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Resolution of the Photosystem I and Photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature

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Abstract

Green leaves illuminated with photosynthetically active light emit red fluorescence, whose time-dependent intensity variations reflect photosynthetic electron transport (the Kautsky effect). Usually, fluorescence variations are discussed by considering only the contribution of PSII-associated chlorophyll *a*, although it is known that the fluorescence of PSI-associated chlorophyll *a* also contributes to the total fluorescence [Aust. J. Plant Physiol. 22 (1995) 131]. Because the fluorescence emitted by each photosystem cannot be measured separately by selecting the emission wavelength in in vivo conditions, the contribution of PSI to total fluorescence at room temperature is still in ambiguity. By using a diode array detector, we measured fluorescence emission spectra corresponding to the minimal (F_O) and maximal (F_M) fluorescence states. We showed that the different shapes of these spectra were mainly due to a higher contribution of PSI chlorophylls in the F_O spectrum. By exciting PSI preferentially, we recorded a reference PSI emission spectrum in the near far-red region. From the F_O and F_M spectra and from this PSI reference spectrum, we derived specific PSI and PSII emission spectra in both the F_O and F_M states. This enables to estimate true value of the relative variable fluorescence of PSII, which was underestimated in previous works. Accurate separation of PSI–PSII fluorescence emission spectra will also enable further investigations of the distribution of excitation energy between PSI and PSII under in vivo conditions.

Keywords: Chlorophyll fluorescence; Energy distribution; PSI; PSII

1. Introduction

In 1931, Kautsky and Hirsch [1] reported for the first time that the intensity of the red fluorescence emitted by Chl varied during exposure of intact leaves to strong blue light after a short period of adaptation to darkness. This effect, known as 'Kautsky effect' or 'variable fluorescence', was studied extensively in the following decades (reviewed in Ref. [2]). In 1963, Duysens and Sweers [3] formulated the 'quencher' hypothesis, which remains today the basic frame of interpretation of variable fluorescence in relation to photosynthetic activity. According to the modern formulation of this hypothesis, the intensity of Chl fluorescence is affected by the redox state of the electron carrier Q_A, a plastoquinone that undergoes a one-electron photoreduction in the PSII RC. In the light, the energy of antenna Chl excited states is trapped by the P680 Chl special pair of RC and Q_A is reduced by excited P680 via a pheophytin molecule. Photochemical reactions in the RC, leading to Q_A reduction, compete with the dissipation of Chl excited state energy through fluorescence emission. Therefore, reaction centres with oxidised Q_A quench efficiently the fluorescence of PSII Chls, whereas those with reduced Q_A do not (reviewed in Ref. [4]). On a time scale of less than one second, continuous actinic light causes an increase of the fluorescence intensity from a low (F_O) to a high (F_M) level due mainly to the process of Q_A photoreduction. Additional factors such as the redox state of secondary plastoquinone acceptors also influence to a lesser extent the fluorescence yield in this period [5]. During longer exposure to light, Chl fluorescence slowly declines in response to Q_A reoxidation.

Nowadays, Chl fluorescence measurements are extensively used as a noninvasive method to investigate plant

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stress induced by high photon flux, heat, drought or various pollutants, which affect the efficiency of the photosynthetic apparatus [6,7]. The F_V/F_M ratio, or relative variable fluorescence where $F_V = F_M - F_O$, is widely used as an indirect estimation of the PSII photochemical yield ('relative PSII efficiency', [8]). In most cases, fluorescence is measured in a fixed wavelength interval that is selected by optical filters in order not to overlap with the emission spectrum of the excitation light source. In most available commercial devices, fluorescence is measured either in a narrow wavelength window centered at 690 nm or through a high-pass filter which transmits only the fluorescence emitted beyond 710 nm. It is known since early studies [9,10], however, that the wavelength dependence of the fluorescence emitted at room temperature is complex. In leaves of higher plants, two main emission bands occur at 685 and 735–740 nm [6]. It is generally assumed that most of this fluorescence arises from PS II Chls, with the 685 nm band arising from the main transitions and the 735–740 nm band arising from vibrational sublevels whose relative intensities are increased in vivo through self-absorption in the shorter wavelength region. Some fluorescence of PSI Chls also contributes to the fluorescence emitted at physiological temperatures [4,8]. Its spectrum strongly overlaps with the one of PSII fluorescence, but it is generally admitted that the relative intensity of PSI emission is highest around 720 nm [9,10]. It is, however, impossible to ascribe specific emission bands to one or the other photosystem. Only at low temperature (77 K) can distinct PSI and PSII emission bands be resolved [2,11]. The complete emission spectra of PSI and PSII in vivo at room temperature are still unknown and the relative contribution of each photosystem to total fluorescence could not be measured quantitatively until now. In contrast to the general assumption that PSI fluorescence is very low compared to the one of PSII, indirect evidence was presented that the contribution of PSI fluorescence could be of the order of 30% in F_O measured at wavelengths higher than 700 nm in C3 plants [12].

Whatever the degree to which PSI Chl contribute to the fluorescence measured at physiological temperatures, there is little doubt that the rapid fluorescence rise at the onset of actinic illumination is essentially caused by electron transport within the PSII RC [4,8,11]. PSI shows little change in fluorescence intensity in response to trap closure, most likely due to effective quenching by $P700^+$ [4]. Therefore, if PSI emitted significant fluorescence in vivo, the intensity of this fluorescence would be affected only indirectly by increased excitation migration from PSII to PSI upon Q_A photoreduction (a phenomenon termed 'spill-over', [13]) and, to a minor extent, by increased reabsorption of PSII fluorescence by PSI.

A substantial PSI contribution to Chl fluorescence in vivo would have consequences on the significance of the F_V/F_M ratio. In such hypothesis, the F_V/F_M ratio would not simply reflect relative PSII efficiency since it would depend also on the relative contribution of PSI fluorescence in F_O and F_M, which would in turn depend on energy distribution and self-absorption.

Previous studies on the spectral dependence of variable fluorescence have shown that the F_V/F_M ratio is lower when measured in the long wavelength ($\lambda > 700$ nm) rather than in the short-wavelength ($\lambda < 700$ nm) Chl emission band at room temperature [14,15]. Here we have reinvestigated the wavelength dependency of variable fluorescence in the 650–850 nm interval by measuring individual F_O and F_M spectra in intact leaves using a sensitive diode array detector. From these results and from independent measurements of PSI fluorescence, we could derive distinct in vivo PSI and PSII fluorescence emission spectra related to the F_O and F_M states at room temperature. We discuss how PSII efficiency measurements are affected by the substantial contribution of PSI to total fluorescence. The approach developed here to derive PSI and PSII fluorescence spectra during the fluorescence induction process is of potential interest for a noninvasive, direct assessment of the energy distribution between PSI and PSII in vivo.

2. Materials and methods

Green barley leaves were obtained by growing the plants for 7 days in a growth cabinet at a temperature of 23 °C and light intensity of 200 μmol m^{-2} s^{-1}.

Chloroplasts were extracted according to Ref. [16] and PSII particles were isolated according to Ref. [17], except that the second Triton X-100 solubilization step was omitted.

Fluorescence emission spectra were measured from the upper leaf side or from particles suspensions placed in a 1 mm glass cuvette. The exciting light was provided by a stabilized Oriel light source. It was filtered through a combination of broad blue filters (Corning CS4-96 and CS5-59) with maximal transmission at 446 nm and half-band-width of 90 nm, and focused on the leaf sample to produce an actinic light intensity of 560 μmol m^{-2} s^{-1} used for F_M measurements. For the F_O measurements, the exciting light intensity was decreased to 0.18 μmol m^{-2} s^{-1} by neutral filters. Fluorescence was measured at right angle with the excitation light path using an optical spectrometric multichannel analyzer (OSMA, Princeton Instruments) equipped with a 1024 channels intensified diode array detector and a SPEX Model 1681 grating monochromator. A high-pass red filter (maximal transmission at 620 nm) was used to protect the detector from blue stray light. The slit-width was 0.4 mm, which allowed a spectral resolution of 3 nm. Spectra were corrected for a baseline and for the diode-dependent response of the measuring system. The linearity of the detector response as a function of incoming fluorescence intensity was verified using a Chl solution in methanol. Wavelength calibration was performed with an Oriel neon light source (#6032). Monitoring of the fluorescence yield during spectral measurements was done using

a Pulse-Amplitude-Modulated fluorometer (FMS/2S, Hansatech Instruments Ltd.).

In order to measure the PSI fluorescence spectrum at wavelength higher than 730 nm in the diode-array system, PSI was excited by placing in front of the Oriel light source a combination of two narrow interference filters (Spindler & Hoyer) resulting in a transmission maximum at 708 nm and half-band width of 8 nm. The photon flux was about 35 μmol m^{-2} s^{-1} in these conditions. Steady-state fluorescence emission spectra under different excitation wavelengths were measured using a conventional fluorometer (LS50-B, Perkin-Elmer) with excitation and emission slits of 8 and 5 nm, respectively. Additional interference and cut-off filters at excitation and emission sides were used to lower stray light.

3. Results

F_O and F_M spectra of dark-adapted barley leaves were measured under analytical or actinic blue excitation light. Fig. 1 shows the time course of a typical experiment, in which changes in fluorescence yield were monitored with a PAM fluorometer during spectra measurements. The spectrum of the fluorescence emitted by dark-adapted leaves at the F_O state was measured by summing 500 scans (duration of each scan: 0.1 s) recorded in series under an analytical light of 0.18 μmol m^{-2} s^{-1}, which did not cause significant change in the fluorescence yield. After switching off this light, neutral filters in front of the excitation source were removed and the actinic light of 560 μmol m^{-2} s^{-1} was triggered. During the first second of this illumination, a fluorescence rise to a level close to the F_M measured upon

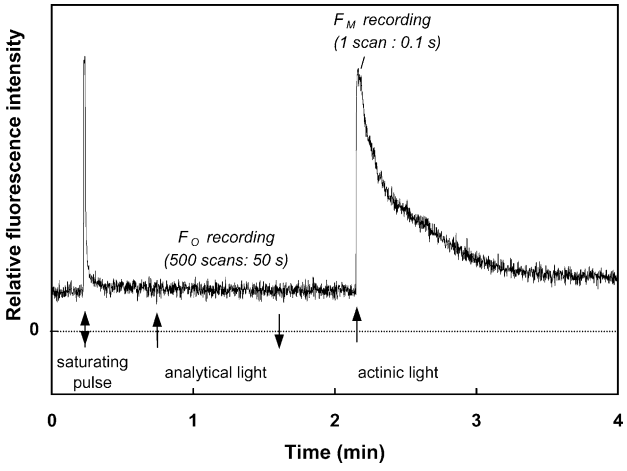

Fig. 1. Time course of a typical experiment in which individual F_O and F_M spectra were recorded in dark-adapted barley leaves. The onset and offset of analytical and actinic blue light (450 nm) used for excitation during the recordings of F_O and F_M spectra are indicated by upward and downward arrows. The changes in fluorescence yield were simultaneously monitored with a PAM fluorimeter, which provided an additional, modulated analytical light at 580 nm and the initial saturating light pulse.

a saturating pulse occurred. The onset of actinic light was synchronized with the recording of 15 spectra of 0.1 s each. The 10th spectrum of the series had the highest intensity and was taken as the F_M spectrum. To compare the relative intensities of the F_O and F_M spectra on a constant excitation basis, the F_O spectrum was divided by the transmission of the neutral filters and by the number of scans. For dark-adapted barley leaves, an average of five experiments of this kind gave the F_O and F_M emission spectra of Fig. 2A. Both spectra showed a maximum at 684 nm and a broad shoulder around 735 nm. The relative intensity of the latter was higher in the F_O than in the F_M spectrum. Weak shoulders around 700 and 720 nm were also detected. They were more clearly visible in the F_O spectrum. As a result of the different shapes of the F_M and F_O spectra, the F_M/F_O and $(F_M - F_O)/F_M$ ratios were strongly dependent on emission wavelength (Fig. 2B,C). Two maxima were observed at 683 and 742 nm, whereas minima occurred at 723 nm and at very long wavelength (beyond 780 nm, not shown).

The differences in the shapes of the F_O and F_M spectra, and the resulting wavelength dependence of the amplitude of relative variable fluorescence, may have in principle two nonexclusive causes. They may originate from spectral heterogeneity resulting in different relative contributions of PSI and PSII in the F_O and F_M spectra. They may also be due to changes in the shape of the fluorescence spectrum of each photosystem during the fluorescence rise from F_O to F_M. In order to distinguish between these two possibilities, we recorded the F_O and F_M spectra of isolated PSII particles from barley. Diluted PSII particles showed a main emission band at 683 nm and a shoulder at 740 nm, which most probably originated from a vibrational sublevel. When increasing the Chl concentration, a slight red shift of the main band occurred and the relative amplitude of the 740 nm side band developed as a true band due to self-absorption (Fig. 3). In the isolated PSII particles the variations of the F_M/F_O ratio as a function of wavelength were not significant compared to those of the intact leaves (Fig. 3, inset). This makes it very unlikely that the wavelength-dependent changes in the amplitude of variable fluorescence observed in the leaf are due to heterogeneous excitation distribution within Chls of PSII with closed or open RC. On the other hand, the intensity of PSI emission should increase much less than the one of PSII emission during the fluorescence rise and the PSI spectrum is not expected to change since fast equilibration of excited states in this photosystem [18] ensures that its emission spectrum is independent of the excitation conditions (either direct excitation or excitation through spill-over). Therefore, the differences between the F_O and F_M spectra of intact leaves must essentially reflect different relative contributions of PSI and PSII fluorescence in the two spectra.

We next tried to decompose the F_O and F_M spectra into their respective PSI and PSII components. For this purpose, we hypothesized that, at each wavelength λ, the F_O

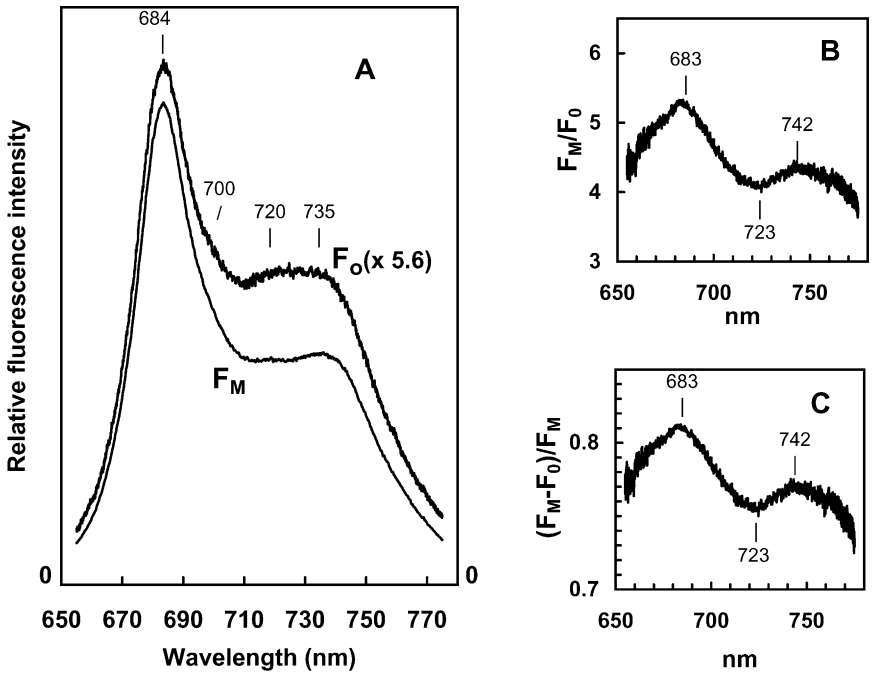

Fig. 2. (A) Comparison of the room temperature fluorescence emission spectra of dark-adapted barley leaves in the F_O and F_M states (note the magnification factor used for F_O). (B) Wavelength dependence of the F_M/F_O ratio. (C) Wavelength dependence of the relative variable fluorescence $(F_M - F_O)/F_M$.

fluorescence is the sum of the fluorescence intensities $F^{\lambda}_{O,I}$ and $F^{\lambda}_{O,II}$, emitted by PSI and PSII Chl:

$$F^{\lambda}_{O} = F^{\lambda}_{O,I} + F^{\lambda}_{O,II} \quad (1)$$

If one admits that the spectrum of F_M is the sum of PSI and PSII spectra with unchanged spectral shapes compared to

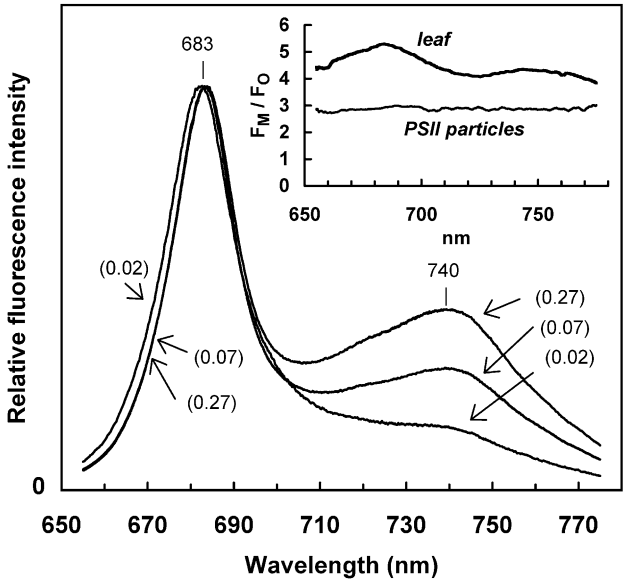

Fig. 3. Room temperature fluorescence emission spectra of increasingly concentrated suspensions of isolated PSII particles (Chl concentrations: 0.02, 0.07 and 0.27 mg ml^{-1}) measured at the F_M state. The spectra were normalized at their maximum. Inset: comparison of the wavelength-dependence of the F_M/F_O ratio in intact leaves and in PSII particles (0.27 mg ml^{-1}).

F_O, but whose respective intensities are increased by respective factors k_I and k_{II}, one can write:

$$F^{\lambda}_{M} = k_I F^{\lambda}_{O,I} + k_{II} F^{\lambda}_{O,II} \quad (2)$$

Eqs. (1) and (2) allow to calculate the PSI and PSII emission spectra from the experimental F_O and F_M, provided that k_I and k_{II} are known. In principle, these constants could be measured from the F_O and F_M spectra if there were specific emission wavelengths for PSI and PSII. This, however, is not the case since the PSI and PSII emission spectra overlap over the entire spectral range.

The value of k_{II} could be measured by an indirect experimental approach. By rearranging Eqs. (1) and (2), a relationship between the experimental F_O and F_M spectra and the spectrum of PSI in F_O was obtained:

$$k_{II} F^{\lambda}_{O} - F^{\lambda}_{M} = F^{\lambda}_{O,I}(k_{II} - k_I) \quad (3)$$

Eq. (3) could be used to determine k_{II}. According to this equation, if the correct value of k_{II} is combined with the experimental F_O and F_M spectra, the calculated spectrum of $[k_{II}F_O - F_M]$ should have the wavelength distribution of a typical PSI emission spectrum at room temperature. In order to test different k_{II} values according to this criterion, it was first necessary to obtain a reference PSI spectrum. Such reference spectrum was obtained at wavelengths higher than 730 nm by measuring the fluorescence emission of the same leaves under 708 nm excitation with the diode array detector. As shown in Fig. 4A, this spectrum differed significantly from the one measured under blue excitation of low intensity (F_O). We verified that the fluorescence emitted

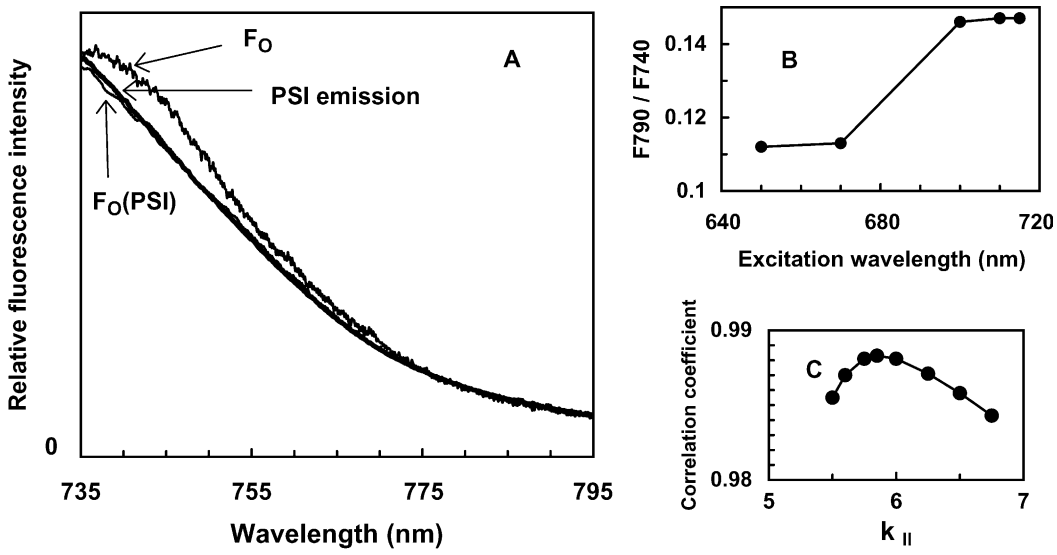

Fig. 4. (A) Comparison of the reference PSI fluorescence emission spectrum obtained with barley leaves under 708 nm excitation with the F_O spectrum under blue excitation and with the calculated $[k_{II}F_O^{\lambda} - F_M^{\lambda}]$ spectrum when $k_{II} = 5.85$ (all spectra were normalized at 735 nm). (B) Excitation wavelength dependence of the $F790/F740$ fluorescence emission ratio measured with a conventional fluorometer. (C) Effect of k_{II} on the correlation between the $[k_{II}F_O^{\lambda} - F_M^{\lambda}]$ spectrum and the PSI reference spectrum.

under 708 nm excitation originated preferentially from PSI by recording emission spectra under weak light of different excitation wavelengths from 650 to 730 nm using a steady-state fluorometer. We measured the $F790/F740$ ratio in these spectra to monitor the shape of the spectra as a function of excitation wavelength (Fig. 4B). This ratio increased abruptly between 680 and 700 nm and remained constant at higher excitation wavelengths. This indicated that light beyond 700 nm excited PSI preferentially. Evidence for this statement can also be found in earlier studies. A curve qualitatively similar to the one in Fig. 4B was found earlier when measuring the excitation partition coefficient between the two photosystems as a function of excitation wavelength at 77 K [19]. The proportion of light absorbed directly by PSI was close to one at 700 nm. At room temperature, action spectra of PSI and PSII activities of isolated chloroplasts indicated that around 710 nm PSII excitation was very low compared to PSI excitation [20,21]. For each leaf sample, we therefore used the emission spectrum under 708 nm excitation as a reference PSI emission spectrum at wavelengths higher than 730 nm to evaluate the k_{II} constant from Eq. (3). The spectrum $[k_{II}F_O^{\lambda} - F_M^{\lambda}]$ obtained with different k_{II} values was compared to the reference PSI spectrum and the correlation coefficient between the two spectra was calculated. As shown in Fig. 4C, this procedure allowed to determine the value of k_{II}. The average k_{II} value found by this method was 5.85 ± 0.45. The spectrum $[k_{II}F_O^{\lambda} - F_M^{\lambda}]$, which, according to Eq. (3), has the same shape as the $F_{O,I}^{\lambda}$ spectrum, was then identical to the reference PSI spectrum after normalization (Fig. 4A).

Since there is no excitation wavelength specific of PSII, the approach used for k_{II} determination could not be applied to determine k_I, the factor by which PSI fluorescence is enhanced during the rise from F_O to F_M under blue light excitation. Spill-over dependent rise of PSI fluorescence under actinic illumination was reported when dark-adapted leaves were illuminated at 77 K [12,22,23]. At this temperature, the intense PSI emission at 735 nm is distinct from the PSII emission bands at 685 and 695 nm. Under saturating light, it was found to increase by a factor of 1.4 in intact leaves of *Nerium oleander* [22] as well as in spinach chloroplasts suspended in high salt medium [13]. This value can be regarded as a maximum k_I value in dark-adapted samples since, even at 77 K, PSII contributes to long-wavelength fluorescence [11] and even a low contribution can give rise to significant variable fluorescence due to the high k_{II} value. On the other hand, equivalent measurements cannot be performed at room temperature since the PSI and PSII fluorescence bands strongly overlap. In the following calculations, we have first considered that PSI fluorescence represents a constant contribution ($k_I = 1$) to the total fluorescence during the rise from F_O to F_M. The effect of k_I being possibly somewhat higher (between 1 and 1.4) has then been evaluated.

By taking k_I equal to 1 and introducing the calculated value of k_{II} in Eq. (2), we derived the PSI and PSII fluorescence spectra in F_O and in F_M for barley leaves over a wavelength range from 650 to 850 nm. This could be done by combining measurements performed using two different monochromator positions. The resulting spectra are shown in Fig. 5. The PSI spectrum showed a maximum at 722 nm and a shoulder around 680 nm, while PSII had a main emission band at 684 nm and a side-band at 738 nm. As expected, the contribution of PSI was much higher in F_O than in F_M. In F_O, it was close to 40% at its emission

maximum at 722 nm. The wavelength-dependence of the PSI contribution to fluorescence is shown in Fig. 6 for F_O, F_M and F_V ($=F_M-F_O$). It was the lowest at 684 nm (position of PSII emission maximum), showed a first maximum at 722 nm (position of the PSI emission maximum) and increased to high values (close to 60% in F_O) in the far-red. The decrease from 660 to 685 nm may be due to a broader PSI emission in this region, but it may also arise from uncoupled Chl with constant fluorescence or from a minor PSII contribution due to residual PSII fluorescence in the reference spectrum taken for PSI (see Discussion).

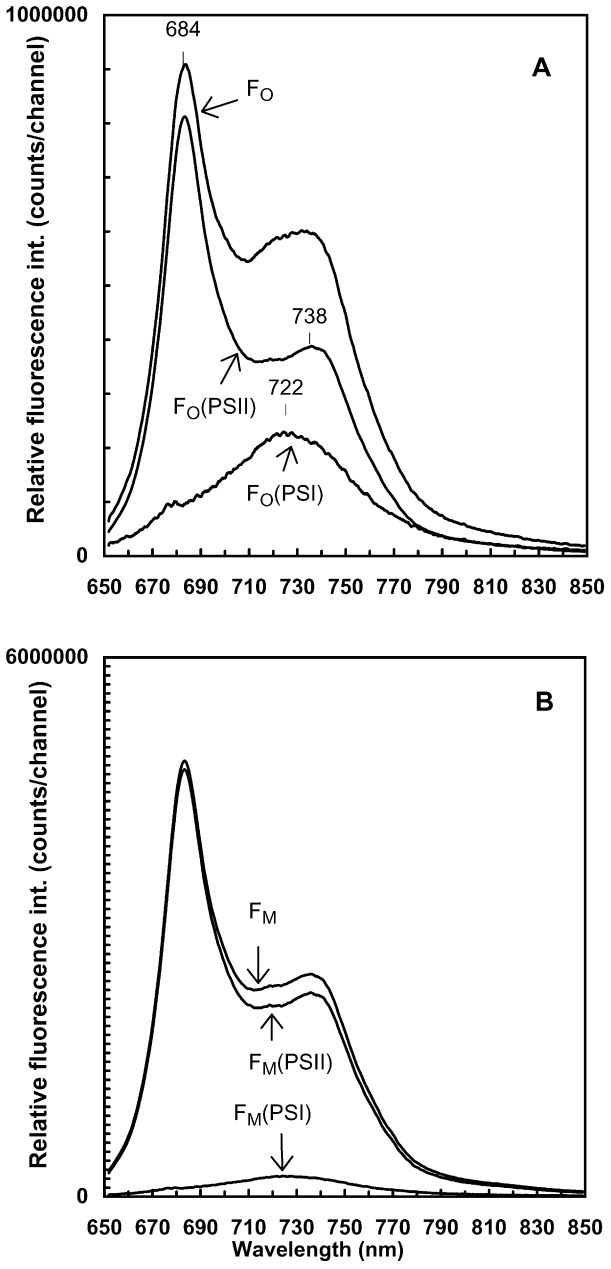

Fig. 5. Resolution of the PSI and PSII fluorescence emission spectra in F_O (A) and in F_M (B) in barley leaves ($k_I=1$, $k_{II}=5.85$).

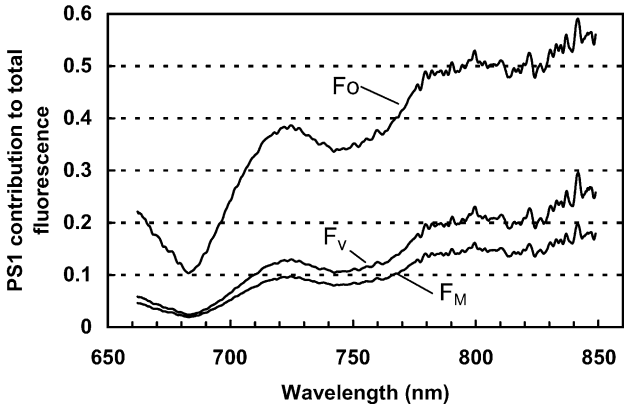

Fig. 6. Relative contribution of PSI fluorescence to F_O, F_M and F_V as a function of emission wavelength in barley leaves ($k_I=1$, $k_{II}=5.85$).

The shape of the PSI and PSII spectra were independent of the assumed k_I value. On the other hand, when a maximum k_I value of 1.4 was assumed instead of 1, only minor effects on the relative amplitudes of the PSI and PSII spectra were found. The contribution of PSI to F_O at 722 nm was increased from 38% to 41%. In F_M, the contribution remained weak (14% instead of 10% at 722 nm). Thus, the estimated PSI contribution was only weakly sensitive to the assumptions made regarding the extent of spill-over.

The same experiments as those reported here for barley were performed also with leaves of geranium and *Lemna gibba*. The results were qualitatively similar but the value of k_{II} was species-dependent. *L. gibba* behaved similarly to barley but geranium leaves, which had a higher Chl content, showed higher contribution of PSI fluorescence to the emission spectra (data not shown). This was most probably related to stronger self-absorption caused by their higher Chl content (70% higher than in barley on a surface area basis).

4. Discussion

The results obtained here confirm that the amplitude of the relative variable fluorescence (defined as $(F_M-F_O)/F_M=F_V/F_M$) depends significantly on the emission wavelength in vivo [9,10]. We have interpreted this dependence as being due to unequal ratios of PSI and PSII fluorescence in the F_O and F_M spectra. This interpretation is supported by measurements made on isolated PSII particles, which did not show significant wavelength dependence of their variable fluorescence. It is noteworthy that in a previous study [24], slight wavelength-dependent changes of the F_M/F_O ratio were reported for PSII particles. What we emphasize here is that the amplitude of such variations are negligible, compared to those found in intact leaves.

Our further quantitative treatment of the data is based on a few principles. First, it is assumed that the shapes of the fluorescence spectra of PSII and PSI are constant during the rise from F_O to F_M. We showed experimental evidence of

this for PSII. We did not consider the possible effects of PSII heterogeneity [25] but, as discussed already in Ref. [12], most of the PSII fluorescence in vivo is expected to arise from the predominant PSII type (PSIIα). Another implicit assumption of our model is that the PSI fluorescence spectrum measured beyond 730 nm under specific 708 nm excitation can be taken as reference to fit the PSI emission spectrum under the 450 nm excitation used as actinic light. Again, this is expected as a consequence of fast equilibration of the Chls excited at 700 nm with the bulk of PSI Chls [18,26].

At wavelengths greater than 700 nm, the shape of the in vivo PSI emission spectrum derived from the F_O and F_M spectra is similar to published spectra of PSI particles isolated by mild solubilisation of thylakoids [26]. This is good confirmation of the validity of our procedure to derive the PSI spectrum. This spectrum is very broad, with a maximum around 720 nm. On the long wavelength side, it extends well beyond 800 nm, where the PSII emission is very low. In this region, PSI contributes by almost 60% to the total fluorescence in F_O. On the short wavelength side, a weak shoulder at 680 nm is found in the calculated PSI spectrum. The spectrum of isolated PSI shows no distinct shoulder at this wavelength, but has a shoulder at 690 nm instead [26]. It is not impossible that the weak 680 nm shoulder found here belongs in fact to PSII, because the reference PSI spectrum under 708 nm excitation may still include a minor PSII contribution. An alternative explanation for the presence of this shoulder could also be that minor amounts of weakly coupled Chl emit constant fluorescence in this region. In such case, this fluorescence would be included in PSI by our procedure. It is noteworthy, however, that the isolated PSII particles did not show significant decrease of relative variable fluorescence in the short wavelength region. Hence, in the hypothesis that uncoupled Chl emitting at 680 nm occurs in vivo, it must have been lost during the isolation of PSII particles.

The calculated in vivo spectrum of PSII has its maximum at 684 nm and a side band around 740 nm, the relative intensity of which must depend on self-absorption in the leaf. The addition of this band and of the 722 nm band of PSI explains the broad emission of the leaf fluorescence in this spectral region already reported earlier [15].

The relatively high contribution of PSI to the leaf fluorescence found here is in line with estimations obtained by other experimental approaches. Fluorescence lifetimes studies with pea chloroplasts indicated that the contribution of PSI fluorescence in the 730 nm region could be as high as 30% at F_O [4,27]. We find a value close to 40% at that wavelength (Fig. 6). PSI contribution at room temperature was also estimated indirectly on the basis of a linear relationship between the intensity of PSI fluorescence at 77 K and the variable fluorescence measured at room temperature beyond 720 nm in leaves [12]. In that study, a contribution of about 30% at wavelengths higher than 700 nm was estimated.

The consequences of a high PSI contribution to the fluorescence of intact leaves are of two kinds. The first consequence is a significant underestimation of the relative PSII efficiency (estimated as F_V/F_M), especially when the fluorescence is measured in the region of the PSI band at 720 nm or at wavelengths greater than 780 nm. From our measurements, we calculated the apparent PSII efficiency as the relative variable fluorescence emission from PSII:

$$\phi_{(PSII)} = (F_{M,II} - F_{O,II})/F_{M,II} = 1 - 1/k_{II} \quad (4)$$

According to this evaluation, $\phi_{(PSII)}$ was 0.83 in barley. This value is significantly higher than the values of 0.81 or 0.75 obtained from the F_V/F_M measured at 685 or 720 nm, respectively (Fig. 2C). Estimations of $\phi_{(PSII)}$ on the basis of variable fluorescence measured at wavelength higher than 710 nm (as with several commonly used commercial fluorometers) are therefore significantly biased due to PSI contribution to the measured fluorescence.

The second, and perhaps more significant, consequence of the PSI contribution to fluorescence is that any alteration of the F_V/F_M ratio observed upon changing experimental conditions can reflect either a change in PSII efficiency (as usually considered), or a change in the relative PSI fluorescence intensity caused by changes in the distribution of excitation energy between the two photosystems (state transitions). Since, as this study has clearly shown, PSI and PSII fluorescence spectra overlap over the entire spectral region of Chl emission, methods based on simultaneous detection at two wavelengths [28] can give indications on changes in the total emission intensities due to the two photosystems, but do not provide true measurements of the specific contribution of each photosystem. With the approach developed in this study, it should be possible in the future to measure quantitatively how various factors affect energy distribution between the two photosystems at room temperature in vivo by analyzing the emission spectra in terms of PSI and PSII contributions.

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