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Greening of peas: parallel measurements of 77 K emission spectra, OJIP chlorophyll *a* fluorescence transient, period four oscillation of the initial fluorescence level, delayed light emission, and P700*

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Abstract

Irradiation of etiolated leaves leads to their greening. Although this problem has a long history, the question of whether the intermittent irradiation (IMI) grown plants have fully functional reaction centres as well as the oxygen clock, before exposure to continuous irradiation (CI), had not been resolved. To answer this question, as well as to analyze the development of the two photosystems, the following parallel measurements were made: (*1*) Emission spectra at 77 K; (*2*) OJIP chlorophyll (Chl) *a* fluorescence transient; (*3*) period 4 oscillation in the flash number dependence of initial fluorescence F_0 (at 50 μs) and F_J (at 2 ms); and (*4*) P700. In the 1-ms-flash (FL) grown pea, that has a different biogenesis of the photosynthetic apparatus, delayed light emission (DLE) and Chl *a* fluorescence transient were measured in parallel. Quantitative analysis of Chl *a* fluorescence values provided the following conclusions: (*1*) IMI, not FL, plants have almost fully developed reaction centres and the oxygen clock. (*2*) Further greening of IMI plants under CI involves two phases: (*a*) during 3-4 h of CI, the number of PS2 units and connectivity between them

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Abbreviations: Chl, chlorophyll; CI, continuous irradiation; DLE, Delayed Light Emission, F_0, initial Chl *a* fluorescence level; FL, one-ms flashes given every 15 min; F_M, maximum Chl *a* fluorescence level; IMI, intermittent irradiation, 2 min irradiation and 118 min dark cycles; J and I, intermediate steps of Chl *a* fluorescence rise between F_0 and the so-called P level or the F_M level; OEC, oxygen evolving complex; PS, photosystem.

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increase, and then (*b*) light-harvesting antenna increases. (*3*) In FL, 10 min CI activates fully the oxygen clock.

Additional key words: intermittent irradiation; photosystem 2; *Pisum sativum*.

Introduction

The development of the photosynthetic apparatus has been widely studied in both higher plants and algae. In complete darkness, angiosperm plastids develop only to etioplasts that lack chlorophyll (Chl). Irradiation of etiolated seedlings initiates Chl biosynthesis through the enzymatic photoreduction of protochlorophyllide to chlorophyllide (Boardman *et al.* 1978, Sironval and Brouers 1984, Fujita 1996, Adamson *et al.* 1997). During the greening process, the synthesized pigments are incorporated into the appropriate proteins, embedded in thylakoid membranes. Thus, the photosynthetic apparatus is built up resulting in a gradual development of the photosynthetic activity. The development of the photosynthetic apparatus depends on the quality and quantity of radiation (Pauncz *et al.* 1992, Tripathy and Brown 1995, Barnes *et al.* 1996). Irradiation of etiolated leaves with continuous "white light" converts protochlorophyllide to Chl *a,* which triggers the biosynthesis of other pigments and proteins for membrane formation. The activation of Hill activity has been observed after a few minutes to several hours (Ogawa *et al.* 1973, Franck *et al.* 1984) and all the photosynthetic activities are developed within 10 to 15 h (Boardman *et al.* 1978).

In contrast to the greening under continuous irradiation (CI), intermittent irradiation (IMI), consisting of alternated irradiation periods of few min and longer dark periods, induces a different development. The IMI plants are suggested to contain fully active photosynthetic reaction centres but are devoid of most of the light-harvesting LHC2b, the Chl *a/b*-binding antenna proteins, also called the CAB-proteins (Argyroudi-Akoyunoglou and Akoyunoglou 1970, Armond *et al.* 1976, Tzinas *et al.* 1987, Briantais 1994, Jahns and Krause 1994, Jahns and Schweig 1995, Hértel *et al.* 1996). Indeed, the leaves exposed first to IMI synthesize only the PS2 core antenna but when they are subsequently exposed to CI, the synthesis of LHC2b and grana formation occurs. On the other hand, if the plants are grown under a millisecond flash regime (1 ms flash after every 15 min of darkness, FL), the chloroplasts of such leaves lack Hill activity with H_2O as electron donor and do not show Chl *a* variable fluorescence (Remy 1973, Strasser and Sironval 1972, 1973, 1974, Strasser and Butler 1976a,b) suggesting the absence of an active oxygen evolving system. However, full primary photochemistry could be measured at 77 K (Strasser and Butler 1976b) as well as the presence of C550 (a monitor of PS2 charge separation at the acceptor side) and Cyt b_{559} (Strasser and Cox 1974). Exposure of such flashed leaves to CI rapidly induces O_2 evolution, a maximum rate being reached within 6 min, although the quantity of total Chl does not change within that time (Strasser and Sironval 1972, 1974, Remy 1973, Phung-Nhu-Hung *et al.* 1974). A similar result with flashed wheat leaves (1 ms flash after every 5 min) was reported

by Inoue *et al.* (1974) and Inoue and Ichikawa (1975). The photoactivation of the latent water-splitting system in flashed leaves has been correlated with Mn^{2+} incorporation in the water splitting system in green algae as well as wheat leaves (Cheniae and Martin 1973, Remy 1973).

Barthélemy *et al.* (1997) and Mysliwa-Kurdziel *et al.* (1997) have published results on Chl *a* fluorescence transient (the so-called Kautsky effect; see Govindjee 1995), currently labeled as OJIP transient (Strasser *et al.* 1995), the lifetime of Chl *a* fluorescence, and the polypeptide pattern during the greening of barley etioplasts. These results suggest that there are two phases in the greening process, the first one (about 3-4 h of CI) includes increases in the inner antenna and the reaction centres, and the second one is restricted to increases in the light-harvesting complexes 1 and 2. In this paper, we provide new results beyond these studies on IMI and barley plant. We have used pea plants for the following parallel measurements. In addition to the OJIP Chl *a* fluorescence transient (providing information mainly on the electron acceptor side of PS2; see Lavorel and Etienne 1977, Govindjee 1995), we present here parallel measurements on the emission spectra at 77 K (providing information on the pigment protein complexes; see Briantais *et al.* 1986), period 4 oscillation in the flash number dependent values of F_0 (at 50 μs, the initial minimum Chl *a* fluorescence level, when Q_A, an electron acceptor of PS2, is in the oxidized state) and F_J (at 2 ms); delayed light emission (DLE, providing information on the concentration of separated charges in PS2; see Lavorel 1975, Jursinic 1986) and absorption changes at 820 nm (providing information on P700, the PS1 activity; see Parson and Ke 1982, Schreiber *et al.* 1988). Some of these crucial results were compared with those in another system, the 1-ms-flash (with 15 min dark interval) grown system (FL) that has a different biogenesis of the photosynthetic apparatus. A preliminary account of our results was already published (Govindjee *et al.* 1998).

Materials and methods

Plants: Most experiments were performed with the primary leaves of *Pisum sativum* (L.). Seeds, soaked for 24 h in tap water, were sown in commercial soil at about 22 °C. First, seedlings were kept in complete darkness for 8 d to obtain the etiolated plants. To obtain the IMI plants, they were irradiated by 2 min of "white light" (40 W *Sunlux* incandescent lamps, 40 μmol m^{-2} s^{-1} at the leaf level) and 118 min dark cycles (Argyroudi-Akoyunoglou and Akoyunoglou 1970, Armond *et al.* 1976) for 2 to 5 d. For the FL plants, seeds and seedlings were kept for 10 to 15 d directly in the flash regime (1 ms saturating "white light" flashes, given after every 15 min of darkness, Sironval *et al.* 1969). Normal mature green plants were cultivated in the greenhouse, under well-watered conditions, for 2 weeks under sunlight at 22/18 °C (day/night).

Chl *a* fluorescence: Measurements at room and low temperature were done on whole leaves by a fluorometer (plant efficiency analyzer, for short *PEA*; *Hansatech Instruments*, King's Lynn, Norfolk, UK). Irradiation was provided by an array of six

light emitting diodes (LEDs, peak 650 nm) focused on the leaf surface to provide homogeneous irradiation over the exposed area of about 4 mm diameter. The irradiance was 600 W m^{-2}. Chl *a* fluorescence was detected using a PIN photocell after it passed through a long pass filter (50 % transmission at 720 nm), as described in Strasser *et al.* (1995). The software used to draw the models was developed by Roland Rodriguez in the Laboratory of Bioenergetics, University of Geneva.

Low temperature (77 K) fluorescence spectra were recorded by a laboratory built multi-branched fiberoptic system. The leaf discs were excited by 633 nm HeNe laser (40 W m^{-2}). Chl *a* fluorescence passed through a red cut-off filter (*Corning CS 2-64*) and a monochromator (*Jobin Yvon H 10 vis*, 0.5 mm slit, equipped with scan controller) before its spectrum (in the 600 to 800 nm range) was measured by a photomultiplier (*S-20 Hamamatsu R928*). The signal was digitized on line, and stored in the core memory for data handling (Strasser and Butler 1976b).

Measurement of period four oscillation using Chl *a* fluorescence transient: This oscillation related to the electron donor side, discovered by Delosme (1972), was measured according to B.J. Strasser (1997) using the *PEA* and a single-turnover xenon flash system. One of the 650 nm LEDs from the *PEA* head was replaced with the fiber optic connected with a xenon flash radiation (*LS-1130-5, EG & B Electro-optics*, USA). The flash and the fluorometer were triggered by Digital Delay/Pulse Generator (*DG535 Stanford Research System*). Chl *a* fluorescence induction was measured 100 ms after the last flash (about ~10 μs xenon flashes at 1 Hz, half rise time 5 μs). Dark-adapted leaf was clamped on the *PEA* head with a leaf clip for Chl *a* fluorescence induction measurements. The dark adapted leaf was excited with red actinic radiation and the Chl *a* fluorescence induction signal was measured. The same leaf was left, in the dark, on the *PEA* head for 2 min to permit reopening of the reaction centres and then again Chl *a* fluorescence induction was measured after the 1st flash on the same spot. In this way the same spot of the leaf was used to measure the Chl *a* fluorescence induction kinetics after different number of pre-flashes. The spacing between two consecutive flashes was 1 s.

Induction of oxygen evolution measurements: A leaf disc was placed directly on the membrane of a Clark type oxygen electrode as described by Strasser and Sironval (1972). In this way the oxygen exchange in actinic radiation and darkness can be measured in the time span of about 15 min.

Delayed Light Emission (DLE) and, in parallel, prompt Chl *a* fluorescence were measured simultaneously by using a multibranched fiber optic system. The excitation radiation was 632.8 nm from a He-Ne-Laser with an irradiance of 100 W m^{-2} at the surface of the leaf. Chl *a* fluorescence was measured through a 680 nm interference filter (*Baird Atomic B10*) with an *S20* photomultiplier (*EMI 9558B*). With a single disc phosphoroscope (Strasser 1974), samples were excited during light-dark cycles with a frequency of 100 cycles per s. The ratio of duration of irradiation to the duration of dark was 9 : 1. The modulated DLE was also registered with a delay of 0.4 or 0.8 ms with a *S-20* photomultiplier protected with a red cut-off filter *RG 665* (*Schott*, Germany).

P700 absorption change measurements: Using the same *PEA* head and the leaf clip used for Chl *a* fluorescence induction, radiation induced absorption changes at 820 nm were measured with the *Hansatech P700* measuring system. This was done by replacing one 650 nm LED from *PEA* head with a 820 nm LED. Another 650 nm LED from the *PEA* head was replaced with the fiber optic connected with far-red radiation (2 W m^{-2}, 715 nm interference filter *B40, Baird Atomic Co.*, in combination with a far red cut-off *RG 690 Schott* glass filter). This 715 nm radiation was used as the actinic radiation. A 4 mm diameter area of the lower side of the leaf was excited with 820 nm weak measuring radiation. The transmitted radiation was monitored on the opposite side by a photodiode screened by a 820 nm interference filter. The absorbance change was stored in digital form with 12 bit resolution and recorded with a chart recorder (*Goerz Metrawatt, 120*). These measurements monitor the oxidation of the reaction centre Chl of PS1, the P700 (Schreiber *et al.* 1988).

Chlorophyll *a* and *b* contents of the leaves were determined spectroscopically with 80 % (v/v) acetone extract according to the equations of Porra *et al.* (1989).

Results and discussion

Low temperature Chl *a* fluorescence spectra: These have been used extensively to characterize the various states under which protochlorophyllide accumulates in darkness as well as in the conversion of protochlorophyllide to Chl *a* (Boardman *et al.* 1978, Sironval and Brouers 1984). In order to characterize our samples and to obtain information on the appearance of the various pigment complexes, 77 K emission spectra of the leaf samples during the greening process were obtained (Fig. 1). Etiolated leaves confirmed a clear protochlorophyllide (PChlide) emission peak at 657 nm (curve *A*) which shifted to 677 nm, a chlorophyllide (Chlide) emission peak (curve *B*) after brief exposures to green radiation during the watering of the plants (Fig. 1, *left*). This is a change that was reported 43 years ago (Shibata 1957). This phototransformation leads to the formation of a small amount of Chlide under our green radiation (Boardman *et al.* 1978, Sironval and Brouers 1984).

When etiolated pea plants are kept in IMI and transferred to CI, they green. At the same time, the amount of several pigment-protein complexes increases. This can be judged from 77 K emission spectra (Fig. 1 and its legend). There are several peaks: F685, F696, F720, and F730. The F685 originates from CP-43 of PS2 core, F696 from CP-47 of PS2 core, F-720 from the PS1 core (the combined inner antenna and the reaction centre 1), and F-730 from the light-harvesting complex of PS1, LHC1 (for the origin of the various fluorescence bands at 77 K see Govindjee and Yang 1966, Briantais *et al.* 1986, Dekker *et al.* 1995). With increasing CI, increases in the following pigment-protein complexes are inferred (Fig. 1, Table 1): (*a*) the PS1 core, as evidenced by increases in the ratio of F720/F685; (*b*) the PS2 core, that includes CP-47, as evidenced by increases in F696/F685; (*c*) LHC1, as evidenced by greater increases in F730/F685 over that in F720/F685; and (*d*) LHC2b, as inferred from

large increases in the ratios of F730 and F720 to F685 due to increased reabsorption of fluorescence with increased antenna size. The shift of the emission band from 720 to 730 nm is a clear indication of increases in LHC1 during the greening process (also see Castorinis *et al.* 1984). The major change during the greening process in CI is an increase in the antenna size, but initially increases in the amount of reaction centres also occur. This emphasizes the biphasic nature of the process by which pigment-protein complexes are assembled.

After 48 h in IMI, CI treatment led to a gradual increase in F696/F685. Although the IMI-grown plants have considerable activity of PS1 (Akoyunoglou 1981), the maximum at 730 nm (F730), which is mainly due to the antenna Chl *a* of PS1, now known to be LHC1a (for the early literature, see Butler and Kitajima 1975, Strasser and Butler 1976b, 1977), is not present in IMI leaves. However, an emission band is clearly present at 720 nm (F720) that originates in the core of PS1 that includes the reaction centre Chls (Dreyfuss and Thornber 1994, Špunda *et al.* 1997). After transferring these plants to CI, the ratio of the F730 to F685 and F696 increases with

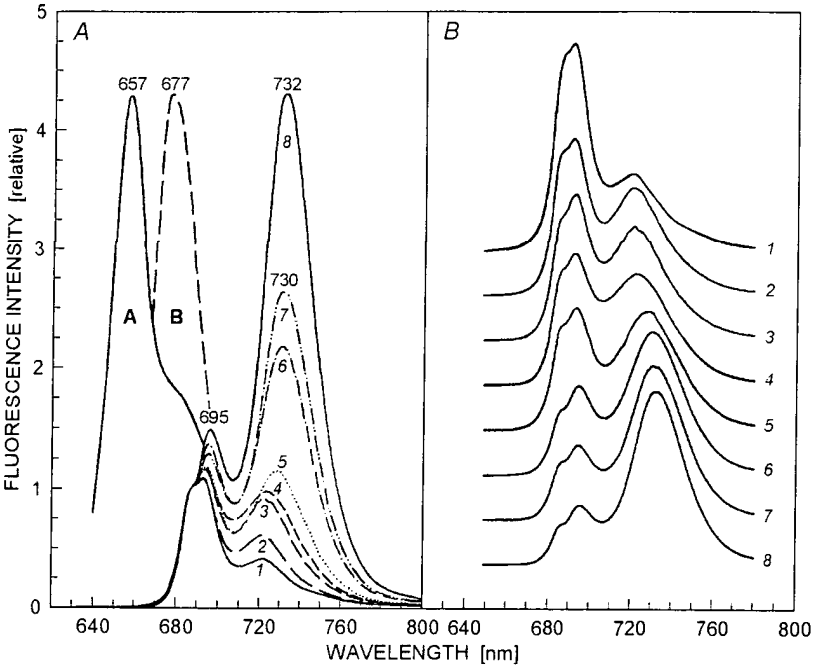

Fig. 1. Low temperature (77 K) emission spectra of leaves from etiolated pea plants exposed to intermittent irradiation (IMI) and then transferred to continuous irradiation (CI) as follows: (*1*) 24 h IMI, (*2*) 48 h IMI, (*3*) 48 h IMI + 1 h CI, (*4*) 48 h IMI + 2 h CI, (*5*) 48 h IMI + 4 h CI, (*6*) 48 h IMI + 6 h CI, (*7*) 48 h IMI + 8 h CI, (*8*) 48 h IMI + 48 h CI. The leaves were dark adapted for 10 min before freezing them to 77 K. Excitation at 633 nm by a HeNe laser of 40 W m^{-2}. The spectra are normalized at 687 nm (*left*), or plotted on equal area basis and staggered for clarity reasons (*right*). In the *left panel* two additional spectra (of adjusted amplitudes) are also presented: spectrum *A* is from an etiolated leaf and spectrum *B* from an etiolated leaf which was exposed shortly to green radiation showing the radiant energy induced protochlorophyllide to chlorophyllide transformation.

Table 1. Total chlorophyll (Chl) content [mg kg^{-1}(fr.m.)] and selected fluorescence parameters during continuous irradiation, CI. Etiolated pea plants exposed to 48 h intermittent irradiation regime, IMI, were used. The fluorescence parameters are: At 77 K, the fluorescence intensities at 696, 720, and 730 nm, normalized to the intensity at 687 nm. The wavelength at which the peak of the long-wavelength band appears, is also listed. At room temperature, the ratio F_V/F_M, where F_M is the maximal fluorescence and F_V is the maximal variable fluorescence, the relative variable fluorescence V_J (at 2 ms) and V_I (at 30 ms), calculated from the general formula, $V_t = (F_t - F_0)/(F_M - F_0)$, are also shown. M_0 is the initial slope of the relative variable fluorescence [$M_0 = (F_{300\mu s} - F_{50\mu s})/(F_M - F_{50\mu s})$]. PI_{CS} is the performance index and has been calculated according to Appendices *C* and *D*.

CI time [h]	Chl	77 K				Room temperature					
		F696/F687	F720/F687	F730/F687	Peak wave-length	F_M	F_V/F_M	V_J	V_I	M_0	PI_{CS}
0	162	0.972	0.597	0.456	721.0	248	0.58	0.49	0.62	0.70	130
2	245	1.106	0.931	0.872	722.8	349	0.60	0.48	0.63	0.76	185
4	404	1.149	0.949	1.133	729.0	395	0.67	0.55	0.65	1.29	268
6	471	1.356	1.491	2.158	731.0	483	0.74	0.55	0.73	1.46	467
8	527	1.275	1.622	2.602	731.0	569	0.76	0.54	0.74	1.14	751
48	1038	1.473	2.307	4.189	732.4	883	0.78	0.47	0.82	0.97	1062

increasing CI, as shown in Fig. 1 (note that the greening increases as the numbers on the curves increase). During the greening process, the long-wavelength emission band shifts from 720 to 730 nm starting at about 4 h of CI (curve *5*); after 48 h of CI (curve *8*), the emission spectrum is similar to that of a fully developed green leaf (see also Table 1). The development of each emission peak during greening is demonstrated in Fig. 1*B* which plots the spectra on an equal area basis. A clear relative decrease in the peaks at 687 nm (F685) and 695 nm (F696) and an increase at 730 and 735 nm (F730) is obvious. In 48 h of CI, several fold increase in the F730/F687 ratio was observed (Fig. 2). Our results are in agreement with the earlier observations regarding the increase of the F730 relative to F690 and the shift of the long-wavelength band from 720 to 730 nm (Mullet *et al.* 1980, Castorinis *et al.* 1984, Dreyfuss and Thornber 1994, Špunda *et al.* 1997). Our results, however, show and emphasize the biphasic assembly of the complexes: first, during 3-4 h of CI, the core PS1 and PS2 increase (the F696 and F720 increase) and then later, the LHCs increase [shift of peak at 720 to 730 nm (F730); and increase in re-absorption of fluorescence]. Control spectra of diluted leaf powder at 77 K with minimum reabsorption effect (values not shown) confirmed the accumulation of core antenna and LHCs. These conclusions, obtained from 77 K emission spectra, are in agreement with the pigment-protein complexes observed by Mysliwa-Kurdziel *et al.* (1997).

Chlorophyll *a* fluorescence induction kinetics (the so-called Kautsky or OJIP transient): During greening in CI, the pigment binding stabilizes the apoproteins,

which in turn causes the accumulation of pigment-protein complexes. Thus, the photosynthetic apparatus is built up resulting in a gradual development of the

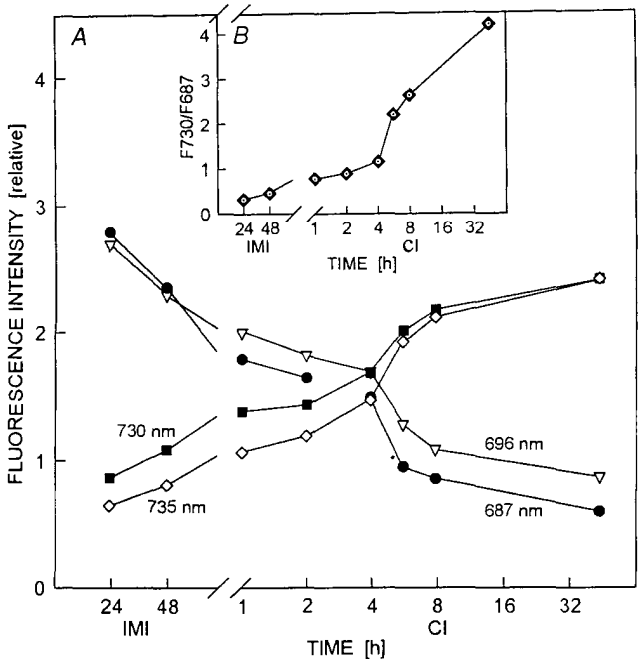

Fig. 2. The relative intensity of chlorophyll *a* fluorescence at 687, 696, 730, and 735 nm and the ratio F730/F687 (*insert*) during the greening process of pea leaves as presented in the right panel of Fig. 1. IMI, intermittent irradiation sample; CI, continuous irradiation. The 48 h IMI sample was transferred to CI.

photosynthetic activity. Chl *a* fluorescence induction kinetics is a good indicator for the functioning of photosynthesis in intact leaves. Although there are earlier reports on the Chl *a* fluorescence kinetics of the greening plants (see Buschmann 1981), the current method of detecting the fluorescence signal by using a shutter-less fluorometer, and plotting values on a logarithmic time scale (see Strasser *et al.* 1995), provides values starting at 50 μs with a resolution of the various steps, not observed otherwise. When green leaves are exposed to the 600 W m^{-2} actinic radiation, Chl *a* fluorescence induction transients follow a pattern of OJIP, where J and I appear as two steps in between the minimum (F_0) and maximum (F_M) levels of fluorescence. Fig. 3*A* shows the Chl *a* fluorescence induction kinetics of greening pea leaves. Plants grown in IMI (48 h) showed variable Chl *a* fluorescence, with F_M/F_0 of ~2.5; after transferring them into CI (from 2 to 48 h), a progressive increase in this ratio was observed (up to F_M/F_0 of ~5.0; Fig. 3*A*). The F_V/F_M ratio increased from 0.58 to 0.78 corresponding to the value of a control green plant (Table 1).

When analyzing the OJIP transient (Appendices *A, B,* and *C*; Fig. 4), one can split the F_0 into two parts, one of which belongs to active PS2, and the other which does not belong to active PS2 ($F_{0(non\text{-}PS2)}$) (see Appendix *B*). When the F_V/F_M ratio was calculated exclusively from F_V and $F_{0(PS2)}$ it ranged from 0.81 to 0.86 for all the samples (Fig. 5) which suggests that plants kept under IMI had fully developed active RCs. A low F_V/F_M ratio is due to the presence of non-Q_A reducing Chl complexes.

This conclusion is consistent with the fact that the F_0 of IMI leaves contains a long lifetime fluorescence component (Karukstis and Sauer 1983) that may originate from

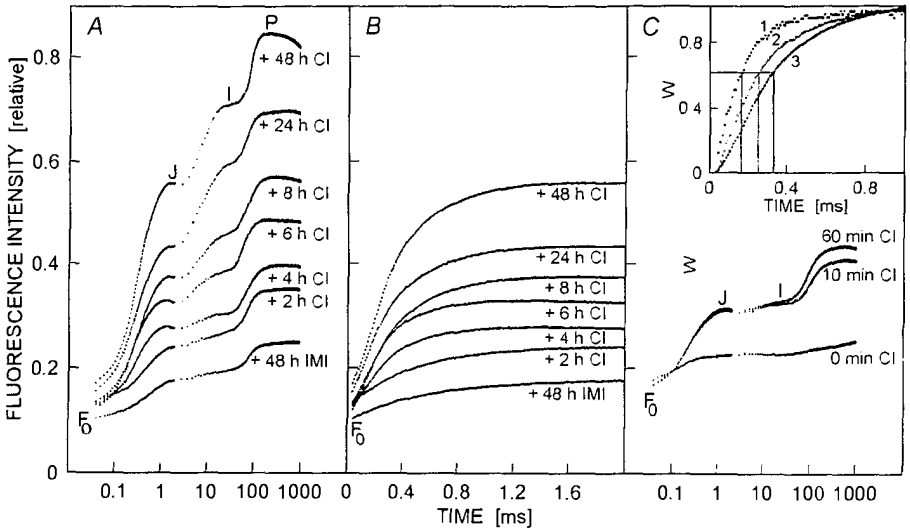

Fig. 3. (*A*) Room temperature chlorophyll (Chl) *a* fluorescence induction kinetics (from 50 μs to 1 s) of pea leaves during greening in intermittent irradiation (IMI) and followed by continuous irradiation (CI). Leaves were dark adapted for 10 min before exposing them to 600 W m^{-2} of red (650 nm) actinic radiation. (*B*) As in (*A*) but from 50 μs to 2 ms on a linear time scale. (*C*) The fluorescence induction curves obtained from the pea leaves grown in flash regime (FL: 1 ms flash - 15 min dark). After 10 d under FL, the plants were transferred to CI for 10 min or 60 min. All transients of *A*, *B*, and *C* are presented as measured on leaves, without any normalization. However, *insert* shows the variable fluorescence intensity normalized to the step J as $W = (F_t - F_0)/(F_J - F_0)$ to compare the fast fluorescence rise of FL (*1*), FL + 10 min CI (*2*), and green leaf (*3*). For the OJIP nomenclature, see text and Strasser *et al.* (1995).

disconnected Chl-protein complexes, and if F_0 is corrected for that component, then the F_M/modified F_0 is already high even in the IMI leaves (J-M. Briantais and I. Moya, personal communication). This means that the quantum yield of photochemistry of individual photosynthetic units is already high, and more of PS2 antenna (both core PS2 and LHC2b/PS2) are added during CI exposure. Chl *a*/*b* ratio was about 25 in leaves grown in IMI which decreased to about 3 in mature green leaves (data not shown).

The Chl *a* fluorescence induction showed a clear O to J step. The fast O-J phase is due to the net photochemical reduction of Q_A to Q_A^-. This phase is also influenced by the S-state of the donor side of PS2 (Delosme 1967, Schreiber and Neubauer 1987, Hsu 1993, B.J. Strasser 1997) and the reaction on the acceptor side of PS2: $Q_A^-Q_B$ to $Q_AQ_B^-$. 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 centres as well as due to the different redox states of the RC of PS2 which reduces the PQ pool (Strasser *et al.* 1995). After transferring the IMI-grown plants into CI, the F_0 to J increases with increasing CI.

When the fast phase of the Chl *a* fluorescence induction kinetics of IMI-grown plants was plotted on a linear time scale, an exponential increase in the Chl *a*

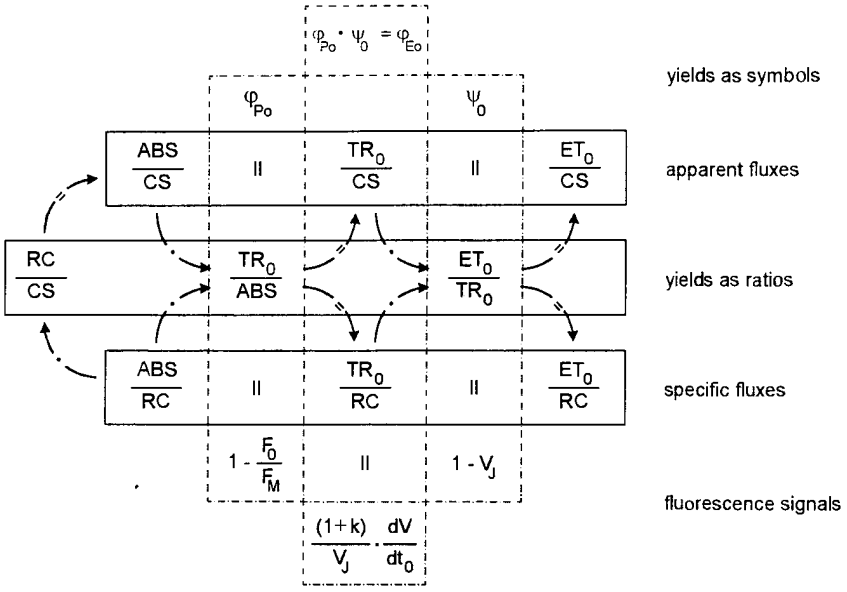

Fig. 4. Correlation and interconnection of the biophysical expressions used to analyze the chlorophyll *a* fluorescence transient, OJIP, that we call the JIP-test for short (see Appendix *A*). The derivation of the yields ($\varphi_{Po} = TR_0/ABS$, $\psi_0 = 1 - V_J$, $\varphi_{Eo} = ET_0/ABS = (\varphi_{Po}\ \psi_0)$ and the fluxes (ABS/RC, TR_0/RC, ET_0/RC) requires the three experimental signals (*hatched areas*): (*1*) the fluorescence intensity ratio of minimal to maximal fluorescence, F_0/F_M; (*2*) the relative variable fluorescence $V_J = (F_{2ms} - F_0)/(F_M - F_0)$; (*3*) the slope $(dV/dt)_0$ of the relative fluorescence rise at the onset of actinic radiation. The description of the apparent fluxes (or phenomenological activities) requires a proportionality factor, which corresponds to ABS/CS. This expression can be derived from transmission or reflection measurements or it can be substituted, as an approximation, by the fluorescence intensities F_0 or F_M.

fluorescence from F_0 to J level was observed up to ~0.4 ms (Fig. 3*B*). However, when IMI-grown plants were transferred into CI, the Chl *a* fluorescence rise from F_0 to J showed a deviation from the exponential towards the sigmoidal shape which indicates the possibility of connectivity or grouping between PS2 units or the probability of exciton transfer among PS2 units (labeled as G) during the greening process. A method has been developed to calculate this G, the overall probability of connectivity between PS2 units, from the experimental OJIP Chl *a* fluorescence transient (see Appendix *D*). Plants grown under IMI showed G = 0 (Fig. 6). It suggests that IMI grown plants had PS2s that did not exchange excitation energy with each other (that is, they had zero connectivity). However, PS2s became connected and exchanged excitation energy within a few hours after transferring them in to CI (Fig. 6).

Although $(F_J - F_0)/F_0$ increased when the IMI grown plants were transferred into CI, insignificant differences in the relative variable fluorescence at 2 ms [$V_J = (F_{2ms}$

$- F_0)/(F_M - F_0)]$ is observed (Table 1). This observation is in qualitative agreement with a report by Barthélemy *et al.* (1997). However, a 40 % increase in the

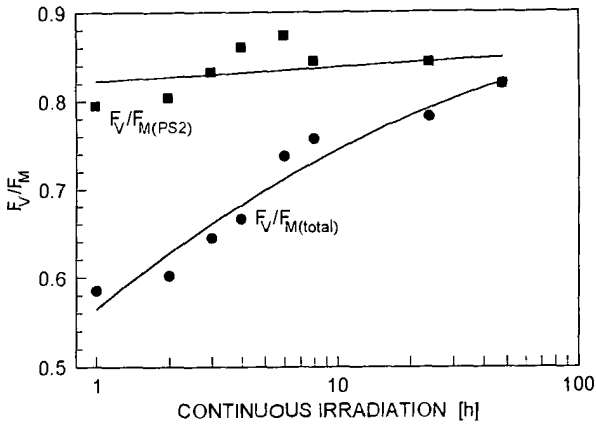

Fig. 5. Fluorescence ratios $F_V/F_{M(total)}$ and $F_V/F_{M(PS2)}$ (corrected for F_0 of disconnected units, *etc.*) during greening, calculated from the fluorescence transients of Fig. 3*A* according to Appendix *B*.

V_I [$= (F_{30ms} - F_0)/(F_M - F_0)$] level is recorded during the greening process (Table 1). On the other hand, due to the reduction and, thus, the filling-up of the PQ pool, the J-I-P transient developed progressively during the first hours of greening. The I step is not obvious in IMI-grown plants. Insignificant difference in V_I was observed between IMI-grown plants and after they were transferred for 2 h in CI. However, after 6 and 48 h of CI, more than 18 and 32 % increase in the relative variable fluorescence at the I level [$V_I = (F_{30ms} - F_0)/(F_M - F_0)$] was observed.

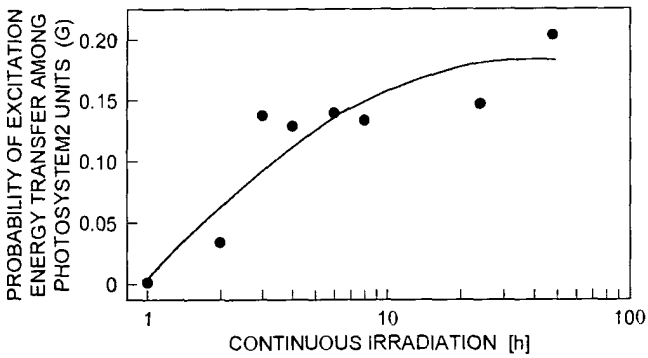

Fig. 6. The probability (G) of excitation energy transfer among photosystem 2 units, during greening in continuous irradiation, CI of 48 h intermittent irradiation, IMI, grown pea leaves. Values were obtained from Fig. 3*A* and calculated according to Appendix *D*.

FL-grown (under 1 ms flash regime after every 15 min darkness) plants contained more Chl [about 250 mg(Chl) kg^{-1}(fr.m.)] than the IMI-grown plant [162 mg(Chl) kg^{-1}(fr.m.), see Table 1]. However, the FL plants showed a variable fluorescence curve of different kind than the green or green-induced leaves (Fig. 3). FL plants showed a variation in their room temperature fluorescence only from 0 to the J step (about 2 ms) or to K step (about 300 μs), *i.e.*, with only a single turn over photochemical phase of low amplitude. These results are in contrast with previous results (Strasser and Sironval 1972, 1973, 1974) where FL plants did not show at

room temperature any appreciable variable Chl *a* fluorescence, however, normal variable fluorescence could be measured at 77 K (Strasser and Butler 1976b). In the previous experiments (Strasser and Sironval 1972, 1973, 1974) a slow shutter [ms]

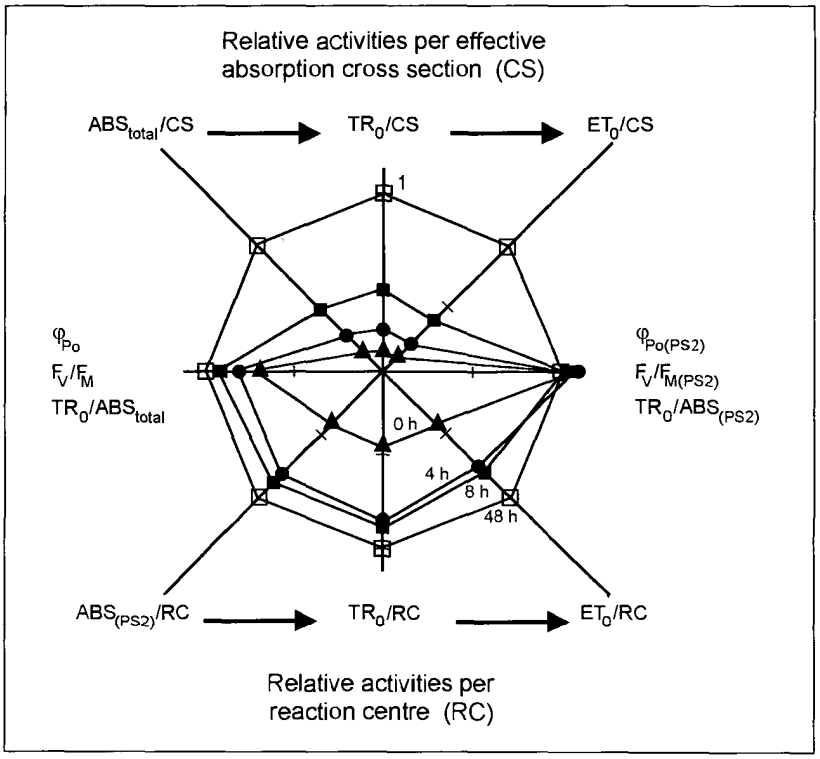

Fig. 7. Spider plot presentation of the apparent activities (ABS/CS, TR_0/CS, and ET_0/CS), the specific activities (ABS/RC, TR_0/RC, and ET_0/RC), as well as the quantum yields of primary photochemistry TR_0/ABS_{total} and $TR_0/ABS_{(PS2)}$. The parameters were calculated from the fluorescence transients of Fig. 3*A* according to Appendices *A* and *C* (also see Table 2). The values at different stages of greening were normalized over the corresponding values at 48 h continuous irradiation, CI.

was used to measure the room temperature variable fluorescence and it was not possible to measure the early events before 2 ms. When these flashed leaves were transferred to CI, the variable Chl *a* fluorescence of the OJIP type could be induced at room temperature within 10 min (Fig. 3*C*; also see Strasser and Sironval 1972, 1973, 1974). After 60 min exposure of FL leaves under CI the F_M/F_0 was equivalent to that obtained in 48 h IMI plants. In all likelihood, the antenna size was still small. The time needed to accumulate LHC2 for FL plants is similar to IMI-grown plants.

Using an analysis of the OJIP rise (that includes the main steps J, I, and P), several expressions of PS2 activities such as (*a*) rate of absorption (ABS) of photons per RC (ABS/RC); (*b*) rate of trapping (TR) of excitons per RC (TR_0/RC); and (*c*) the rate

Table 2. Summary of the JIP-test formulae using the experimental data extracted from the fast fluorescence transient O-J-I-P. * = absorbed radiation per cross section (ABS/CS) is measured as the radiation absorbed (J_a) by absorption measurements, or approximated by F_0 or F_M indicated by the index CS_0 or CS_M. k stands for $G \times (F_V/F_0)$, G is the overall grouping probability. If energetic co-operativity is not considered (no grouping, G = 0), the constant k becomes zero.

Extracted and technical fluorescence parameters		I
F_0	$F_{50\mu s}$, fluorescence intensity at 50 μs	
F_{150}	fluorescence intensity at 150 μs	
F_{300}	fluorescence intensity at 300 μs	
F_J	fluorescence intensity at the J-step (at 2 ms)	
F_M	maximal fluorescence intensity	
t_{Fmax}	time to reach F_M [ms]	
V_J	$(F_{2ms} - F_0)/(F_M - F_0)$	
Area	area between fluorescence curve and F_M	
$(dV/dt)_0$ or M_0	$4\ (F_{300} - F_0)/(F_M - F_0)$	
Quantum efficiencies or flux ratios		II
φ_{Po} or TR_0/ABS	$(1 - F_0/F_M) = F_V/F_M$	
φ_{Eo} or ET_0/ABS	$(1 - F_0/F_M)\ \psi_0$	
ψ_0 or ET_0/TR_0	$1 - V_J$	
Activities per reaction centre (RC)		III
ABS/RC	$(1 + k)\ M_0\ (1/V_J)\ (1/\varphi_{Po})$	*
TR_0/RC	$(1 + k)\ M_0\ (1/V_J)$	
ET_0/RC	$(1 + k)\ M_0\ (1/V_J)\ \psi_0$	
DI_0/RC	$(ABS/RC) - (TR_0/RC)$	
Activities per effective absorption cross section		IV
ABS/CS	F_0 or F_M or J_a	*
TR_0/CS	φ_{Po} (ABS/CS)	
ET_0/CS	$\varphi_{Po}\ \psi_0$ (ABS/CS)	
DI_0/CS	$(ABS/CS) - (TR_0/CS)$	
RC/CS	(ABS/CS)/(ABS/RC)	
Vitality indexes		V
PI_{ABS}	$(RC/ABS)\ [\varphi_{Po}/(1 - \varphi_{Po})]\ [\psi_0/(1 - \psi_0)]$	
PI_{CS}	PI_{ABS} ABS/CS	*
Driving forces		VI
DF_{ABS}	$\log(PI_{ABS})$	
DF_{CS}	$\log(PI_{ABS}) + \log(ABS/CS)$	*

of electron transfer beyond Q_A^- per RC (ET_0/RC) (see bottom of a plot, that we call a spider plot, Fig. 7, and the left panels of what we call the pipe-line model, Fig. 8), as well as the same PS2 activities per leaf area or per effective absorption cross-section (see top of the spider plot of Fig. 7 and the right panels of the pipe-line model of Fig. 8) can be calculated (see Appendices *A*, *B*, and *C*, Table 2, and the legends of Figs. 7 and 8). An expression for the relative density of RCs per leaf area or leaf cross

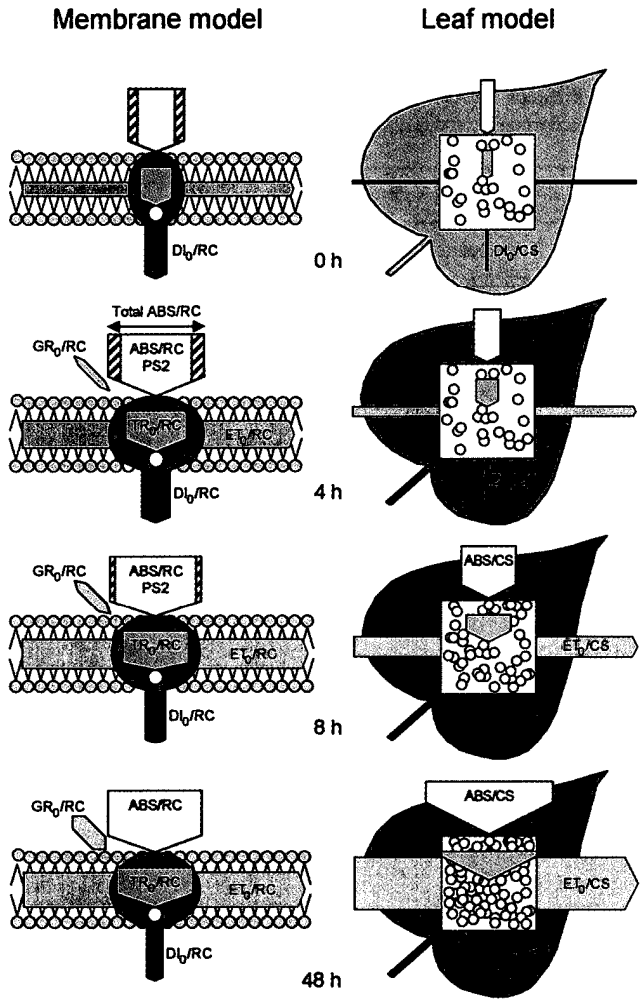

Fig. 8. An energy pipeline model of greening pea leaves under continuous irradiation, CI, from 0 to 48 h following 48 h intermittent irradiation, IMI. The membrane models (*left*) show the specific activities (per reaction centre, RC). The leaf models (*right*) show the phenomenological fluxes or apparent activities (per cross section, CS). The arrows of the membrane models (*left*) indicate the fluxes for light absorption (ABS), excitation energy trapped (TR_0), energy dissipation (DI_0), probability of grouping (excitation energy migration between PS2-units, GR_0) and electron transport (ET_0) beyond Q_A^-. The oval shape in the membrane model has the same width as the arrow of the ABS/RC to indicate the average antenna size. The hatched parts of the absorption flux arrows and of the antenna correspond to absorbing pigments which are not involved in the reduction of Q_A to Q_A^-. The value of each activity is shown by the width of the corresponding arrow. The coloration in the leaf models indicates the pigment concentration per cross section (ABS/CS). The density of Q_A reducing photosynthetic units, per cross section, is demonstrated by the density of small circles in the leaf models. The detailed calculation of each expression is given in Appendices *A-D* and Table 2.

section (RC/CS) was also calculated (see Appendix *A* and Table 2). After transferring the IMI-grown plants into CI, the specific activities (ABS/RC, TR_0/RC, ET_0/RC) at time zero were almost constant. However, several fold increases in the same activities per leaf area or leaf cross section (ABS/CS, TR_0/CS, and ET_0/CS) were observed. The number of RC/CS also increased after transferring the IMI-grown plants to CI. All the above reported kinetics which describe greening can be quantified by a so-called performance index, PI, expressed on an absorption basis (PI_{ABS}) or on a leaf area or active cross section basis (PI_{CS}) (see Appendix *C* and Tables 1 and 2).

Delayed Light Emission (DLE): In mature green leaves, an anti-parallel correlation between the intensities of Chl *a* fluorescence and DLE has been observed, but only during a part of the induction period starting with the first DLE peak where the fluorescence intensity shows a minimum (Malkin *et al.* 1994). Fig. 9*A* shows the DLE at 0.4 (*top*) and 0.8 (*middle*) ms and prompt fluorescence (F, *bottom*) after the irradiation in FL leaves. In agreement with the results in Fig. 3*C*, there was very little variable Chl *a* fluorescence. Further, DLE also did not show any variations with time (unlike mature greened plants). However, with increasing time of irradiation up to 7 min, the DLE intensity decreased. This is easily understood because DLE is a measure of the concentration of the separated charges in PS2 (see Lavorel 1975), and

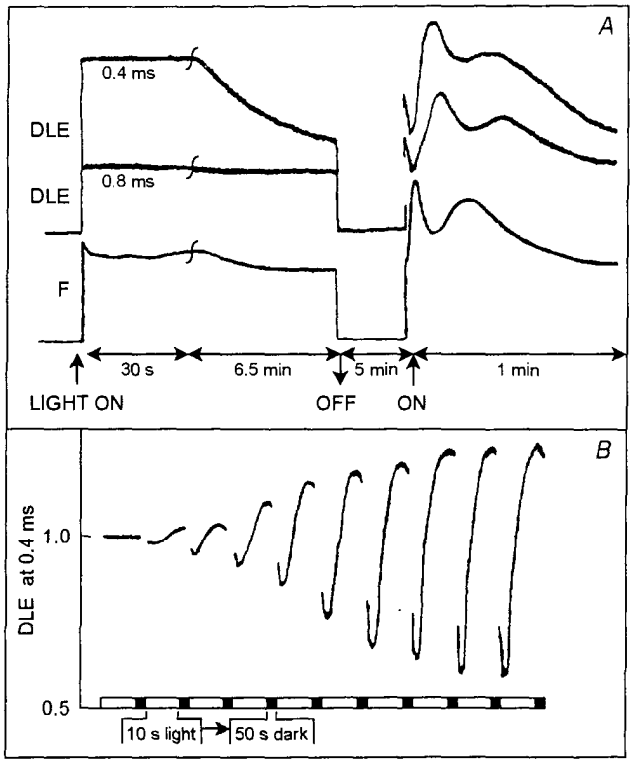

Fig. 9. (*A*) Delayed Light Emission (DLE) from FL grown leaves measured at 0.4 ms (*top left*), at 0.8 ms (*middle left*), and from the same leaves after they had been irradiated for 7 min and then dark adapted for 5 min (*right top* and *middle*). The simultaneously recorded chlorophyll *a* fluorescence transient (F) is also shown (*bottom*). (*B*) The 0.4 ms DLE of FL-grown leaves, during successive irradiation periods of 10 s separated by dark intervals of 50 s.

when the donor side of PS2 is non-functional, the separated charges may live longer and be detected at higher concentration. As the donor side becomes functional, the positive charges are removed and DLE decreases. Thus, the decrease in DLE during the 6.5 min (see top curve in left side of Fig. 9*A*) is interpreted as reflecting the formation of the water oxidation system on the donor side of PS2. After a 5-min dark adaptation, all is normal in the FL samples that already had 7 min of irradiation: the normal DLE induction curve as well as the Chl *a* fluorescence induction curve is observed (see right side of Fig. 9*A*). Fig. 9*B* shows the amplitude of the 0.4 ms DLE in 10 dark-light periods (the duration of each light period was 10 s of modulated actinic radiation of 100 Hz with a light to dark ratio of 9 : 1; the dark period was 50 s). The starting DLE intensity at the beginning of each light period decreases as the number of periods increases, and at the same time, the variable component of DLE (maximum DLE level *minus* minimum DLE level) increases reflecting the formation of functional oxygen evolving PS2 centres.

Induction of oxygen evolution and period 4 oscillation due to the water-splitting system during the greening process: Joliot *et al.* (1969) and Kok *et al.* (1970) showed that the yield of O_2 from chloroplasts or green algae, exposed to a series of saturating flashes, shows a damped oscillation with a period of four when the oxygen yield/flash is plotted as a function of flash number. As stated earlier (Delosme 1967, Schreiber and Neubauer 1987), the donor side of PS2 plays an important role in Chl *a* fluorescence changes. The oxygen evolving complex (OEC) is in S_1 state after long dark adaptation (Vermaas *et al.* 1984, Styring and Rutherford 1987). After exposing photosynthetic samples to different number of single turnover flashes, the system moves into the next S-state. Therefore, the Chl *a* fluorescence induction measured after 0, 1, 2, 3, and 4 preflashes should be affected by the PS2 centres with their OEC in S_1, S_2, S_3, and S_0 before measurement. The S_4 state has a very short life of about 1 ms and cannot be measured in the time course of the given experiment. It has been shown by other techniques, that, in the dark, S_2 and S_3 decay in tens of seconds to S_1, and S_0 converts to S_1 with $t_{1/2}$ of about 20 min (Vermaas *et al.* 1984, Styring and Rutherford 1987). Changes in the initial fluorescence and the fluorescence kinetics related to the OEC have been reported in thylakoid or algal suspensions (Hsu 1993, Lavergne and Leci 1993). But this method had not been applied directly on intact leaves. Although an indirect method has been developed by Shinkarev *et al.* (1997) for measuring the kinetics of oxygen evolution in leaves, very few experiments have exploited the use of fluorescence to monitor the "oxygen clock" [see, *e.g.*, Delosme 1971, Joliot *et al.* 1971, Eaton-Rye and Govindjee 1988 (see their Fig. 2 for period 4 oscillations), B.J. Strasser 1997, Govindjee *et al.* 1998]. Here, we have measured period 4 oscillations directly on the intact IMI leaves and those treated with CI by using a shutterless fluorometer (*PEA*) which provides the first reliable data point at 50 μs and allows data accumulation over several order of magnitude of time (B.J. Strasser 1997, Strasser and Strasser 1998). The same sample was used to record the oscillations after giving 1 to 10 flashes.

In the experiments of Fig. 10, a green pea leaf was dark-adapted for 10 min and Chl *a* fluorescence induction kinetics were measured by exposing the leaf to 600 W m^{-2} actinic radiation after a given number of pre-flashes. The spacing between the flashes was 1 s. After each actinic irradiation of only 1 s duration, a 2 min dark adaptation was given to permit reopening of the reaction centres. A 5 min dark adaptation gave identical results. Thus, 2 min dark adaptation was sufficient. Fig. 10 shows the pattern of flash-induced Chl *a* fluorescence oscillation in green pea leaves at different times of the Chl *a* fluorescence induction curve. The Chl *a* fluorescence yield measured at 50 μs has a periodicity of four with maxima after 2nd and the 6th flash, and minima after the 4th flash. The amplitude of the oscillation of four decreases during the fast fluorescence rise with time. For example, the amplitude of oscillation at 200 μs is very small and at 280 μs the oscillation is totally absent. At longer time of the fluorescence kinetics, *i.e.*, for example after 1 ms, the oscillation appears again, but in the opposite direction (the mimima after 2nd and the 6th flash and maxima after 4th flash).

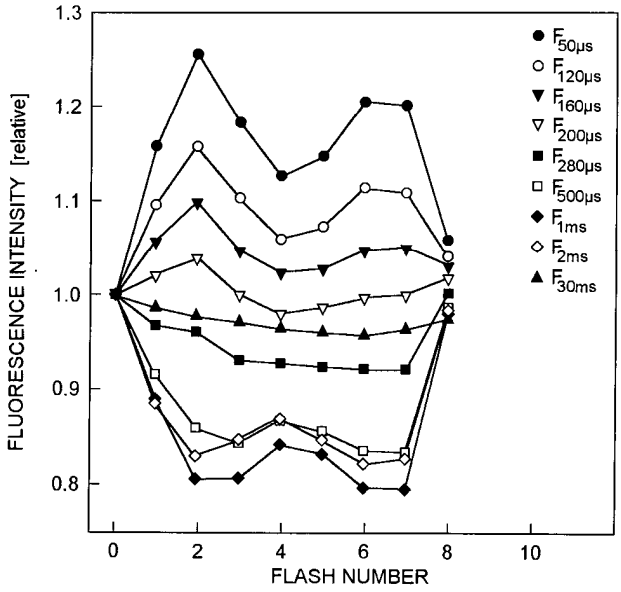

Fig. 10. The chlorophyll *a* fluorescence intensity of green pea leaves at the indicated times of the OJIP-rise, as a function of the number of saturating pre-flashes. Leaves were dark adapted for 10 min prior to the onset of the actinic irradiation. All fluorescence intensities are expressed relatively to the corresponding value of the sample that did not receive any pre-flash.

The first phase of the transient (O-J phase) was distinctly affected by the flash number (Fig. 11). The changes in F_0 and F_J oscillated with a period of 4, reflecting the existence of the O_2 evolving clock. The results in Fig. 11*A* show clear changes in the flash number dependence of the initial fluorescence, F_0, and F at 2 ms (F_J). A pronounced oscillation with a periodicity of 4 flashes is present. The initial level of fluorescence F_0 shows maxima after the 2nd and the 6th flash, and the minima after zero (dark adapted state), the 4th, and the 8th flash. These results reflect a slightly higher Chl *a* fluorescence yield in states S_2 and S_3 with respect to the S_0 and S_1 states (cf. Joliot *et al.* 1971, Delosme 1972). The amplitude of fluorescence intensity after the flashes 2 and 4, the ratio of fluorescence after flash 2 to that after the flash 4 (Fig.

11*B*) increases with increasing greening time; this can be directly correlated with increased Hill reaction rates during the greening of the plants observed by, *e.g.*, Akoyunoglou (1981). The increases in oscillations, observed here during the greening process, were correlated with the increases in the total chlorophyll concentration (data not shown). The J level of Chl *a* fluorescence showed a period 4 oscillation in the opposite direction (Fig. 11*A*, *bottom*) in greening pea leaves as well.

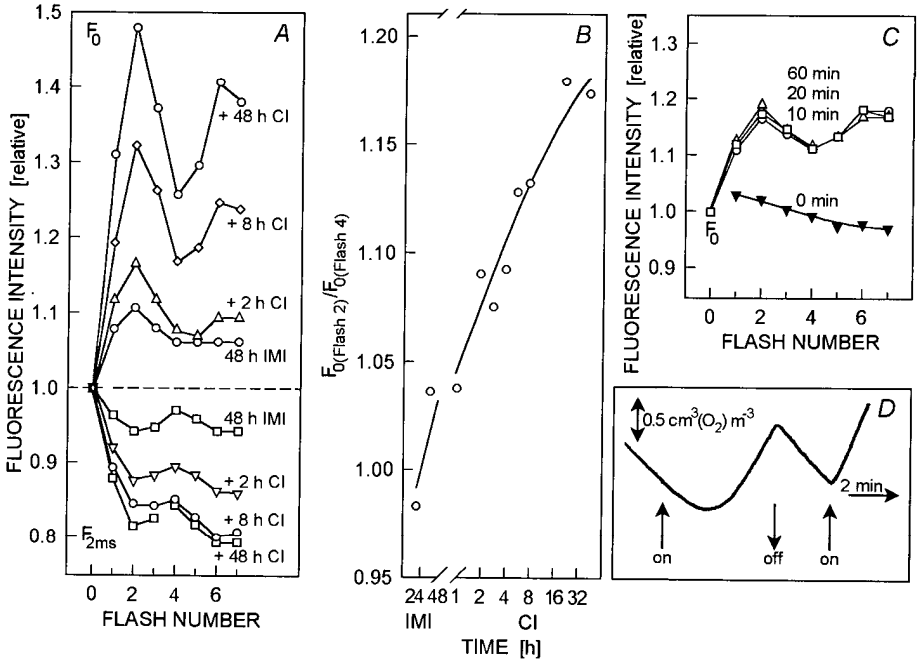

Fig. 11. (*A*) The chlorophyll *a* fluorescence intensity at 50 μs (F_0) and 2 ms (F_J) of the OJIP-rise *versus* the number of pre-flashes, in pea plants during greening as in Fig. 3*A*. (*B*) Ratio of the initial fluorescence intensities (F_0) of leaves exposed to 2 and 4 pre-flashes, during greening (as in Fig. 3*A*). (*C*) The initial fluorescence intensity (F_0) of FL-grown leaves transferred (as in Fig. 3*C*) to continuous irradiation, CI, for 0 up to 60 min, *versus* the number of saturating pre-flashes. For details concerning the pre-flashing procedure, see Materials and methods. (*D*) Induction of oxygen evolving capacity in FL-grown pea leaves (as described in the legend of Fig. 3*C*), during exposure to CI up to 6 min. Note that the oxygen evolution starts immediately upon actinic irradiation after dark period of 3 min.

Plants kept under FL regime (1 ms flash after 15 min of dark during 10 d; see "0 min CI" curve) did not show the period four oscillation (Fig. 11*C*), and no O_2 evolution was measured (Fig. 11*D*). However, when these plants were transferred to CI (*e.g.*, 5 to 10 min), a clear period 4 oscillation as well as a net O_2 evolution (Fig. 11*D*) was observed. The capacities of net oxygen evolution, appearance of period four oscillation and variable OJIP fluorescence as well as variable DLE is induced during the first irradiation with CI. These capacities remain induced even after a dark period of several min. These results are in agreement with those on DLE (Fig. 9) and

the earlier conclusions of Strasser (1973) and Strasser and Sironval (1972, 1973, 1974).

Changes in P700 during the greening process: The formation of the oxidized reaction centre Chl *a* of PS1, $P700^+$, measured as 820 nm absorption changes, increased after transferring the IMI-grown plants to CI (Fig. 12*A*). However, on a Chl basis, the ratio of $P700^+$/[Chl] decreased due to the increased antenna size during the greening process. An increase in PS1 activity on fresh mass basis has been reported in other systems (Akoyunoglou 1981). Leaves grown under ms flashed regime also showed the same trend for the development of the fraction of $P700^+$ (values not shown) under actinic radiation. During 60 min of greening of leaves, grown under ms flashed regime, no change in the induced absorption changes at 820 nm across the leaf was observed (Fig. 12*B*). However, already with 5-10 min of CI a steady build-up of variable Chl *a* fluorescence (Figs. 3*C* and 12*B*), variable DLE (Fig. 9*B*), oxygen evolution (Fig. 11*C*), and a periodicity of four of the initial fluorescence, F_0 (Fig. 11*C*) were observed.

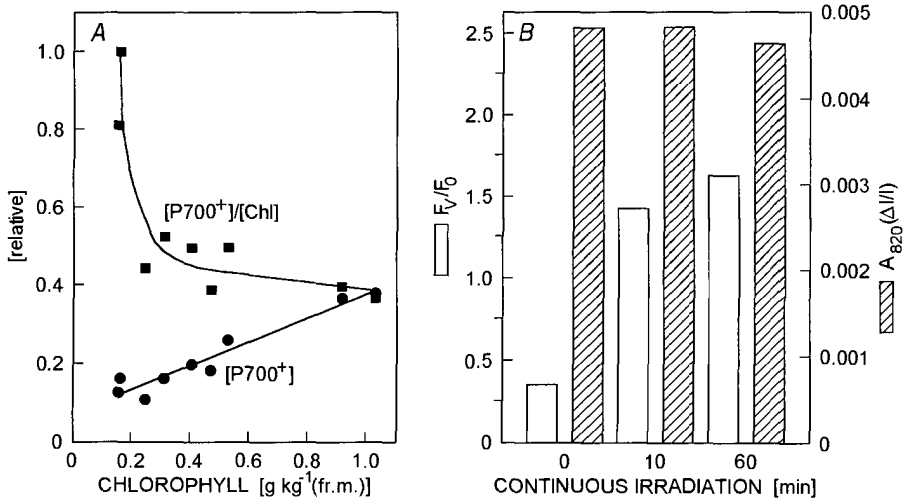

Fig. 12. (*A*) The development of active PS1 reaction centres [$P700^+$] measured by the irradiation-induced absorption change at 820 nm across the leaf, during greening in CI of IMI-grown pea leaves. [$P700^+$] and the ratio [$P700^+$]/[Chl] are plotted *versus* the chlorophyll (Chl) concentration of the leaf. (*B*) The F_V/F_0 ratio (*open bars*) and the irradiation-induced absorption change at 820 nm, A_{820} (*hatched bars*) of FL-grown pea leaves transferred to CI.

Concluding remarks: In conclusion, the results of this paper show new details on the development of the PS2 apparatus through measurements by an intrinsic and sensitive probe of photosystems: emission of Chl *a* fluorescence and DLE. The etiolated plants contain only protochlorophyllide as can be seen in 77 K emission spectra. In plants kept under FL and IMI, and then transferred to CI, the development of PS2 is different. Based on parallel measurements on the OJIP Chl *a* fluorescence transient, the 77 K emission spectra, the period 4 oscillations in the initial

fluorescence level and DLE, the progressive developmental processes are as follows (Fig. 13): Plants kept under FL have fully developed small PS2 units (with inner antenna; reaction centre Chls; electron acceptors Q_A and Q_B; and the donor Y_Z), but without the light-harvesting complex, LHC2, and the oxygen evolving complex, OEC, is not functionally connected to the PS2 (*Box 1*, Fig. 13). They do not show period 4 oscillation and variable OJIP Chl *a* fluorescence at room temperature. However, after few min of CI, FL-grown plants have fully functional RCs, with OEC, and still in small units, but are devoid of LHC (*Box 2*). They are able to evolve O_2 (inferred from period 4 oscillations in F_0) and show normal variable fluorescence at room temperature. Within a few hours under CI, in both IMI- and FL-grown plants, further assembly of core PS1 and PS2 takes place and in later hours LHCs increase (*Box 3*). PS2 units are initially disconnected (separate package model), but later, after they are transferred to CI, they form groups which exchange excitation energy (grouping) and are thus connected photosystem (*Box 4*).

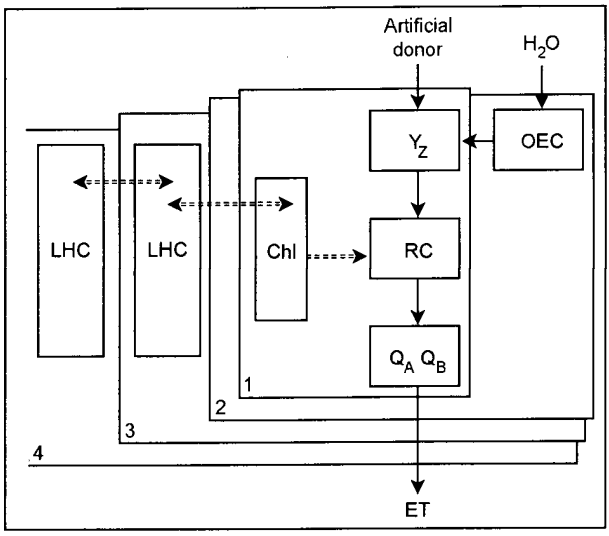

Fig. 13. Stages of photosystem 2 development under different irradiation conditions. (*Box 1*) Plants grown under flash-irradiation regime (FL: 1 ms irradiation - 15 min dark): accumulation of photochemically active units without oxygen evolving capacity and without Chl *ab*-LHC. (*Box 2*) Plants grown under FL and then transferred to continuous irradiation (CI) for 4-5 min, as well as plants grown under intermittent irradiation (IMI: 2 min irradiation - 118 min dark): accumulation of photochemically active units exhibiting oxygen evolution, without Chl *ab*-LHC. (*Box 3*) Plants grown under FL or IMI and then exposed to CI for 2-3 h: formation of fully developed photosynthetic units (PSUs) containing Chl *ab*-LHC complex, of separate package type, *i.e.*, exhibiting no or very little energy migration (grouping) between the LHC. (*Box 4*) Fully green leaves forming groups of PSUs with energetic cooperativity (grouping) between the LHCs. ET, electron transport; LHC, light-harvesting complex; OEC, oxygen evolving complex; Q_A and Q_B, bound plastoquinones; Y_z, primary electron donor to P680.

Appendix *A*

Summary of the analysis of the OJIP transient (modified from Strasser and Strasser 1995 and Strasser *et al.* 1996)

The Chl *a* fluorescence induction kinetics, OJIP, were analyzed according to the formulae presented in the Table 2. Section I contains the selected original values, as well as the relative variable fluorescence V_J (at the J-step, *i.e.*, at 2 ms), and the initial slope $(dV/dt)_0$ (also labelled as M_0) of the relative variable fluorescence intensity *vs.* time [ms^{-1}]. These values were used for the calculation of different parameters as summarized in Table 2.

Section II: The flux ratios or yields, *i.e.*, the maximum quantum yield of primary photochemistry ($\varphi_{Po} = TR_0/ABS$); the efficiency ($\psi_0 = ET_0/TR_0$) that a trapped exciton can move an electron into the electron transport chain beyond Q_A^-; the probability ($\varphi_{Eo} = ET_0/ABS = \varphi_{Po}\ \psi_0$) that the energy of an absorbed photon by the antenna is conserved in the electron transport beyond Q_A^-.

Section III: The specific energy fluxes (activities per reaction centre) at the onset of irradiation, for absorption (ABS/RC), trapping (TR_0/RC), and electron transport (ET_0/RC).

Section IV: The corresponding activities per excited cross section or per active measured leaf area (ABS/CS, TR_0/CS, and ET_0/CS), as well as the amount of active reaction centres per excited cross section (RC/CS).

The key expressions of the analysis of the OJIP Chl *a* fluorescence transient, also labelled as the JIP-test, are the maximum energy flux trapped by the open RCs (TR_0/RC), and the efficiency (ψ_0) that a trapped exciton can move an electron into the electron transport chain further than Q_A^-. These expressions are derived from the fluorescence transient OJIP by the five biophysical equations of the energy flux theory of R.J. Strasser, as summarized in Srivastava *et al.* (1997, 1998):

(*1*) φ_{Po} (the maximum trapping flux per absorption or maximum quantum yield of primary photochemistry) is:

$$\varphi_{Po} = TR_0/ABS = 1 - (F_0/F_M) = F_V/F_M$$

(*2*) V_t (the relative variable fluorescence *versus* the fraction of closed RCs) is:

$$V_t = (Q_A^-)/Q_{A(total)})/[1 + k\,(1 + Q_A^-/Q_{A(total)})] \text{ where}$$

(*3*) k (the curvature constant k of the hyperbola V_t *versus* $Q_A^-/Q_{A(total)}$) is:

$$k = G\ \varphi_{Po}/(1 - \varphi_{Po}) = G\ F_V/F_0,$$

where G is the overall grouping probability (Strasser 1981) which can be measured according to Appendix *B*.

(*4*) TR_0/RC (the specific maximum energy flux trapped by the open RCs) is:

(*a*) under single turnover conditions (*e.g.*, with DCMU)

$$TR_0/RC = (dQ_A^-/Q_{A(total)})/dt_0 = (dV/dt)_0\ (1 + k)$$

and for experiments *in vivo* or *in vitro*;

(*b*) under multiple turnover conditions, it is:

$$TR_0/RC = (dV/dt)_0\ (1 + k)/V_J$$

(*5*) ψ_0: the efficiency that a trapped exciton (TR_0) can move an electron (ET_0) into the electron transport chain further than Q_A^-

$$\psi_0 = ET_0/TR_0 = 1 - V_J$$

All the expressions used to analyze the OJIP transients are summarized in the Table 2. The correlations between the equations, which link the biophysical expressions to the experimental signals, are shown in Fig. 4. The experimental requirement to execute the JIP-test is an accurate data acquisition of the fluorescence rise in the μs range.

Appendix *B*

$F_V/F_{M(PS2)}$ for the fluorescence which originates only from PS2

Our assumptions are: (*a*) the measured F_0 of the 48 h-irradiated samples belongs entirely to PS2; (*b*) all measured variable chlorophyll (Chl) *a* fluorescence originates from functional Q_A reducing PS2. Based on these assumptions, the $F_{0(PS2)}$ can be calculated for each sample as follows:

$F_0 = F_{0(PS2)} + F_{(non\text{-}PS2)}$, or as a fraction $r_0 = F_{0(PS2)}/F_0$
$F_M = F_{M(PS2)} + F_{(non\text{-}PS2)}$, or as a fraction $r_M = F_{M(PS2)}/F_M$

where $F_{(non\text{-}PS2)}$ originates from several sources: PS1, the antenna that are still not connected to the RCs or that are connected to the RCs which however are not able to reduce Q_A to Q_A^-, or from free Chl, or from other pigments than Chl.

The apparent quantum yield of photochemistry is

φ_{Po} = trapping/absorption(total) = $TR_0/ABS_{(total)} = F_V/F_{M(total)}$

But the quantum yield of photochemistry of the PS2 unit is

$\varphi_{Po(PS2)}$ = trapping which forms Q_A^-/absorption by PS2 = $TR_0/ABS_{(PS2)} = F_V/F_{M(PS2)}$,

where F_M of PS2 is $F_V + F_{0(PS2)}$.

The analysis of the Chl *a* fluorescence transient, OJIP, gives the possibility to calculate the expression RC/CS (cf. Appendix *A* and Table 2). If we assume that (*a*) the irradiated leaf area is constant (CS is constant); and (*b*) all F_0 of a reference sample (*e.g.*, a greened sample at 48 h) belongs to PS2, the $F_{0(PS2)}$ can then be calculated for each sample as follows:

$$F_{0(PS2)(t)} = \frac{RC_{(t)}/CS_0}{RC_{(ref)}/CS_0} F_{0(ref)}$$

where (t) refers to the time of greening under continuous irradiation.

$\varphi_{Po(PS2)(t)}$ can be calculated as

$$\varphi_{Po(PS2)(t)} = \frac{F_{V(t)}}{F_{M(PS2)(t)}} = \frac{F_{V(t)}}{F_{V(t)} + F_{0(PS2)(t)}} \qquad \text{(Fig. 4)}$$

From the relationship $F_{V(t)} = F_{V(PS2)(t)}$ follows

$$\frac{F_V}{F_0} = \frac{F_V}{F_{0(PS2)}} \frac{F_{0(PS2)}}{F_0}$$

$$\downarrow \quad \downarrow \quad \downarrow$$

$$\frac{\varphi_{Po}}{1 - \varphi_{Po}} = \frac{\varphi_{Po(PS2)}}{1 - \varphi_{Po(PS2)}} r_0$$

and after rearrangement we find the two absorption fluxes, $ABS_{(total)}$ and $ABS_{(PS2)}$ of the pipe line model as follows:

$$\frac{\varphi_{Po(t)}}{\varphi_{Po(PS2)(t)}} = \frac{1 - \varphi_{Po(t)}}{1 - \varphi_{Po(PS2)(t)}} r_0 = r_M$$

$$\downarrow$$

$$\frac{TR_0/ABS_{(total)(t)}}{TR_0/ABS_{(PS2)(t)}} = \frac{ABS_{(PS2)(t)}}{ABS_{(total)(t)}} = \frac{1 - \varphi_{Po(t)}}{1 - \varphi_{Po(PS2)(t)}} r_0 = r_M \qquad \text{(Fig. 7)}$$

Appendix *C*

Vitality indexes

The product of the three independent parameters, RC/ABS, φ_{Po}, ψ_0 has been used as an expression which represents an index combining functional and structural criteria of PS2, denoted as structure function index for the reaction chain which is driven by primary photochemistry (SFI_{Po}) (Tsimilli-Michael *et al.* 1998). The non-photochemical or dissipation related parameters have been denoted as SFI_{No} (Strasser *et al.* 1999). On the basis of total chlorophyll (Chl) absorbed we can write:

$SFI_{Po(ABS)} = (Chl_{RC}/Chl_{tot})\ \varphi_{Po}\ \psi_0$ and accordingly

$SFI_{No(ABS)} = [1 - (Chl_{RC}/Chl_{tot}]\ (1 - \varphi_{Po})\ (1 - \psi_0)$

where Chl_{tot} refers to the total Chl *a* concentration ($Chl_{tot} = Chl_{antenna} + Chl_{RC}$).

The combination of both structure function indexes leads to the expression performance index, PI_{ABS} (Clark *et al.* 1999, Strasser *et al.* 1999). Based on the absorption of antenna Chls of PS2, labeled as $Chl_{tot} - Chl_{RC} = ABS$ we can denote as

$$PI_{ABS} = SFI_{Po}/SFI_{No}$$
$$= [Chl_{RC}/(Chl_{tot} - Chl_{RC})]\ [\varphi_{Po}/(1 - \varphi_{Po})]\ [\psi_0/(1 - \psi_0)]$$

or in terms of the expressions used in the JIP-test

$$= (RC/ABS)\ [\varphi_{Po}/(1 - \varphi_{Po})]\ [\psi_0/(1 - \psi_0)]$$

Based on the leaf area or active cross-section (CS) we define:

$$PI_{CS} = ABS/CS\ PI_{ABS} = (ABS/CS)\ (RC/ABS)\ [\varphi_{Po}/(1 - \varphi_{Po})]\ [\psi/(1 - \psi_0)]$$
$$= (RC/CS)\ [\varphi_{Po}/(1 - \varphi_{Po})]\ [\psi_0/(1 - \psi_0)]$$

After substitution of the biophysical expressions with the experimental signals the performance indexes can be calculated as follows (see Table 2):

$$PI_{ABS} = [(V_J/M_0)\ (F_V/F_M)]\ (F_V/F_0)\ [(1 - V_J)/V_J]$$
$$PI_{CS} = F_M\ [(V_J/M_0)(F_V/F_M)]\ (F_V/F_0)\ [(1 - V_J)/V_J]$$

According to the Nernst's equation the logPI can be defined as a photosynthetic driving force (DF) of the sample, which can be deconvoluted into partial, driving forces.

$$DF_{ABS} = \log(PI_{ABS}) = \log(RC/ABS) + \log[\varphi_{Po}/(1 - \varphi_{Po})] + \log[\psi_0/(1 - \psi_0)]$$
$DF_{CS(M)} = \log(PI_{CS(M)}) = \log F_M + \log(PI_{ABS})$ or
$DF_{CS(0)} = \log(PI_{CS(0)}) = \log F_0 + \log(PI_{ABS})$ not used in this paper.

The formulae defining the vitality indexes are included in the Table 2 sections V and VI, along with the formulae describing other parameters of the JIP-test.

Appendix *D*

The Overall Grouping Probability G (modified from Strasser 1981)

The grouping probability G was calculated from the experimental values $F_{50\mu s}$ for F_0, $F_{100\mu s}$, $F_{300\mu s}$, F_{2ms}, and F_M of the O-J-I-P fluorescence transient as follows:

$$G = \frac{W_E - W}{W\ (1 - W_E V_J)} \quad \frac{F_0}{(F_M - F_0)\ V_J}$$

where: $V_J = (F_{2ms} - F_{50\mu s})/(F_M - F_{50\mu s})$, $W = (F_{100\mu s} - F_{50\mu s})/(F_{2ms} - F_{50\mu s})$,
$W_E = 1 - [(F_{2ms} - F_{300\mu s})/(F_{2ms} - F_{50\mu s})]^{(100\mu s - 50\mu s)/(300\mu s - 50\mu s)}$,
$W_E = 1 - [(F_{2ms} - F_{300\mu s})/(F_{2ms} - F_{50\mu s})]^{1/5}$

For all the calculations $F_{0(PS2)}$ was used instead of F_0 which was measured at $F_{50\mu s}$. $F_{0(PS2)}$ was calculated according to Appendix *A*. For details, see Strasser and Tsimilli-Michael (1998) and Stirbet *et al.* (1998).

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