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Spectroscopic investigations of fluorescence behaviour, role and function of the long-wavelength pigments of Photosystem I

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A method was established for quantitative evaluation of fluorescence spectra of intact leaves without expensive sample preparation. Fluorescence induction and excitation effects were used to evaluate the contribution of Photosystem I to the long-wavelength fluorescence of intact leaves at room temperature which appeared to be about 25%. To characterize its spectral line-shape we studied low-temperature fluorescence. The connection of these three methods made it possible to determine the quantitative spectra of both photosystems in intact leaves. Investigations of fluorescence decay kinetics on isolated Photosystem I subchloroplast particles provided information about functional aspects of the long-wavelength pericentral antenna pigments. It appeared that these pericentral pigments are not essential for transferring excitation energy toward photochemically active reaction centres. Only if the reaction centres were closed the pericentral antenna would act as a protection guard against photodestruction.

Introduction

The photosynthetic apparatus of green plants contains a number of functionally different pigment-protein complexes which absorb light and deliver excitation energy to the reaction centres (RCs) in which the photochemical separation of charges occurs. The spectral characteristics of the pigments are sensitive and informative attributes which can provide useful information for understanding the nature of the photosynthetic processes.

The fluorescence spectra of in vivo chlorophyll at different temperatures were studied. The primary concern was the determination of individual bands in the overall spectrum with the aim of identifying which bands belong to which chlorophyll species among the light-harvesting antenna (LHA), Photosystem I (PS I) or Photosystem II (PS II). It is widely believed now that fluorescence at 680 nm (F_{680}) is emitted by the LHA [1–3], fluorescence at 685 nm (F_{685}) belongs to the D_1-D_2-cytochrome *b*-559 complex which is the reaction centre of PS II [4] and fluorescence at 696 nm to the antenna of PS II, presumable to the CP47-complex [4], and to the antenna core of PS I [5–8]. In fact a distinct maximum at 681 nm was observed at room temperature in the fluorescence spectrum of an isolated LHA preparation [9]. In the low-temperature fluorescence spectra of PS II RC two marked maxima at 686 nm and 694 nm were observed [10].

Similarly, the fluorescence spectrum of PS I RC at room temperature shows a band at 695 nm [11]. Thus, the nature of the components responsible for the shortwave fluorescence spectrum (up to 700 nm) is believed to be well established. In addition to the main fluorescence bands (F_{680}, F_{685}, F_{695}) observed at room temperature pratically all the photosynthetic membranes and their fragments show luminescence at longer wavelengths, namely, 710–770 nm. Its intensity strongly increases at low temperatures, especially in samples enriched in PS I. This luminescence exhibits a broad band consisting of at least two components with maxima at 720–725 nm and around 745 nm [12]. They are commonly ascribed to the inner and pericentral antennae of PS I (F_{720}, F_{745}).

The goal of the present work was to investigate the origin of the long-wavelength luminescence from intact

Abbreviations: Chl, chlorophyll; F_{xyz}, fluorescence at *xyz* nm; LHA, light-harvesting antenna; PS, Photosystem; RC, reaction centre; λ_E, excitation wavelength.

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leaves, chloroplasts and isolated photosystems and to elucidate the role of the long-wave species of PS I in the photosynthetic reactions.

Methods and Materials

Stationary measurements

For preventing disturbing effects of sample preparation all stationary spectroscopic experiments were carried out on intact leaves. On the other hand, the reabsorption of fluorescence within strongly absorbing samples, such as intact leaves, distorts the fluorescence spectra measured in the common way. Therefore, we developed a special experimental arrangement and a numerical method to correct for the reabsorption effect. An experimental setup was made for measuring absorption and fluorescence at the same point of the sample only by switching the shutters in the optical pathway.

Fluorescence was excited by a continuous-wave argon ion or a helium neon laser and by a pulsed, tunable dye-laser (pulse duration, 5 ns; spectral bandwidth less than 0.1 nm; repetition rate, 12 p.p.s.) for measuring excitation spectra. An optical multichannel analyser (OMA-2, Princeton Applied Research) was used to record the spectra, control the measurement and compute data. Low-temperature measurements down to 27 K were carried out with a directly cooled neon cryostat [13].

The correction for the effects of fluorescence reabsorption within the sample is an indispensable condition for quantitative investigations of fluorescence spectra of intact leaves. In the case of the validity of the Lamber–Beer law, for measurements in the same direction as excitation the fluorescence spectrum $F(\lambda)$ measured out of the sample can be described by an expression given below. $E^x(\lambda, x)$ is the primary x within the sample; $\mathscr{K}(\lambda)$ is the absorption spectrum, $I(\lambda_E)$ is the excitation intensity and d is the thickness of the sample:

$$F(\lambda) = \int_0^d I(\lambda_E)\, e^{-\mathscr{K}(\lambda_E)x} E^x(\lambda, x)\, e^{-\mathscr{K}(\lambda)x}\, dx$$

On the assumption that $E^x(\lambda, x) = E(\lambda)$, there is a simple solution of the integral equation which was computed in the form:

$$E(\lambda) = \frac{F(\lambda)}{\sum_{i=0}^{n} P(n)\, e^{-(\mathscr{K}(\lambda_E)+\mathscr{K}(\lambda))\frac{i}{n}}}$$

in which $P(n)$ is only a proportionality factor. We found $n = 12$ sufficient to obtain good quantitative results. This method was calibrated and tested on chloroplast suspensions and on leaves of different optical densities. It works correctly for leaves of a maximum absorption ($-\ln(I/I_0)$) at 680 nm up to 1.5. Fig. 1 demonstrates the power of this method for two different geometries of exciting fluorescence. This method enables an investigation of such complex objects as intact leaves without expensive sample preparation. On the other hand, to work efficiently it requires a sophisticated experimental arrangement and numerical data processing at a computer-aided spectroscopic measuring system.

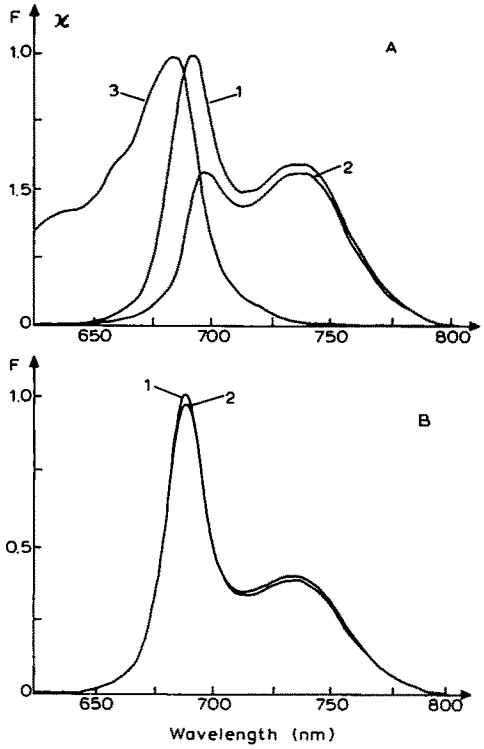

Fig. 1. Spectra measured at a single point of an intact leaf. A, before correction for reabsorption; B, after correction for reabsorption; 1, fluorescence spectrum measured with backward excitation; 2, fluorescence spectrum measured with forward excitation; 3, absorption spectrum.

A numerical deconvolution of the measured spectra was carried out with a computer program based on the principle of systematically checking the free parameters of a superposition of gaussian bands by the least-sqare test [13].

Kinetic measurements

Fluorescence decay kinetics were measured on a pulsed fluorometer described elsewhere [14]. Excitation was given by single, second harmonic pulses from a Nd laser (530 nm). The pulse duration was about 2 ps, the pulse power less than 10^{13} photons per cm^2 per pulse and the repetition rate 0.5 p.p.s. Interference light filters were used to separate the desired spectral regions. A streak camera with inherent averaging facilities was used for recording data. The light on the streak camera screen was detected with a vidicon. Data processing and

fluorometer control were carried out by a computer. Means were provided to ensure rigorous control of the excitation pulse quality through the selection of pulses by duration and energy, which enabled measurements of relative quantum yields.

Materials

Wheat (*Triticum aestivum* ssp. *vulgare*) was brougt to germination on wet cotton wool at 22° C for 24 h and then put into soil. The seedlings were grown at 20° C under weak light conditions and normal day-night cycles. Spectroscopic investigations were carried out at 7–9-day-old primary leaves at a distance of 2 cm from the top.

Chloroplasts were isolated from cooled spinach leaves. The leaves were homogenized in a buffer containing 350 mM NaCl, 50 mM phosphate buffer (pH 7.4) and 200 mM EDTA. The supernatant was centrifugated at 200 g for 1.5 min. The precipitate was resuspended in 200 mM sucrose, 3 mM KCl, 50 mM Tris-buffer (pH 8.0) and washed out twice in the same mixture. For low-temperature measurements 60% glycerol (by volume) was added to the sample.

The isolation of subchloroplast particles enriched in RC's (PS I-110 complex) was performed by the method of Bengis and Nelson [15]. A chlorophyll suspension (chlorophyll content 1 mg/ml) was diluted with a 10% digitonin solution to a concentration of 1%. After incubation for 1 h at 4° C, the mixture was fractionated by centrifugation at $30\,000 \times g$ for 10 min. The supernatant was mixed with 1.5% dry NaCl and the mixture diluted with 10% digitonin solution to a final concentration of 0.1 M. After overnight incubation in the cold, the mixture was centrifuged at $30\,000 \times g$ for 10 min. The precipitate was discarded and the supernatant centrifuged at $150\,000 \times g$ for 2 h. If not in immediate use, the suspension was stored frozen in liquid nitrogen.

PS I-65 and PS I-40 complexes were obtained from PS I-110 complex in a further purification procedure by adding 20% Triton X-100 of a concentraton of about 4% and incubating overnight in the cold. The preparation was desalted on Sephadex G-50 and then dialysed against 50 mM Tris-HCl buffer (pH 8.0) equilibrated with 0.2% Triton X-100 applied to DEAE cellulose column. Depending on the type of desired preparation (PS I-65 or PS I-40), the sample was washed on the column with different amounts of the above buffer containing Triton X-100. The resultant RC-enriched preparations were diluted with a linear NaCl gradient from 0 to 400 mM in the same buffer with Triton X-100.

Comparison of PS I-110, PS I-65 and PS I-40 absorption spectra shows that PS I-65 has a decreased content of pericentral (longwave) forms of Chl-*a*, whereas PS I-40 had no pericentral forms of Chl-*a* as compared with PS I-110 [16].

Experimental results

Stationary low-temperature measurements

The PS I fluorescence is well known to rise strongly at low temperatures. Therefore, we measured the fluorescence spectra of leaves at temperatures down to 27 K. Considering the long-wavelength band arround 735 nm homogeneous its behaviour differs from those of the short wavelength bands. The specific features of the 735 nm band are:

- the band maximum shifts from 735 nm at 300 K to about 745 nm at 27 K;
- the bandwidth decreases from 55 nm to 35 nm;
- the band is asymmetric in pattern; the asymmetry varies with temperature.

These features can be understood if one assumes an inhomogeneous character of this band. From a second derivative calculation of the fluorescence band around 735 nm we found at all temperatures significance for the existence of two components with different dependence on temperature (Figs. 2 and 3). For getting more detailed information the set of spectra was investigated by means of a component analysis [17,18]. The band was found to consist of at least two independent components with maxima at 731 nm and 747 nm, independent of temperature. Their bandwidths show the same temperature dependence as the other fluorescence components at shorter wavelength (for more detailed information, see Ref. 13).

The component at 747 nm showed a significant asymmetry of its lineshape. For practical reasons this component was described numerically by two gaussian bands with a constant ratio of their amplitudes and fixed positions at 747 nm and 769 nm, respectively (see Table II). But there is no evidence for the existence of these two independent components or substructures of this band.

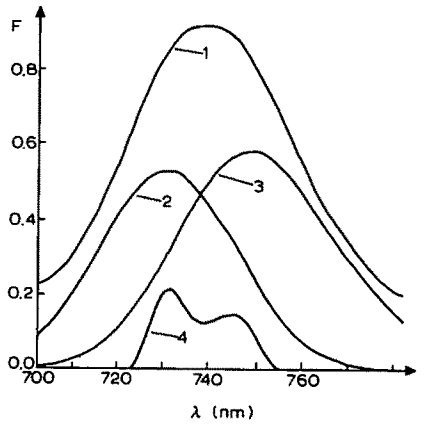

Fig. 2. Long-wavelength fluorescence of a primary wheat leaf at a temperature of 150 K. 1, integral long-wavelength fluorescence band; 2, component at 731 nm; 3, component at 747 nm; 4, negative second derivation of lineshape 1.

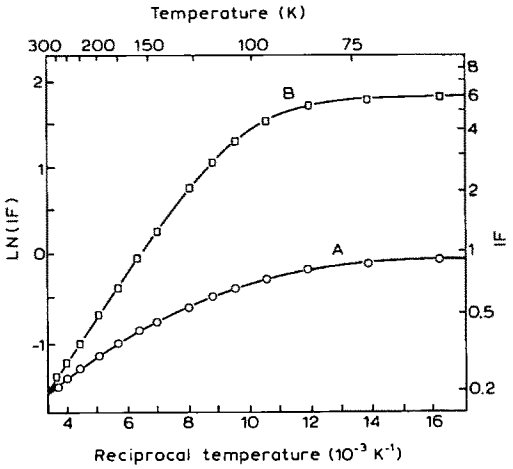

Fig. 3. Temperature dependence of the integral intensities (IF) of the fluorescence components at 731 nm (A) and at 747 nm (B).

The intensities of the 747 nm and 731 nm components showed different temperature dependences as shown in Fig. 3. The semilogarithmic plot demonstrates that the temperature dependence of the 747 nm component can be described by a activation energy of (78 ± 5) meV. The fluorescence intensity of this band was fitted to $F_{747}(T) = (0.16 + 805\ e^{-78\ meV/kT})^{-1}$. This value is in good aggreement with the results obtained on isolated PS I particles described in the subsection kinetic measurements and with previous results [19,20]. This suggests that the 747 nm band can be attributed to PS I.

The band at 731 nm could not be described by a simple activation energy, because there is no linear region in Fig. 3. But its amplitude is in an approximately constant ratio with the 685 nm fluorescence bands belonging to PS II [13]. This supports the assumption that the component at 731 nm is the envelope line of the vibrational satellites of the PS II bands around 685 nm. Fig. 3 demonstrates that Photosystem I has a significant contribution to the fluorescence of an intact leaf, even at room temperature.

Excitation spectra of fluorescence

The excitation spectra of intact leaves were used as another independent approach to evaluate the contribution of PS I to the integral fluorescence at room temperature. The conventional way of measuring excitation spectra by recording the fluorescence intensity at a fixed wavelength as a function of excitation wavelength may generate artefactual results in such optically dense samples as intact leaves. Varying the excitation wavelength leads to significant changes of the penetration of exciting light and in the reabsorption effect. For this reason, we measured the whole fluorescence spectrum at every excitation wavelength, removed the reabsorption effects and deconvoluted these spectra into two components according to Table I.

Varying the excitation wavelength causes no changes of the band position or bandwidth but affects the ratio

TABLE I

Major components of the fluorescence of an intact leaf at room temperature

Γ, half-width

Component	λ_0 (nm)	Γ (nm)
F_{685}	684.2 ± 0.2	19.6 ± 0.3
F_{735}	736.0 ± 2.0	53.0 ± 2.0

of their amplitudes. The ratio $Q(\lambda_E) = F_{685}(\lambda_E)/F_{735}(\lambda_E)$ is plotted in Fig. 4. This ratio shows a clear maximum at excitation wavelengths around 475 nm.

For evaluating the contribution of PS I ($L(\lambda_E)$ to the integral long-wavelength band F_{735}, we made the following assumptions:

- the PS I fluorescence is represented by one band around 735 nm ($F_{I\,735}$),
- the PS II fluorescence consists of a band at 685 nm ($F_{II\,685}$) and its vibrational satellite at about 735 nm ($F_{II\,735}$) with a constant ratio (H),
- the levels of the constant fluorescence of the two photosystems correspond to the activity of the photosystems measured by the O_2-evolution according to Ried [21], $R(\lambda_E)$, the ratio of the activities of PS II and PS I as a function of the excitation wavelength, is proportional to the ratio of the constant fluorescence of PS II and PS I.

This led to a system of equations:

$$F_{735} = F_{I\,735} + F_{II\,735}$$

$$F_{735} = L(\lambda_E) \cdot F_{I\,735}$$

$$F_{II\,685} = H \cdot F_{II\,735}$$

$$Q(\lambda_E) = F_{II\,685}/F_{735}$$

$$R(\lambda_E) = P \cdot (F_{II\,735} + F_{II\,685})/F_{I\,735}$$

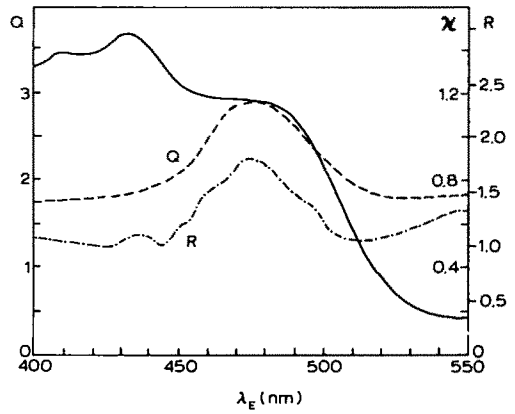

Fig. 4. $\mathscr{K}$, absorption spectrum of an intact leaf; Q, ratio of the amplitudes (F) of the deconvoluted fluorescence bands F_{685} and F_{735} (according to Table II); R, ratio of the activation spectra of PS II and PS I measured by O_2-evolution according to Ried [21].

We computed the experimental data and obtained the following results for the fluorescence contributions at room temperature:

(a) The ratio $F_{II\,685}/F_{II\,735}$ is about 3 : 1.
(b) The share of PS I ($L(\lambda_E)$) for balanced supply of both photosystems with excitation energy ($R(\lambda_E) \approx 1$) is 0.4–0.5.
(c) $L(\lambda_E)$ decreases to 0.2–0.3 at excitation in the range between 460 nm and 490 nm which is characterized by a predominant excitation of PS II.

The extreme excitation of PS II is due to the specific absorption of chlorophyll *b* and of the carotenoids which contribute predominantly to PS II excitation [22]. A narrow-band excitation around 475 nm oversteps the range of the in vivo regulation mechanism leading to a unbalanced supply of the two PS's with excitation energy resulting in a significant rise of the fluorescence yield and in a decrease of the efficiency of photosynthesis as shown by Heath [23].

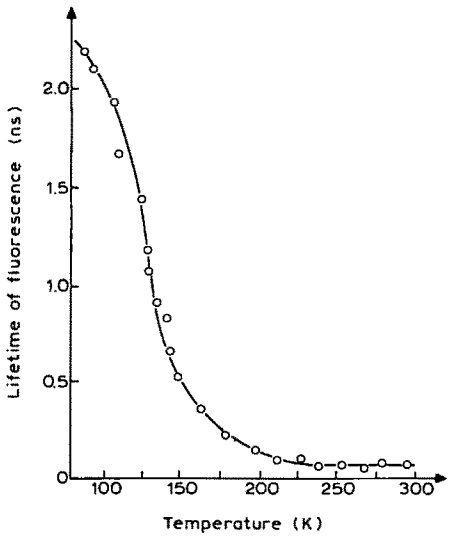

Fig. 6. Temperature dependence of the lifetime of fluorescence emission from pericentral chlorphylls of subchloroplast particles PS I-110.

Fluorescence induction

The light-induced variation of the fluorescence yield is an effect often investigated to study the primary processes of photosynthesis (for an example, see Ref. 21). Its variations reflect the adaptation of the photosynthetic apparatus to the beginning illumination.

We studied this effect as a third independent method for evaluating the fluorescence of PS I at room temperature only. We modified the usual experimental method by recording total fluorescnece spectra with the OMA-system with a repetition rate up to 5 per s. The set of spectra was corrected for reabsorption and deconvoluted into two gaussian bands (see Table I). Fig. 5 demonstrates the ratio of these two bands F_{685} and F_{735} the dark-adapted leaf.

The evaluation is based on the assumption that PS I does not emit variable fluorescence and that no state 1–state 2 transition takes place within the first ten seconds of illumination [21]. This led to a system of equations similar to the case of the excitation spectra (subsection Excitation spectra of fluorescence).

$$F_{735} = F_{I\,735} + F_{II\,735}$$

$$F_{735} = L(\lambda, t) \cdot F_{I\,735}$$

$$F_{II\,685} = H \cdot F_{II\,735}$$

$$Q(t) = F_{685}/F_{735}$$

$$F_{I\,735} = \text{constant}$$

We computed the experimental data and obtained results which confirm the conclusions drawn from the above-discussed methods. We got a value of 0.4 for the share of PS I on the long wavelength band in the stationary case ($t \rightarrow \infty$) and for excitation at 515 nm.

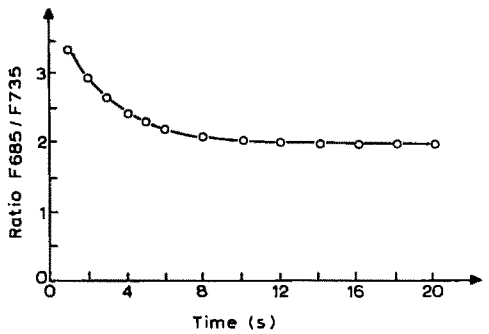

Fig. 5. Ratio of the F_{685} and F_{735} fluorescence bands deconvoluted according to Table I after beginning illumination of a dark-adapted leaf.

Kinetic measurements

To define the nature of the long-wave fluorescence of the pigment apparatus in higher plants we investigated the temperature dependence of the F_{696} and F_{735} fluorescence from PS I-enriched subchloroplast particles. PS I particles were used to measure the lifetime of the Chl-*a* fluorescence from the internal antenna (τ_{ia}) and from pericentral forms (τ_{pf}). According to its spectra of fluorescence τ_{ia} was measured at 696 nm and τ_{pf} at 750 nm.

As shown in Fig. 6 the temperature dependence of the fluorescence lifetime of PS I-110 subchloroplast particles can be described by the expression:

$$\tau_{750\,nm} = (0.45 + 830 \cdot e^{-73\ meV/kT})^{-1}$$

This is an exponential dependence with an activation energy of about 73 meV.

We have measured lifetime of fluorescence from Chl *a* of the internal antenna of all preparations (PS I-110, PS I-60 and PS I-40). Under conditions where the RC's were in a photoactive state (state P-700), τ_{ia} appeared to be about 30 ps in all preparations used, consistent with results obtained earlier [22]. After oxidation of the RC's (state P-700$^+$), we found τ_{ia} to be 60 ps in preparations having the full set of pericentral forms (PS I-110). This number increased to 400 ps in preparations devoid of pericentral antenna (PS I-40).

Discussion

Investigations of fluorescence induction and excitation effects of intact leaves at room temperature made it possible to evaluate the contribution of PS I to the integral fluorescence. We found that the PS I fluorescence consists of a band around 740 nm. In the case of balanced excitation energy supply to both photosystems, the contribution of PS I to the total fluorescence is about 25% (Table II).

To get more information about the spectral characteristics of the PS I emission band, we studied the temperature dependence of the fluorescence down to 27 K. Two bands at 731 nm and 747 nm were resolved by their different temperature dependencies. Both bands had approximately the same amplitudes at room temperature. Fitting their temperature dependencies we got an activation energy of 78 meV for the component at 747 nm. This value is in good agreement with the activation energy measured on isolated PS I particles. Therefore, the component at 747 nm could be attributed to PS I.

Fig. 7 shows the results of these three independent methods. The lineshapes of the fluorescence bands can be described by the expressions given in Table II.

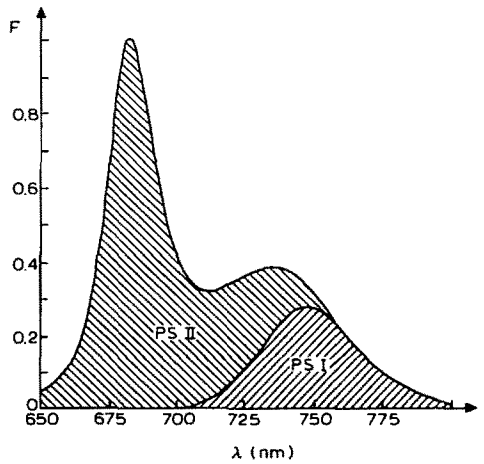

Fig. 7. Total fluorescence of an intact leaf at room temperature (upper line) and the estimated shares of PS I and PS II ($\lambda_E = 515$ nm). Upper spectrum: total fluorescence.

TABLE II

Numerical description of the fluorescence line shapes of PS I and PS II of an intact leaf at room temperature ($\lambda_E = 515$ nm)

F is the amplitude of the band calibrated against the fluorescence intensity at 685 nm; IF is the integral fluorescence of the band calibrated against the overall fluorescence of sample, Γ, half-width.

Component		λ_0 (nm)	Γ (nm)	F	IF
PS I	1	747.0±1.3	36.0±1.5	0.23	0.19
	2	769.0±2.0	36.0±1.5	0.06	0.05
					0.24
PS II	3	660.0±1.5	18.0±1.3	0.07	0.02
	4	684.3±0.2	19.6±0.3	0.91	0.40
	5	705.0±1.6	33.0±1.6	0.23	0.18
	6	731.2±0.8	32.0±1.1	0.21	0.16
					0.76

We found that the PS I lineshape has a significant asymmetry. But there was no evidence for the existence of distinguishable subbands. Therefore, the two bands (Table II) have to be considered a mathematical description of PS I lineshape, only.

The situation with PS II is similar. Its four bands (Table II) were necessary to fit the major fluorescence band at 685 nm and its vibrational satellite. It is not useful considering these four gaussian bands independent components or real subbands of the PS II fluorescence at room temperature. Subbands were evident only at temperatures below 160 K. Below 160 K the integral band around 685 nm splits into subbands. These subbands can be associated with pigments that function as intermediates in energy transfer of PS II (as an example, see Ref. 23). Our detailed results about this topic will be published next.

To get information about role and function of the long-wavelength pigments of PS I we studied the fluorescence decay kinetics of chloroplasts and isolated PS I subchloroplast particles with different ratios Chl *a* to P-700. On the basis of these results several important conclusions can be made about the role of the pigments surrounding the RCs. The fact that with photoactive RCs the fluorescence decay time of the internal antenna (τ_{ia}) is 30 ps regardless of the degree of enrichment in pericentral forms suggests that the excitation energy is probably transferred directly to the RC without involving the pericentral pigments. When the RC is oxidized and cannot trap the energy from the internal antennae, the value τ_{ia} becomes a direct function of the amount of pericentral Ch's available: τ_{ia} is 60 ps in their presence and 400 ps in their absence. It follows from the above that the pericentral chlorophylls of PS I act as excitation energy trap only when the RC is oxidized. The question which immediately arises, is: what are the ways of deactivation of the pericentral pigments? It has been deomonstrated on intact leaves as well as on isolated PS

I subchloroplast particles that the temperature dependence of pigment excitation may be expressed as:

$$\tau_{750\ nm} = (A + B \cdot e^{-E/kT})^{-1}$$

with E about 75 meV. The exponential pattern of the temperature dependence of excitation lifetime or fluorescence intensity is appropriate for cases where the energy transfer to the RC or internal antennae involves the anti-Stokes mechanism or where deactivation occurs by internal conversion [26]. Let us consider the two cases.

Recently, we have investigated the fluorescence intensity of PS I-110 subchloroplast particles as a function of the state of the reaction centres [16]. The fluorescence of pericentral Chl *a* after RC oxidation rises a factor of only 1.45. From these results, it is clear that the main path for energy deactivation of the pericentral antenna states is not anti-Stokes energy migration toward the RC, but internal conversion.

We cannot, however, exclude the possibility of anti-Stokes energy transfer from the pericentral pigments to photoactive RC after the direct excitation of the pericentral antenna. The rate constants for excitation transfer to the RC in this situation may be determined from measurements on the dependency of τ_{pf} on the RC state, when excitation falls within the absorption band of the pericentral forms of chlorophyll *a*. These experiments are in progress.

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