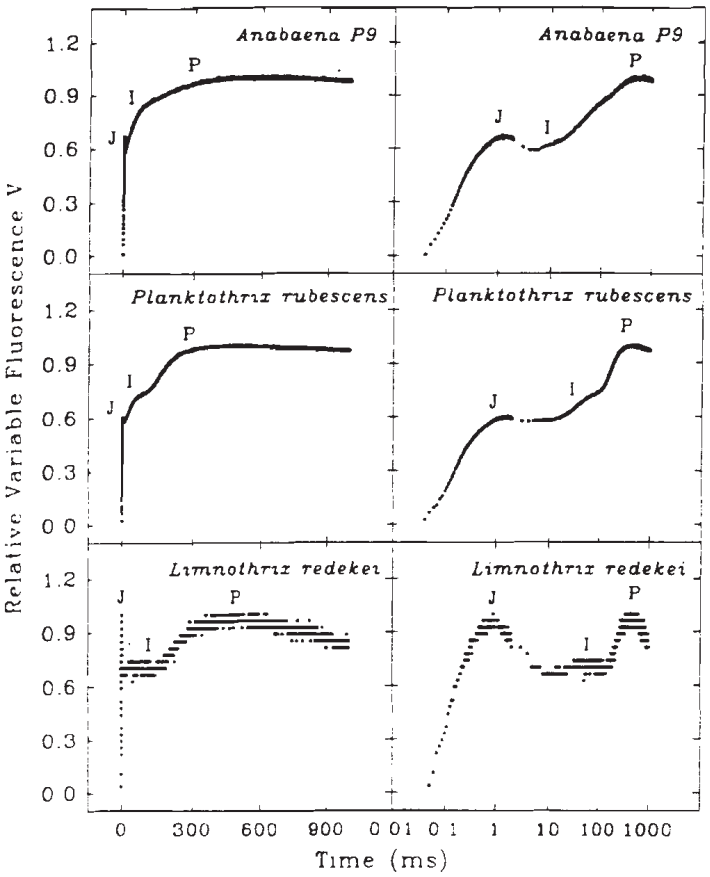

Figure 11 Relative variable Chl *a* fluorescence $V_t = (F_t - F_0)/(F_m - F_0)$ of dark-adapted cyanobacterial cells in sterile liquid culture. (Top): *Anabaena* P9 (F_0 = 535, F_m = 776). (Middle): *Planktothrix rubescens* (F_0 = 510, F_m = 828). (Bottom): *Limnothrix redekei* (F_0 = 305, F_m = 332). The curves on the left are on linear and those on the right side are on logarithmic time scales.

ed in proof); both PSI and phycobilins do not contribute to the variable fluorescence.[46] In spite of this problem, Mohanty and Govindjee[47,48] were able to discover Chl *a* fluorescence transients of cyanobacteria: OIP. The importance of cyanobacteria in site-directed mutagenesis[49] has renewed our interest in their fluorescence transients. We show, for the first time, the complete O, J, I, P transient in cyanobacteria. Figure 11 shows the Chl *a* fluorescence induction transients of three species of cyanobacteria. The left panels are on linear and right panels are on logarithmic time scales. Here again, prominent J, I and P levels are observed. In *Anabaena* P9 and *P. rubescens* the relative amplitude of J level was very similar to those in leaves, but in *L. redekei* it is even higher than the P level. We suggest that the P level is not always the maximum level (F_m) in cyanobacteria due to high PSI activity. Therefore, the J level could be higher than the P level.

DISCUSSION

We provide here a brief description of the fast rise of the Chl *a* fluorescence transients. Most of the fluorescence at room temperature is from the PSII antenna (Fig. 1). The general behavior of the oxygen-evolving organism is that its Chl *a* fluorescence transient, starting at the initial fluorescence F_0, passes through two intermediate steps F_J and F_I before it reaches a maximum F_P. If the ratio of F_P/F_0 does not increase any more with increasing light intensity, the considered F_P has reached its maximal relative intensity and F_P is then called F_m (or F_{max}). According to Butler,[10,11] the initial fluorescence F_0 originates in antenna chlorophylls connected to open RC with all Q_A in the oxidized state (see Fig. 1). In intact cells and thylakoids this F_0 contains a component of PSI Chl *a* fluorescence but is usually small except in cyanobacteria. In contrast, the maximum fluorescence F_m originates in the same antenna chlorophylls connected to the closed RC with all Q_A in the reduced state. Therefore, the fluorescence intensity at any given time between F_0 and F_m includes two fluorescence components in the Butler model: (1) due to the fluorescence of the sum of all PSII with an open RC, F_t^{open} and (2) due to the fluorescence of the sum of all PSII with a closed RC, F_t^{closed}. Therefore the experimentally measured variable Chl *a* fluorescence Fv_t [$= (F_t - F_0)$] does not belong to a specific biochemical species of the RC. Fv_t is a useful technical expression, which is linked to the two biochemical states of the RC II in terms of being open or closed. The fluorescence intensity F at any time of the transient can be expressed technically as the sum of the two components F_0 and Fv_t or biologically as the sum of the two components F_t^{open} and F_t^{closed}.

Therefore

$$F_t = F_0 - Fv_t$$

$$= F_t^{open} - F_t^{closed} \qquad \text{(see Strasser}^{50}\text{)}$$

where,

$$F_t^{open} = F_0(1 - V_t)$$

and

$$F_t^{closed} = F_m V_t.$$

The relative variable fluorescence V_t, where $V_t = (F_t - F_0)/(F_m - F_0)$, is a measure of the fraction of closed RC, B_t. Therefore

$$V_t = B_t = [Q_A^-]_t/[Q_A]_{(total)}.$$

When no energy transfer from one photosynthetic unit (PSU) to a neighboring unit occurs then V_t is equal to the fraction of closed RC. Otherwise the correlation of V_t *versus* B_t is hyperbolic (for a comparison of different interpretations, see Strasser *et al.*[47]).

It is assumed that only the PSII component of F_0 is being used.[50] The above expression is a straightforward consequence of the basic concepts of Butler,[11] as to how the variable fluorescence may be described.[10] The introduction of two biochemical states (open and closed RC), which differ in their fluorescence yield, allowed a quantitative description of the measured variable Chl *a* fluorescence. Although Butler (personal communication to R.J.S.) was aware of the simplifications in this concept, it was then the best available concept that could be experimentally tested.

We believe, as postulated earlier,[2,3,40] that the fluorescence induction transient, measured in continuous light, reflects the filling up of the electron acceptor side of PSII (Q_A, Q_B and PQ pool) with electrons from the donor side of PSII. The O to J phase is the photochemical phase, leading to the reduction of the quencher Q_A to Q_A^-; however, it includes

the influence of the S-state through the equilibration on the donor side of PSII, as $P680^+$ is also a quencher of Chl *a* fluorescence. We speculate that the intermediate steps I and the final P reflect a certain heterogeneity, which is due to the existence of fast and slow reducing PQ centers as well as due to different redox states (*e.g.* $Q_A Q_B^{2-}$ or $Q_A^- Q_B^{2-}$) of the RC complex, which reduce the PQ pool.

Chlorophyll *a* fluorescence transients, measured over many orders of magnitude in time, provide an experimental approach to visualize and to analyze PSII reactions leading to the filling up of the PQ pool. All the individual segments of the induction curve F_0–J–I–P presented in this paper have been reported separately under specific conditions by other authors in the past: Delosme[37] and Genty *et al.*[38] reported a very fast fluorescence rise (in the microsecond range) using very high light intensities. Our intensity dependence data (Fig. 6) suggest that our "J" is "I" of Delosme.[37] Table 1 shows the equivalence of our terminology to those used earlier. Sometimes we have observed an additional fast step in the fluorescence transient around 200 μs that we had labeled with the next letter of the alphabet (K).[51,52] Such transients would be labeled as F_0–K–J–I–P. However, we do not further discuss it here.

The F_0–J–I–P fluorescence transient has been observed in all oxygen-evolving plants and cyanobacteria examined in this paper. The transient reflects the kinetics and heterogeneity involved in the filling up of the PQ pool with the electrons. Thus, transients can be used as a sensitive tool to investigate the photosynthetic apparatus *in vivo* under physiological conditions. Watching fluorescence signals is like listening to the stethoscope by medical doctors.[52] In both cases dissipation signals provide us with information on what could be happening inside a living system. The proof of the conclusions made by Chl *a* fluorescence measurements must always involve other measurements and additional biochemical and biophysical analysis. Obviously, to find precise answers, other parallel tests and measurements (*e.g.* individual partial reactions, O_2 evolution, absorption spectroscopy and EPR spectroscopy) must be made to diagnose the problem.

Depending on the experimental conditions, the O–J–I–P transients can also provide information about the donor side of PSII.[28,29] Under other conditions, these signals can be used to analyze the heterogeneity of different types of PSU such as inactive and active centers.[31]

The experiments reported here became extremely easy to perform due to the development of a small, portable and affordable PEA instrument. Since PSII in many situations is highly sensitive to environmental stress, the O–J–I–P fluorescence induction curve is expected to be highly useful in the screening, in a short time, of many cultivars. This procedure has already been used in our laboratory to describe urban trees under stress, the reaction of plants in the field to elevated CO_2 levels, mycorrhiza on oak trees *in vitro*, Fabaceae with and without nodules, salt, heat, drought and UV stress[53] on higher plants, and underwater fertilization of Caribbean sea-grasses.

Chlorophyll *a* variable fluorescence, first used as an empirical signal as it was 63 years ago by Kautsky and Hirsch[1] (see evolution of these concepts by Govindjee[54]), with a high time resolution over several orders of magnitude is on the way to becoming a heavily used and sensitive probe in plant biology, agronomy, forestry and horticulture. A lot of what we are able to do today technically, and what we already are able to understand, is due to the creative work of several pioneers in this field. W. L. Butler was for sure one of them." We dedicate this paper to him.

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Note added in proof We cite papers by Tel-or and Malkin (58) and Mimuro and Fujita (59) showing that the high F_0 in cyanobacteria (Fig. 11) is due to preponderantly high PSI Chl *a* fluorescence in these organisms.

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