THE "OXYGEN CLOCK" IN GREENING PEA LEAVES AS PROBED BY THE PERIOD FOUR OSCILLATIONS IN THE FLUORESCENCE INTENSITY AT 50 μs AND 2 ms AFTER PRE-FLASHES DURING THE OJIP TRANSIENT

Govindjee[1], Alaka Srivastava[2], and Reto J. Strasser[2]
[1]UIUC, Urbana, IL 61801-3707, USA, [2]Lab.of Bioenergetics, University of Geneva, CH-1254, Jussy, Geneva, Switzerland, gov@uiuc.edu

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1. Introduction

Plants kept under IML (intermittent light), with 2 min light and 118 min dark cycles, contain active photosynthetic reaction centers and capacity to evolve oxygen, but are devoid of most of the Chl *a/b* antenna protein complexes (1-4). If the plants are grown under a one-ms light flash given every 15 min of darkness (FL), the chloroplasts of such leaves lack Hill activity and they do not show the Chl *a* variable fluorescence (5,6). Exposure of these flashed leaves to continuous light (CL) rapidly (within minutes) induces O_2 evolution. In a series of saturating light flashes, the amount of O_2 released exhibits a periodicity of four in mature plants (7). The development of the "oxygen clock" in greening pea leaves was probed by period 4 oscillation of the Chl *a* fluorescence yield (8,9) measured at 50 μs and 2 ms during the OJIP fluorescence rise (10). Changes in 77K emission spectra (11), and P700 absorbance changes, during the development of greening process, were also measured in order to characterize our system, and to compare our results with those of the others.

2. Material and Methods

Pea (*Pisum sativum*) plants were grown at 25°C either under IML cycles, i.e., 2 min of white light and 118 min dark (1) or kept directly under 1 ms saturating white light flashes given after every 15 min in darkness (FL) (5). The greening process was performed under CL (40 W Sunlux incandescent light, 40 μmol $m^{-2}s^{-1}$ at the leaf level). Room temperature Chl *a* fluorescence transients were measured on leaves attached to the plant by a fluorometer (plant efficiency analyser, PEA; Hansatech Ltd., King's Lynn, Norfolk, UK) (10). The 77 K fluorescence spectra were recorded by exciting the leaves with 633 nm HeNe laser (40 Wm^{-2}) (11). Oscillation in the Chl *a* fluorescence yield was determined according to B. Strasser (9) using the PEA fluorometer. Xenon lamp provided the single-turnover saturating flashes for the S-state transitions. Light induced absorption changes at 820 nm were measured with the Hansatech $P700^+$ measuring system in combination with the PEA head, replacing one 650 nm LED with a 820 nm LED. The Chl *a* content of the leaves was determined according to Porra et al.(12).

3. Results and Discussion

When IML grown plants are transferred to CL, the amount of several pigment protein complexes increases. This can be judged from 77K emission spectra (Fig.1), where F685

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is known to originate from CP-43; F696 from CP-47; F-720 from the PSI core (the combined inner antenna and the reaction center I); and F-730 from LHCI. With increasing CL, increases in the following pigment protein complexes are observed (Fig.1, Table 1). (a) the PSI core, as evidenced by increases in the ratio of F720/F688; (b) the PSII core, that includes CP-47, as evidenced by increases in F696/F688; (c) LHCI, as evidenced by greater increases in F730/FF688 over that in F720/F688; and (d) LHCIIb, as inferred from large increases in the ratio of F730 & F720 to F685 due to increased reabsorption of fluorescence with increased antenna size. The shift of the emission band from 720 nm to 730 nm is a clear indication of increases in LHC I during the greening process (also see ref. 3). The major change during the greening process in CL is an increase in the antenna size, but initially some increases in the amount of reaction centers also occur. The $P700^+$ also developed during the greening process. However, on the chlorophyll basis, the amount of $P700^+$ decreased due to the increased antenna size during the greening process (inset in Fig.1).

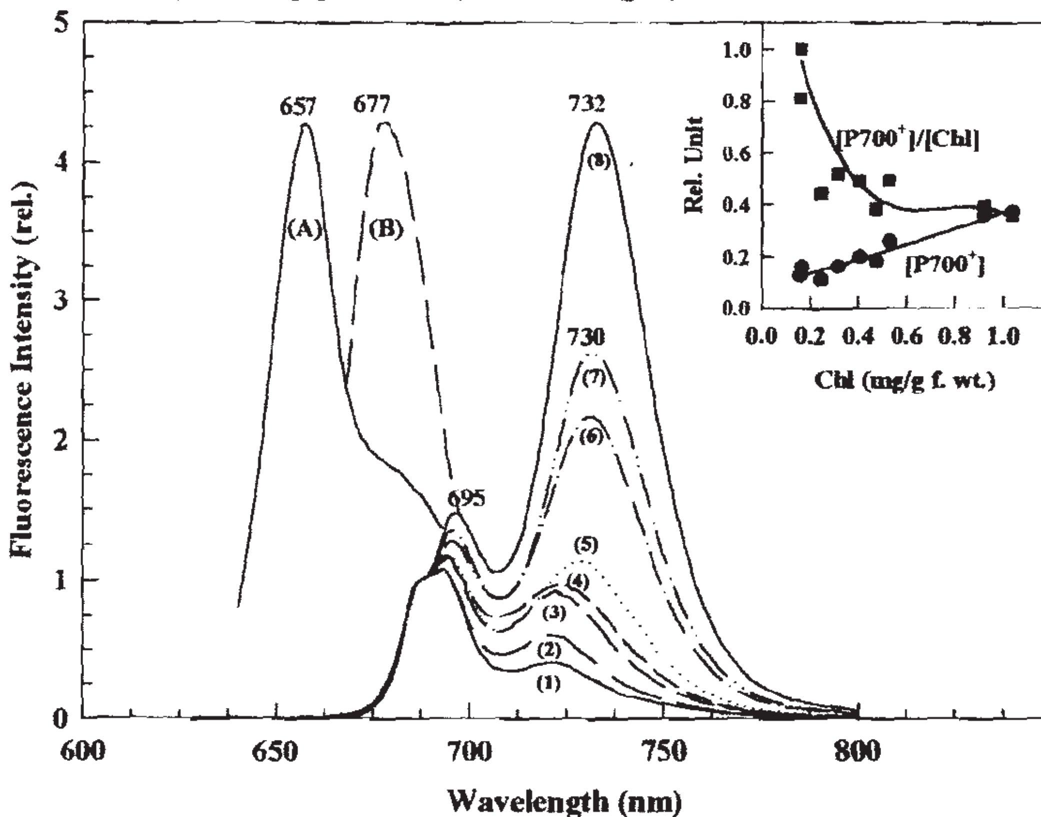

Figure 1. 77K emission spectra of pea leaves during greening in IML and CL. Leaves were dark adapted for 10 min before freezing. Data was normalized at 685 nm. From bottom to the top, spectra are for pea leaves exposed to (1) 24 h IML; (2) 48 h IML; (3) 48 h IML+ 1 h CL; (4) 48 h IML+ 2 h CL; (5) 48 h IML +4 h CL; (6) 48 h IML +6 h CL, (7) 48 h IML +8 h CL; (8) 48 h IML +48 h CL. The trace A (peak, 657 nm, protochlorophyllide) is obtained from etiolated leaf and the trace B (peak, 677 nm, chlorophyllide) is also from etiolated leaf but transiently exposed to weak light. The inset shows the development of $P700^+$ during greening.

Plants grown in IML showed variable Chl *a* fluorescence, with F_M/F_0 of ~ 2.5; after transferring them into CL, a progressive increase in this ratio was observed (upto F_M/F_O of ~5.0; Fig. 2A). The F_V/F_M ratio increased from 0.59 to 0.79 (Table 1). J-M.Briantais (unpublished) has shown that the F_O of IML leaves contains a long lifetime fluorescence component, that may originate from disconnected Chl-protein complexes, and if Fo is corrected for that component, then the F_M/corrected F_O is already high in the IML leaves.This means that the quantum yield of photochemistry is already high, and more of PSII antenna (both core PSII and LHCIIb/PSII) are added during CL exposure. As reported recently (13), insignificant difference in the V_J (=$(F_J-F_0)/(F_M-F_0)$) level was observed, but about 40% increase in the V_I (=$(F_I-F_0)/(F_M-F_0)$) level was recorded during the greening process (Table 1). FL grown plants did not show any variable Chl *a* fluorescence (0 min CL, Fig. 2C), but when they were transferred to CL, the variable

fluorescence could be induced within 10 min (Fig. 2C, and ref. 6). However, the F_M/F_O after 60 min CL was equivalent to that obtained in 48 hour IML plants. In all likelihood, the antenna size was still small.

Table 1. Chl content; fluorescence ratios, at 77K, at different λs; and room temperature fluorescence parameters during the greening process of pea leaves in CL after 48 h of IML. The sixth vertical column lists the peak of the long wavelength fluorescence band.

Time in CL (hour)	[Chl] μg/g f. wt.	F696 nm / F688 nm	F720nm / F688 nm	F730nm / F688 nm	Peak Wavelength	F_V/F_M	V_J	V_I
0	162	0.972	0.597	0.456	721.0	0.59	0.50	0.63
2	245	1.106	0.931	0.872	722.8	0.61	0.49	0.64
4	404	1.149	0.949	1.133	729.0	0.67	0.55	0.66
6	471	1.356	1.491	2.158	731.0	0.75	0.55	0.75
8	527	1.275	1.622	2.602	731.0	0.77	0.55	0.75
48	1038	1.473	2.307	4.189	732.4	0.79	0.48	0.87

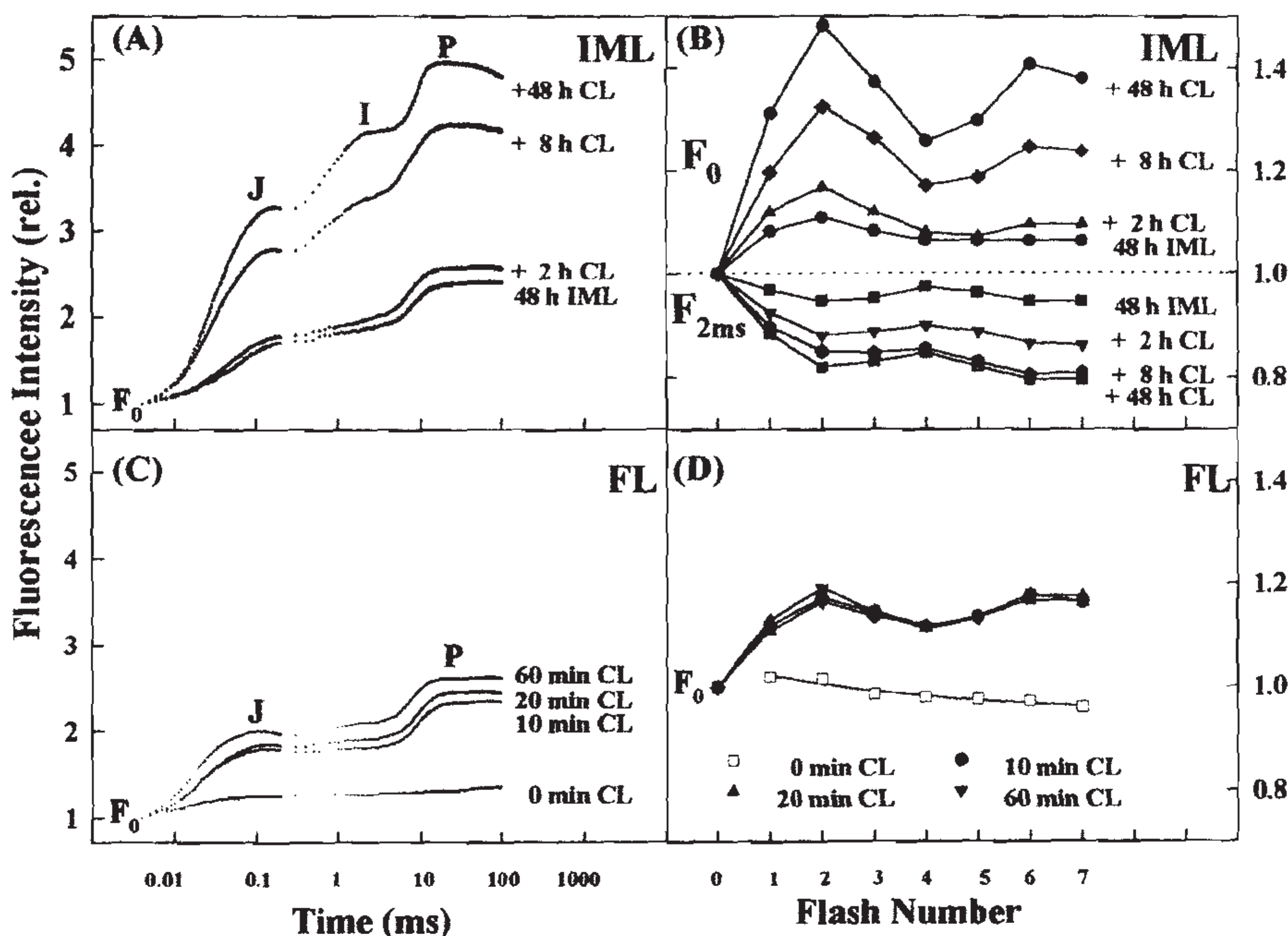

Figure 2. Left panel shows the room temperature Chl *a* fluorescence induction kinetics (OJIP) of pea leaves during greening under CL. Plants were grown either in IML (A) or under FL (C) before being transferred to CL. Right panel shows the oscillation of four as a function of flash number (flashes given prior to the OJIP Chl *a* fluorescence rise) at F_0 (50 μs) (B,D) and F_J (2 ms) (B) during the greening process.

Once the characteristics of the greening samples were established, we measured the development of the "oxygen clock" by measuring oscillations in F_0 and F_J as a function of pre-flashes (9). The first phase of the OJIP transient is distinctly affected by the pre-flash number and the changes in F_0 and F_J follow the period 4 oscillation (Fig. 2B, D). In contrast to O_2 evolution where maxima are at flashes 3 and 7, F_0 shows maxima of the oscillation after 2nd and 6th flashes and minima after 0, 4 and 8 flashes. The amplitude of the ratio of the fluorescence intensity after the 2nd to the 4th flash increases with increasing greening time. This correlates with the increased Hill reaction during greening of the plants (2). The J level had a period 4 oscillation, however, in the opposite direction (also see Strasser & Strasser, these proceedings). When the plants are kept under FL regime, the period four oscillation (0 min CL, Fig. 2D) is absent indicating the lack of O_2 evolution (5,6); this, however, could be recovered within few minutes after CL (Fig. 2D).

Concluding Remark We show here that the non-invasive intrinsic probe of Chl *a* fluorescence (the OJIP transient, 10), and the period 4 flash number dependence (8,9) of F_0 and F_J provide a new and sensitive method for measuring the dynamic properties of the oxygen evolving complex (OEC) *in vivo*. Further application of the analysis of the kinetics of fluorescence decay, after single flashes of light, that can monitor the kinetics of the S_3 to S_0 (14) is also recommended for *in vivo* systems.

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