ELSEVIER

Journal of Photochemistry and Photobiology B: Biology 59 (2000) 103–114

Journal of Photochemistry and Photobiology B:Biology

www.elsevier.nl/locate/jphotobiol

Spectral characterization of chlorophyll fluorescence in barley leaves during linear heating
Analysis of high-temperature fluorescence rise around 60°C

Petr Ilík[a,*], Roman Kouřil[a], Jiří Fiala[a], Jan Nauš[a], František Vácha[b]

[a]*Department of Experimental Physics, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic*
[b]*Institute of Plant Molecular Biology, Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic*

Received 29 August 2000; accepted 22 November 2000

Abstract

The spectral characteristics of chlorophyll fluorescence and absorption during linear heating of barley leaves within the range 25–75°C (fluorescence temperature curve, FTC) were studied. Leaves with various content of light harvesting complexes (green, Chl *b*-less *chlorina f2* and intermittent light grown) revealing different types of FTC were used. Differential absorption, emission and excitation spectra documented four characteristic phases of the FTC. The initial two FTC phases (a rise in the 46–49°C region and a subsequent decrease to about 55°C) mostly reflected changes in the fluorescence quantum yield peaking at about 685 nm. A steep second fluorescence rise at 55–61°C was found to originate from a short-wavelength Chl *a* spectral form (emission maximum at 675 nm) causing a gradual blue shift of the emission spectra. In this temperature range, a clear correspondence of the blue shift in the emission and absorption spectra was found. We suggest that the second fluorescence rise in FTC reflects a weakening of the Chl *a*–protein interaction in the thylakoid membrane.

Keywords: Barley leaves; Chlorophyll; Fluorescence temperature curve; High temperature; Light harvesting complex; Photosystem

1. Introduction

Temperature is one of the abiotic factors influencing the function of photosynthesizing organisms. It is believed that the most thermolabile membrane in a plant cell is the thylakoid membrane of chloroplasts [1]. A very sensitive tool for monitoring the actual state of the photosynthetic apparatus in thylakoid membranes is chlorophyll *a* (Chl *a*) fluorescence [2,3]. By combining temperature control with detection of Chl *a* fluorescence, a sensitive indicator of the thermostability of processes within the thylakoid membrane can be obtained. A frequently used regime is fluorescence detection upon linear heating from room temperature to non-physiological temperatures (55–75°C) (fluorescence temperature curve, FTC). The FTC is measured with leaves, algae, chloroplasts or thylakoid preparations usually under analytic or weak actinic light excitation. Although the shape of individual FTCs depends on the plant material [4], excitation wavelength and intensity [4,5], detection wavelength [6,7], and the rate of heating [6], several characteristic phases in FTC can usually be observed.

In the first phase of FTC (from 25 to about 45–50°C), fluorescence mostly increases up to the first maximum (M1, Fig. 1). Schreiber and Armond [5] found for chloroplasts that the fluorescence rise reflects the rise of the quantum fluorescence yield (absorption changes were negligible). As far-red background illumination does not prevent the appearance of this heat-induced rise [8], these authors assumed that the rise was caused by a functional separation of the primary chinone acceptor (Q_A) from the primary donor (P680) in photosystem II (PSII). In other words, an inhibition of energy conversion at PSII (‘a block’) occurs within this temperature range. Other possible concomitant processes have been reported in the literature, e.g. detachment of light harvesting complexes (LHC) [5]. Although many papers have been published on the molecular mechanism of PSII inhibition in connection with the fluorescence rise [9–15], a definitive solution has not been found. The initial slower rise beginning from

*Corresponding author. Tel.: +420-68-522-2451; fax: +420-68-522-5737.

E-mail address: ilik@risc.upol.cz (P. Ilík).

1011-1344/00/$ – see front matter
PII: S1011-1344(00)00146-9

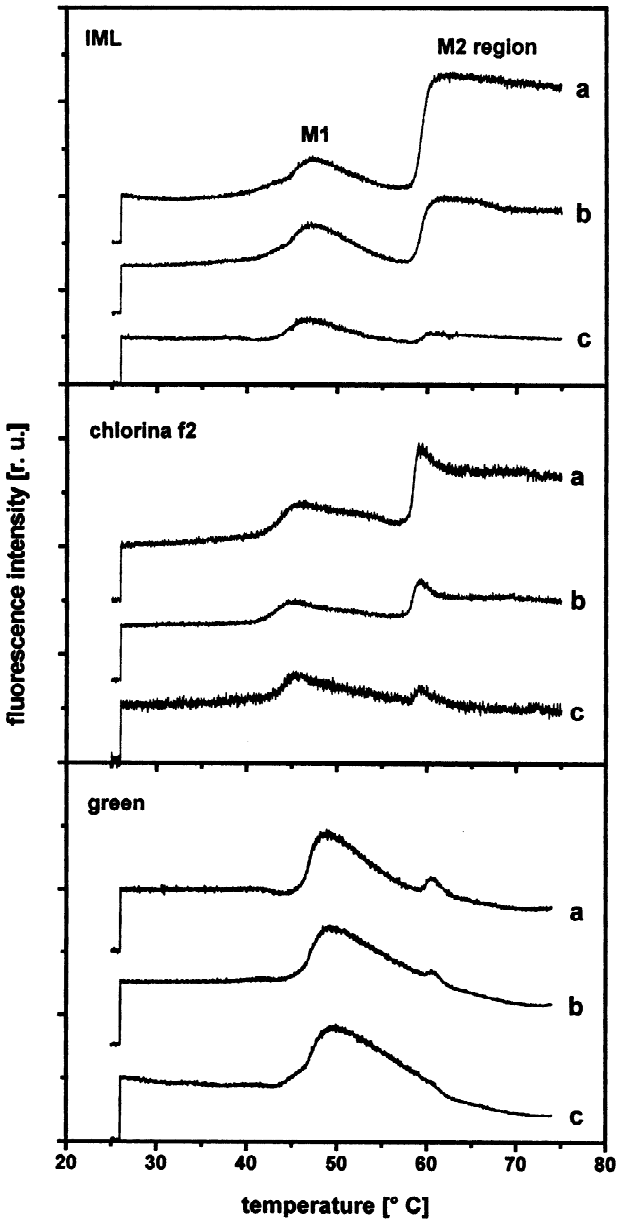

Fig. 1. Typical FTCs of IML (top), *chlorina f2* (middle), and green (bottom) primary barley leaves. Detection wavelength was 675 nm (curves a and c) or 685 nm (curves b). Excitation wavelength was 436 nm (curves a and b) or 475 nm (curves c). Individual leaves were used for different curves. Linear heating rate, 2°C min^{-1}; M1, the first maximum in FTC; M2 region, part of the FTC from the second fluorescence rise to 75°C.

about 30°C is probably due to the inhibition of the PSII oxygen evolving complex, while the following faster phase, terminated at the M1 FTC maximum, reflects rather the inhibition of the acceptor side of PSII [16]. The amplitude of the fluorescence increase to the M1 maximum was shown to be correlated with other fluorescence characteristics reflecting the state of PSII [17]. Another question is whether the detected chlorophyll fluorescence has the same origin during heating. In spite of these ambiguities the critical temperature of the fast fluorescence rise reflects damage to PSII and is used as an indicator of photosystem II thermostability [4]. The critical temperature also reflects changes in thylakoid membrane fluidity varying during plant acclimation (e.g., Ref. [18]). Even a correlation between the critical temperature and the critical temperature of leaf necrosis was found [19].

In the second phase of FTC, beginning at the M1 maximum, the fluorescence intensity gradually decreases. The interpretation of this phase is not clear. At the temperature of the M1 maximum the measured fluorescence level is approximately equal to the maximal fluorescence F_M [5,20] when PSII centers are closed, so the observed second FTC phase reflects a decrease of the F_M level.

Within the temperature range of about 53–63°C (the third FTC phase), a second fluorescence rise (rise to the M2 region, see Fig. 1) can be detected. The threshold temperature of this rise strongly depends on the heating rate [6]. Using preferential excitation of Chl *a* and Chl *b* it was deduced that the rise originates from Chl *a* with strongly weakened assistance of Chl *b* and carotenoids [21–23]. This fluorescence rise is pronounced mainly for samples with a reduced content of LHCs [21–24]. Downton and Berry [21] showed that this rise originates from stroma thylakoids and attributed the emission to photosystem I (PSI). They also proposed using the relative height of this rise for estimation of nonappressed/appressed thylakoids. The interpretation of the second rise was questioned by Gaevsky et al. [22]. They showed with mutants of *Chlamydomonas reinhardtii* lacking PSI or PSII that both photosystems are probably responsible for this second fluorescence rise.

The fourth phase in the FTC represents fluorescence changes beyond the second fluorescence rise (above about 60°C). Usually, fluorescence decreases within this temperature range (e.g., Refs. [6,23,24]), but when samples with a lower LHC content are used the fluorescence is rather constant within 60–75°C [22,23]. The reason for these differences has still not been explained.

In this paper we measured absorption and chlorophyll fluorescence emission and excitation spectra of samples with different LHC content during linear heating up to 75°C. We focused on a comparison of the spectral changes in the first and second fluorescence rise in FTC. A clear blue shift of the spectra is shown for temperatures above 55°C.

2. Materials and methods

2.1. Plant material

Hordeum vulgare L. (cv. Akcent) and the Chl *b*-less barley mutant *chlorina f2* were cultivated in a growth chamber at 20°C in artificial soil composed of perlit and Knopp solution. The light regime was 16 h light (90 μmol

m^{-2} s^{-1} of PAR)/8 h dark. The detached primary leaf segments of 8 day old barley were used for the measurements. Etiolated barley seedlings (*Hordeum vulgare* L. cv. Akcent) for the IML samples were also grown hydroponically in darkness at 20°C for 5 days and then exposed to intermittent light with the regime of 2 min light (45 μmol m^{-2} s^{-1} of PAR)/98 min dark for 41–44 cycles [23]. Alternatively, etiolated barley seedlings were grown for 5.5 days at 25°C according to Ref. [24] and then exposed to continuous light (90 μmol m^{-2} s^{-1} of PAR) for 1.5 h.

2.2. Pigment content

Contents of Chl *a*, Chl *b* and the sum of carotenoids were determined spectrophotometrically in 80% acetone according to Ref. [25]. Mean concentrations ±S.D. were calculated from three to four independent measurements.

2.3. Linear heating

A leaf segment of 1.5 cm length was cut off from the central part of the leaf blade and placed on a sample holder. The holder with the sample was immersed in distilled water and heated at a rate of 2°C min^{-1} from room temperature up to the selected temperature. Linear heating was performed by a computer-controlled electric heater. The water in the vessel was stirred by a magnetic stirrer.

2.4. Fluorescence and absorption measurements during heating

Fluorescence spectra and fluorescence temperature curves (FTC) were measured using a Fluorescence Spectrophotometer F-4500 (Hitachi, Tokyo, Japan). The spectrometer was extended by a laboratory setup with fiber optics allowing measurement outside the sample chamber. Fluorescence was excited and detected on the adaxial leaf side. The dark adapted leaf segments were exposed to light at 436 or 475 nm to reach steady state conditions before the beginning of the measurements. The emission spectra and FTCs were measured with 10 and 5 nm spectral slit-widths for the excitation and emission monochromator, respectively. For measurement of excitation spectra, 5 and 10 nm spectral slit-widths were used, respectively. The scan rate of the spectra was 240 nm min^{-1}. The leaves were exposed to exciting light of 436 nm (10 μmol m^{-2} s^{-1}) constantly during heating.

The absorption spectra of leaf segments were measured during linear heating using a Helios α spectrophotometer (Unicam, Cambridge, UK). The samples were placed in a glass cuvette with distilled water. The spectra were measured at selected temperatures with a 2 nm spectral slit-width.

2.5. High-performance liquid chromatography

The chromatography system consisted of a low-pressure gradient mixing unit GP3 (ECOM, Prague, Czech Republic), a micropump LCP 3001 (ECOM, Prague, Czech Republic) and a photo-diode array detector Waters 996 (Waters, Milford, USA). The samples were injected via an ECOM injector equipped with a 50 μl loop. The separation was run on a reverse-phase C_{18} column, 150×3 mm, particle size 10 μm (Tessek, Prague, Czech Republic).

Leaves were ground in a mortar in 2 ml of 100% acetone in the presence of magnesium oxide. Pigments were extracted for 3 min and the extract was centrifuged at 14 000 *g* for 5 min. The supernatant was collected and filtered through a 0.45 μm PTFE filter membrane (Tessek) prior to injection. The extraction was performed in dim light at 4°C. Pigments were separated using gradient elution. For the first 5 min a linear gradient from 50 to 90% methanol was used followed by isocratic elution by 90% methanol for the next 6 min. Then a linear gradient from 90 to 100% methanol was used for 5 min followed by a linear gradient from 100% methanol to a mixture of methanol and hexane (4:1) for another 6 min. Finally, the column was washed with the methanol–hexane mixture (4:1) until the β-carotene was eluted. The column was pre-equilibrated with 50% methanol for 10 min prior to the injection of the next sample.

3. Results

3.1. FTCs of barley leaves with different LHC content

Three types of barley leaves were used for the study of individual phases of the fluorescence temperature curve, FTC: (i) primary barley leaves of fully green seedlings with Chl $a/b \sim 3$; (ii) intermittent light grown leaves (IML) with significantly reduced contents of light harvesting complexes (LHC) (Chl $a/b \sim 8.7$); and (iii) leaves of *chlorina f2* mutant lacking Chl *b* and specific LHCs [26]. Fig. 1 shows FTCs of these leaves at preferential excitation of Chl *a*, 436 nm (curves a and b), and auxiliary pigments (Chl *b* and carotenoids), 475 nm (curves c). As the wavelength of the fluorescence emission maximum decreases during heating [7], two emission wavelengths were chosen for detection: the first at 685 nm (curves b), where a room temperature emission maximum appears, and the second at 675 nm (curves a and c). The FTCs of all three leaf types show the typical phases described in the Introduction. Also, the temperature regions of these phases are similar, suggesting that the same processes occur in each leaf type during heating.

While the fluorescence level is rather constant within 25–40°C and then sharply increases to the M1 FTC maximum for green leaves, a gradual fluorescence increase from room temperature is evident for IML and *chlorina f2*

leaves at preferential Chl *a* excitation (Fig. 1). The temperature of the M1 maximum was slightly higher in green leaves (49°C) than in *chlorina f2* leaves (46°C) and IML leaves (47°C) (Fig. 1). In green leaves, the first FTC phase appeared at higher temperatures. This indicates a higher PSII thermostability in green leaves than in the other leaf types. The fluorescence rise to the M1 maximum is different on preferential excitation of the auxiliary pigments. A rather constant level with IML, and even a decreasing tendency with green leaves, were detected within the temperature range 25–40°C. The slow decrease could be associated with a heat-induced detachment of LHCII from PSII decreasing the PSII absorption cross section [5].

We concentrated our attention on the differences between the FTCs of the three leaf types above 55°C (Fig. 1). The highest second fluorescence rise in FTC without significant subsequent decrease for IML leaves, a lower second rise with a distinct fluorescence decrease for *chlorina f2* leaves and a small second rise with a pronounced subsequent decay in FTC for green leaves were detected. The rise appeared at slightly higher temperatures in green leaves, indicating again their higher thermostability. As the second fluorescence rise appeared in FTC of Chl *b*-less *chlorina f2*, Chl *b* is not involved in this rise.

As was shown previously for IML barley leaves, there is only a small contribution of auxiliary pigments to the 685 nm fluorescence at the second fluorescence rise in FTC [23] as compared to the first rise to the M1 maximum. Similar results for the 675 nm fluorescence were obtained for green and *chlorina f2* barley leaves (Fig. 1, compare curves a and c). A higher second fluorescence rise was detected for 675 nm than for 685 nm emission, indicating that, in the M2 FTC region, the emission maximum is below 685 nm for all leaf types (Fig. 1, compare curves a and b). This led us to the idea of measuring the full fluorescence emission spectra of the leaves during heating.

3.2. Fluorescence spectra during heating of leaves

The fluorescence spectra were measured at selected temperatures during heating. Fig. 2B shows the emission spectra of the IML barley leaf at distinct points of the FTC (see Fig. 2A) at preferential Chl *a* excitation. The spectra were rather similar to one another up to 55°C. The main spectral change, a shift of the main emission maximum, was seen within 55–61°C, i.e. during the second fluorescence rise in FTC. The difference spectra (Fig. 2C) were calculated from the two consecutive spectra displayed in Fig. 2B. It is evident that the emission spectrum did not change up to 40°C and that the difference spectrum 55−47 (the spectrum detected at 55°C minus that detected at 47°C) and the 47−40 spectrum had a shape very similar to the shape of the room temperature emission spectrum. These results suggest that, within the temperature range 25–55°C, the same emission forms emit fluorescence with various fluorescence intensity. Fig. 2C also shows that an emission form peaking at about 675 nm becomes dominant within 55–61°C. At higher temperatures, no significant spectral change was observed for the IML leaves.

We also measured the excitation spectra during heating of IML leaves at emission wavelength 675 nm (Fig. 2D). However, it was not possible to ensure steady-state conditions in the sample, as the wavelength of the excitation light continuously changed during scanning. In spite of this inaccuracy, no significant spectral changes were detected up to about 55°C (Fig. 2D,E). Again, the main change appeared between 55 and 61°C. Within this temperature range an increase in the excitation range of Chl *a* was detected. This result indicates that some Chl *a* form is responsible for the second fluorescence rise in FTC. Such an increase, but only in relative size, i.e. with respect to the range of auxiliary pigments (450–510 nm), has already been observed in 77 K emission spectra of linearly heated IML leaves [23]. At temperatures above 61°C the excitation spectra did not change (Fig. 2D,E).

The emission spectra of *chlorina f2* and green leaves measured during heating, together with the difference spectra, are displayed in Fig. 3. For *chlorina f2* leaves the spectral changes up to 60°C were approximately the same as for the IML leaves. The fluorescence decrease in FTC above 60°C (Fig. 3A) is accompanied by a decrease in spectrum peaking at about 685 nm (Fig. 3C). The similarity of the spectral change between 55 and 46°C (difference spectrum 55−46) to the change between 67 and 60°C (67−60) (Fig. 3C) indicates that the fluorescence decrease in FTC above 60°C could be a continuation of the progressive fluorescence decrease beginning at the M1 FTC maximum.

This suggestion was strengthened by the measurements with green barley leaves (Fig. 3E,F). For these leaves, two tendencies clearly interfere upon the second fluorescence rise in FTC (Fig. 3F, difference spectrum 61−57). The first tendency is the fluorescence rise of the Chl *a* form probably peaking in the spectrum at about 675 nm as was observed for leaves with a reduced LHC content (see Figs. 2C and 3C). The second tendency is the fluorescence decrease with a spectral maximum at about 685 nm and continuing up to 75°C. A minor, but observable, contribution of the second tendency in the IML leaves can be seen in the difference spectrum (67−61) in Fig. 2C. The larger portion of the second tendency in green and *chlorina f2* leaves is probably associated with the larger content of LHCs in these leaves.

To support the idea that the second fluorescence rise in FTC originates in the emission form with an emission maximum at 675 nm, we also measured the emission spectra of linearly preheated barley leaves at a very early stage of greening (etiolated leaves after 1.5 h of continuous white light illumination). However, the leaves already contain photosystem cores [27]. In such leaves the amount of LHCs was minimal (unmeasurable by native Deriphate-

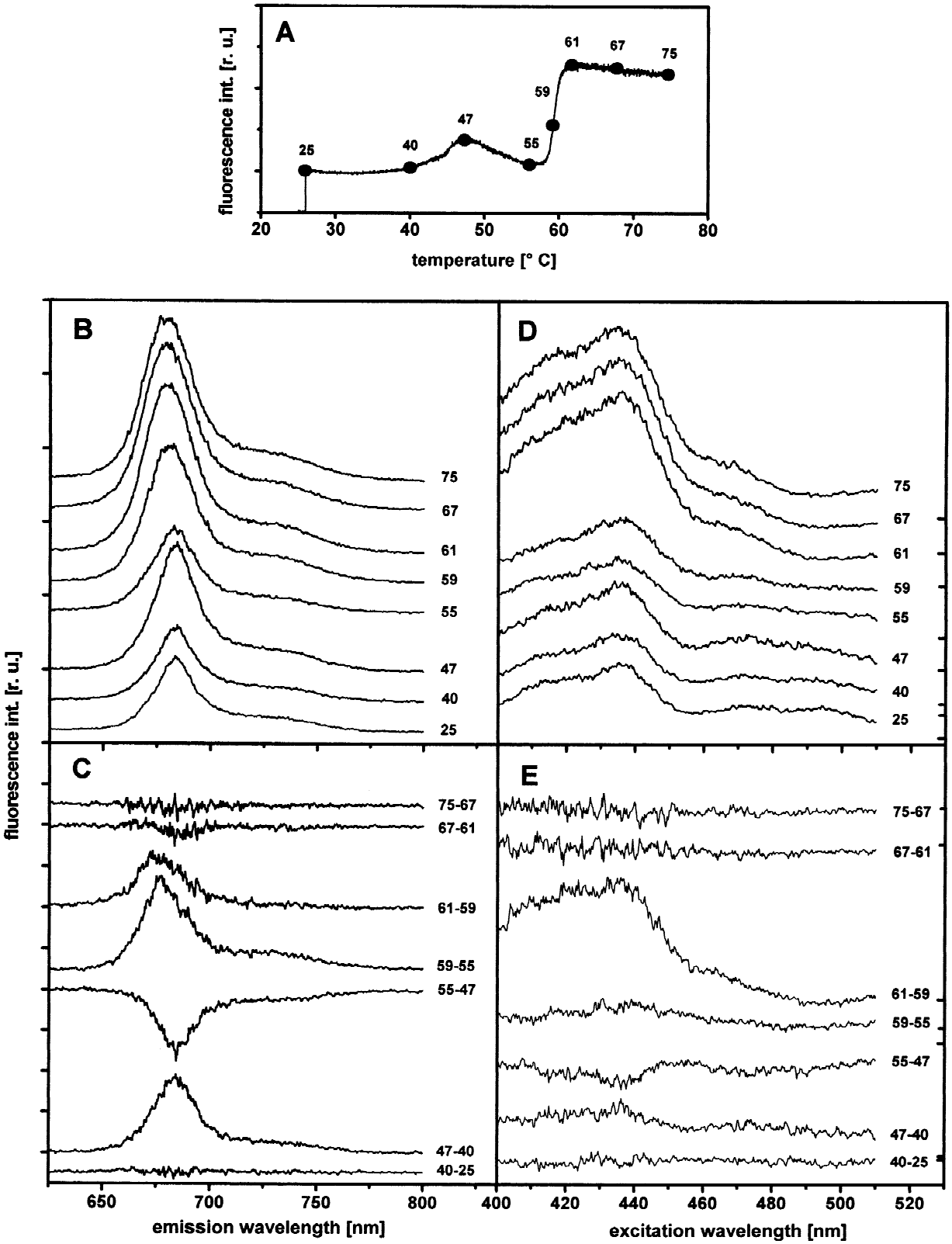

Fig. 2. Fluorescence emission and excitation spectra of IML barley leaves at selected temperatures. (A) The FTC of the IML leaf (from Fig. 1, top, curve a) with selected temperatures indicated. Emission spectra (B) were excited at 436 nm. Excitation spectra (D) were detected at 675 nm. Difference emission (C) and excitation spectra (E) were calculated from spectra (A) and (D), respectively. Given temperatures or temperature differences are indicated. The spectra are vertically shifted. Zero levels of the spectra in (D) and (E) are indicated on the right-hand abscissa in order of the displayed spectra.

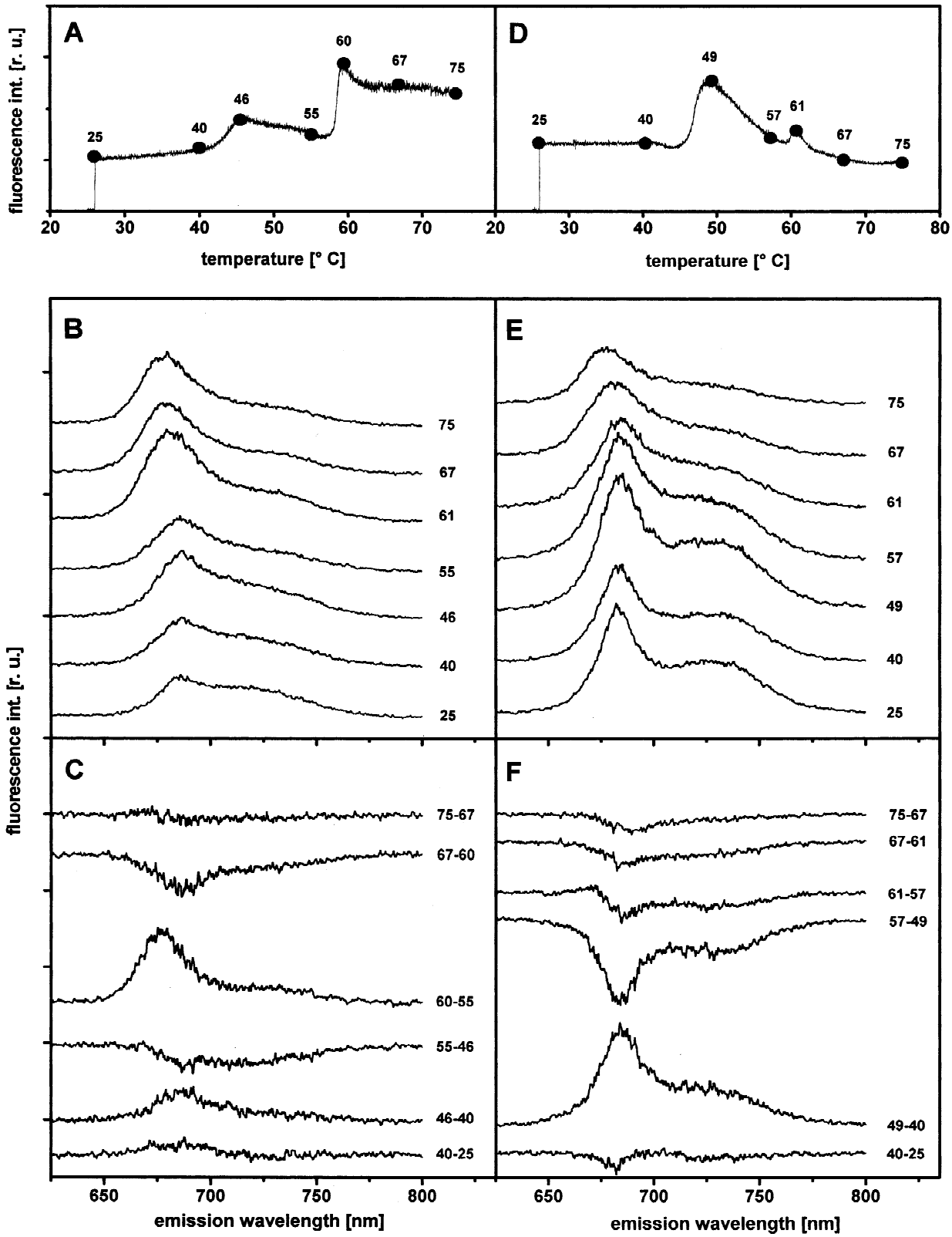

Fig. 3. Fluorescence emission and difference emission spectra of *chlorina f2* (B and C) and fully green barley leaves (E and F) at selected temperatures. The FTCs of *chlorina f2* (A) and green barley leaf (D) from Fig. 1 (curves a, middle and bottom figures) with selected temperatures indicated. The spectra were excited at 436 nm. Given temperatures or temperature differences are indicated. The spectra are vertically shifted.

PAGE), but the leaves still contained a small amount of protochlorophyllide *a* (data not shown). The FTC of this leaf for 675 nm emission reconstructed from the emission spectra measured during heating is displayed in Fig. 4. While the first fluorescence rise in the FTC was absent (PSII function was under development), the second rise was pronounced. The position of the emission maximum decreased from 679 to 675 nm during heating within the temperature range 25–57°C (Fig. 4, insert). At higher temperatures the position remained at 675 nm.

3.3. Absorption spectra during heating of leaves

The absorption spectra of green, IML and *chlorina f2* barley leaves were also measured during linear heating. While the spectra in the Soret region are rather unchanged (data not shown), more pronounced spectral changes were observed in the red region during heating. Fig. 5A shows the absorption spectra of IML leaves in the red region detected at the same selected temperatures as for the fluorescence measurements (see Fig. 2A). A gradual blue shift of the red absorption maximum starting at room temperature was detected, however the main shift appeared within 55–61°C. The large shift is typical for weakening of chlorophyll–protein interactions in pigment–protein complexes (PPCs). This finding strongly suggests that the fluorescence rise to the M2 FTC region is not only associated with the redistribution of excitation energy between PPCs, but with structural changes in PPCs themselves.

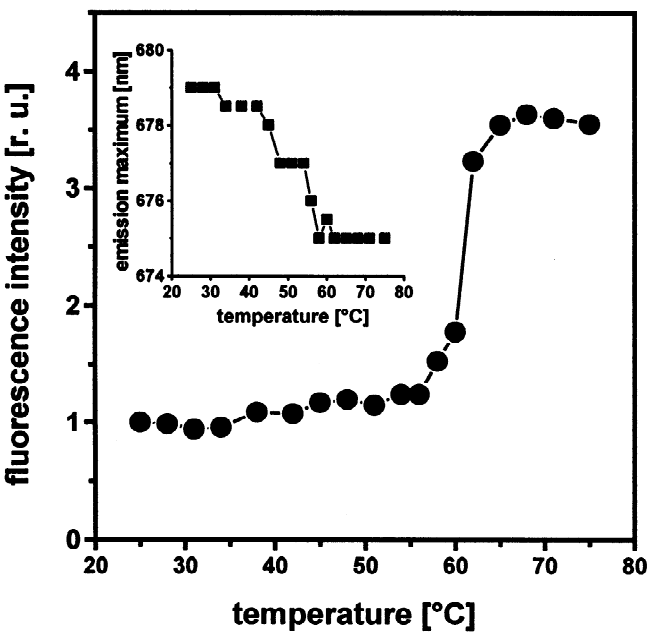

Fig. 4. Typical FTC of barley leaf greening for 1.5 h with continuous light for emission wavelength 675 nm. This FTC was reconstructed from the measurements of emission spectra during linear heating (2°C min^{-1}). The spectra were excited at 436 nm. The position of the emission maximum during heating is shown.

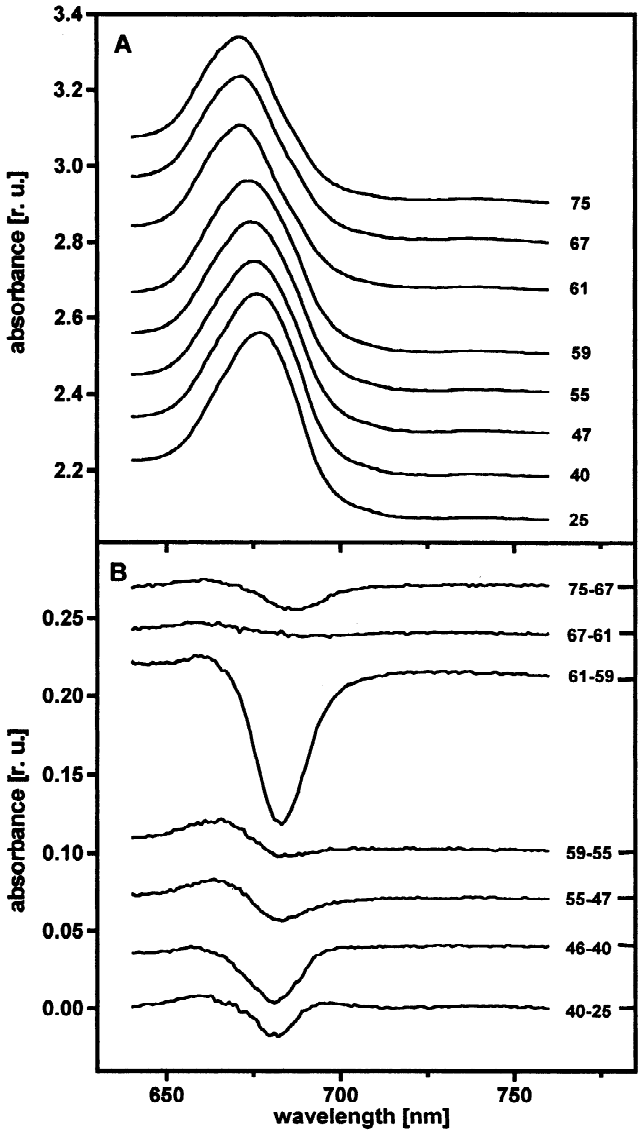

Fig. 5. Absorption (A) and difference spectra (B) of IML barley leaves at selected temperatures (see Fig. 2A). The spectra (A) are not corrected for light distortion. The difference spectra are calculated from the corrected absorption spectra (for details, see Results). Given temperatures or temperature differences are indicated. The spectra are vertically shifted. Zero levels of the spectra in (B) are indicated on the right-hand abscissa in order of the displayed spectra.

One may assume that the observed spectral changes are associated with changes of spectral distortion in the leaf during heating. The spectrum obtained at 25°C is displayed on the real absorbance scale while the others are vertically shifted (Fig. 5A). The measured room temperature absorbance at the red maximum was 2.55. However, the absorbance at 760 nm, outside the photosynthetic pigment absorption spectrum, was also very high, 2.07. This clearly indicates extensive light scattering in the sample [28]. We tried to estimate the changes in light scattering during heating by measurements of absorbance at 750–800 nm, which was approximately constant. In this range the

detected absorbance varied within 2.07–2.10 throughout the heating period. We roughly corrected the absorption spectra for diffusion changes by subtraction of the measured absorbance at 800 nm from the absorbance at the given wavelength. Fig. 5B shows the difference spectra obtained from the corrected absorption spectra. Again, the main spectral shift was seen within the temperature range 55–61°C.

A comparison of changes in the position of the red absorption and emission maxima of all three leaf types during linear heating is presented in Fig. 6. For the IML and *chlorina f2* leaves the major blue shift of both maxima occurred within the temperature range of the fluorescence rise to the M2 FTC region. This clearly shows that the fluorescence rise is associated with structural changes within PPC(s). Slightly higher temperatures of the blue shift in green leaves indicate a possible stabilizing effect of LHCs. The final absorption and emission maxima at 75°C for all three leaf types are approximately at the same wavelength, i.e. at about 671–2 and 679 nm, respectively.

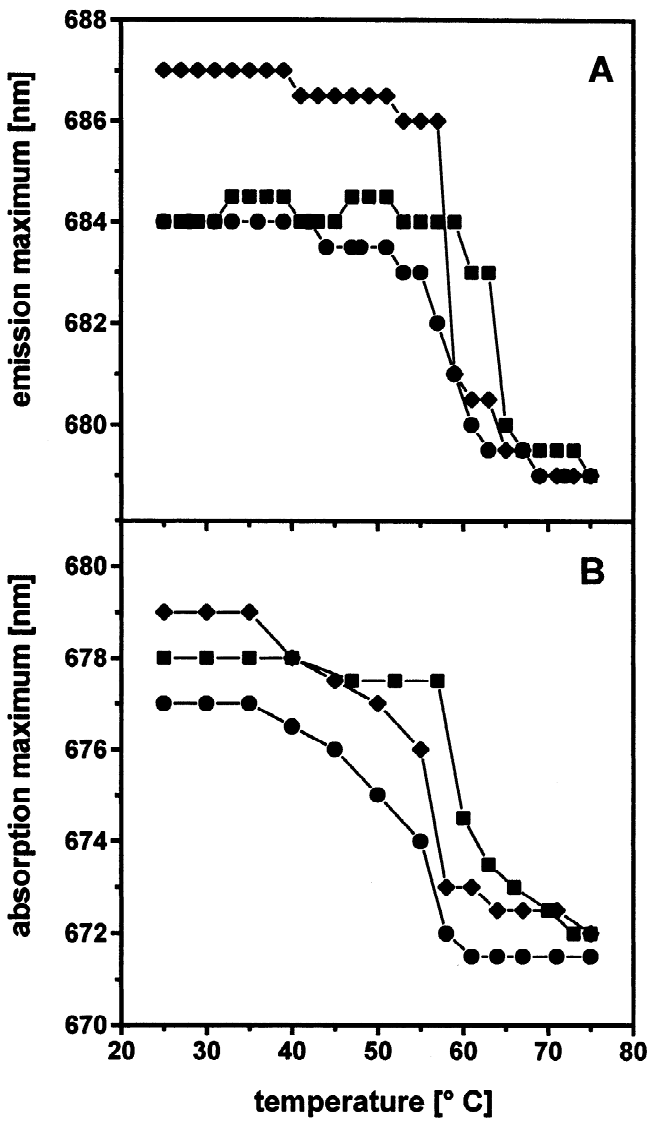

Fig. 6. Positions of the main emission (A) and red absorption maxima (B) of green (squares), *chlorina f2* (diamonds) and IML (circles) barley leaves during linear heating.

Table 1
Chl *a*, Chl *b* and carotenoid concentrations per leaf area of fully green, *chlorina f2* and IML barley leaves unheated and heated linearly to 75°C at a rate of 2°C min^{-1} [a]

		Concentration per leaf area (μg cm^{-2})		
		Chl *a*	Chl *b*	Car
Green	Control	21.3±0.7	7.2±0.3	5.5±0.4
	Heated	21.1±1.0	7.0±0.4	5.4±0.4
Chlorina f2	Control	13.5±0.8	–	3.4±0.1
	Heated	13.4±0.5	–	3.4±0.2
IL	Control	3.3±0.4	0.38±0.06	2.3±0.3
	Heated	3.4±0.2	0.39±0.03	2.3±0.2

[a] Pigments were quantified spectrophotometrically according to Ref. [25]. The S.D. was calculated from three to four independent measurements.

3.4. Changes on the pigment level during heating

The question is whether or not the mentioned structural changes in PPC(s) above 55°C are accompanied by pigment degradation. We estimated the concentration of Chl *a*, Chl *b* and the sum of carotenoids by the spectrophotometric method [25] for unheated leaves and leaves heated to 75°C (Table 1). No significant changes were observed for any of the three leaf types. To detect a possible degradation of Chls to products with a similar absorption spectrum in the red region indistinguishable by the spectrophotometric method, we also used the HPLC method. Up to about 3% degradation of Chl *a* to pheophytin *a* and a 2% degradation of Chl *b* to pheophytin *b* were detected. This conversion could originate in Chl degradation in spots where the leaf segments were cut from the blades. No degradation of Chls to other products such as chlorophyllides were seen for any of the three leaf types. This high Chl stability could be the result of its organization to proton-impermeable domains [29]. The heat conversion of violaxanthin to unknown xanthophylls was observed. For illustration, chromatograms of samples prepared from the control and heated IML leaves are displayed in Fig. 7. To see all pigments in one chromatogram, the Maxplot function was used for the presentation, i.e. each peak is displayed at the wavelength of its global absorption maximum. These findings, together with the data above, reveal that some spectral form of Chl *a* increases its emission in the second fluorescence rise in FTC.

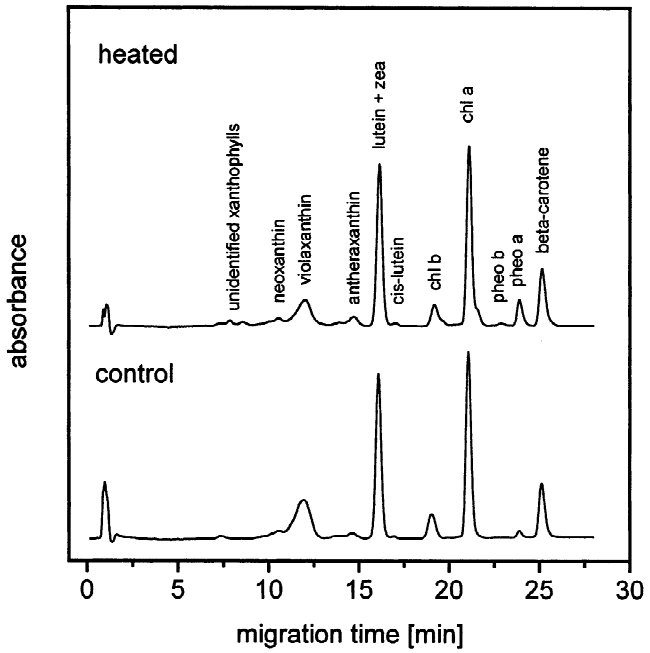

Fig. 7. High-performance liquid chromatography of pigment extracts from IML barley leaves at room temperature (control) and heated linearly to 75°C (heated). Chromatograms are displayed by the Maxplot function, i.e. each peak is displayed at the wavelength of its absorption maximum. Identification of peaks was performed according to their absorption spectra and migration distances.

4. Discussion

4.1. FTC to about 55°C

In plants, chloroplasts, and thylakoid membranes at room temperature the chlorophyll fluorescence is usually thought to originate from Chl *a* in the internal antennae of PSII, CP43 and CP47 (for a review, see Ref. [30]). However, it is probable that outer antenna complexes of PSII contribute to the room temperature spectra [31], as the PSII antenna is a very shallow energy funnel [32]. The question of whether chlorophyll fluorescence has the same origin within the whole temperature interval during heating of these samples has not been elucidated. Briantais et al. [12] applied the reversible radical pair model [33] for modeling the fluorescence rise to the M1 FTC maximum and suggested that the rise could originate from the Chl *a* of some minor LHCII antenna.

Our measurements with the fluorescence emission spectra during linear heating up to 55°C did not indicate any spectral change. The spectra were very similar for barley leaves with different LHC contents (Figs. 2 and 3). Very similar emission, excitation, and absorption spectra at 25–55°C (Figs. 2, 3, 5 and 6) reveal that the changes in fluorescence intensity at these temperatures mostly reflect changes of the fluorescence yield of the emitting forms. The basic features of the FTCs of all three leaf types were similar up to 55°C at preferential Chl *a* excitation (Fig. 1). However, the sharpness of the fluorescence rise to the M1 maximum and the relative height and position of the M1 maximum differed. These details were not studied in this project and may be associated with a stabilizing effect of LHC on the oxygen evolving complex in PSII [35] and the extent of variable fluorescence [17].

The fluorescence decrease detected from the temperature of the M1 FTC maximum to about 55°C can be explained as a continuation of the gradual heat-induced decrease of the maximal fluorescence (F_M) [5,15]. This was originally interpreted as a functional detachment of LHCII from PSII decreasing the absorption cross section of PSII [5]. However, this decrease was also observed with samples with significantly reduced LHCII content (*chlorina f2* barley, IML leaves, stromal thylakoids or PSII cores) (Fig. 1) [4,23,34]. Recently, Pospíšil and co-workers [15,20] have shown that the gradual decrease of F_M can be seen within a much larger temperature range (from about −100°C) and can be modeled as a purely physical effect. In support of this, the phase at 51–55°C of FTC was shown to be reversible [36].

4.2. The second fluorescence rise in FTC

Our HPLC data clearly show that Chl *a* in barley leaves does not degrade significantly during linear heating up to 75°C (see Fig. 7) and thus may be responsible for the second fluorescence rise in FTC. A direct result, the difference excitation spectrum for the IML leaves (see spectrum 61–59°C, Fig. 2E), strongly supports this.

The fluorescence emission spectra and their differences obtained for leaves with reduced LHC contents at 55–61°C showed that the second fluorescence rise in FTC originates from the emission form with an emission maximum at about 675 nm (Figs. 2 and 3). This attribution was strengthened by measurement with barley leaves greening in continuous light (Fig. 4). However, the emission maxima in this temperature range were detected at wavelengths above 675 nm for green, IML and *chlorina f2* leaves. This was most probably due to the overlap of the 675 nm emission with the emission peaking at about 685 nm, as was mainly evident in the difference emission spectra measured on green leaves within 55–61°C (Fig. 3E).

The short-wavelength emission Chl *a* form cannot be attributed to some known intact Chl *a*-containing antenna. Thus, the second fluorescence rise in FTC does not reflect any change in the distribution of excitations between antenna complexes, but rather their structural changes. The correlation between the main shifts of the absorption and emission maxima during linear heating for the used leaf types (Fig. 6) confirms this suggestion.

The sharpness of the second fluorescence rise indicates a

phase transition within the thylakoid membrane. Actually, within the temperature range of this rise, plasmalema and chloroplast membranes lose their semipermeability [37,38], thylakoids burst [39] and form condensed structures [40]. The Q_{10} parameter, describing the kinetics of the process reflected by the fluorescence rise, was estimated to be 7 [41]. This value indicates a protein denaturation process [1] when the Chl–protein interaction changes. The irreversibility of the second fluorescence rise in FTC [41] supports its association with the denaturation of some protein complex with Chl *a*.

Another question is the origin of the fluorescing Chl *a* molecules with weakened interaction with proteins and their interaction with the surroundings at temperatures above 55°C. The denaturation temperature of the isolated PSII core is around 60°C [42], while isolated LHCII denatures above 70°C [43]. Although these temperatures may not correspond to the denaturation temperatures of these complexes in leaves, a higher thermostability of LHCII with respect to the PSII core can be expected. Thus, denaturation of the PSII core seems very likely to proceed during the fluorescence rise to M2 FTC. Gaevsky et al. [22] have shown that the high-temperature rise appeared in *Chlamydomonas* mutants lacking PSII or PSI. Therefore, the Chl *a* from both PS cores are probably responsible for the second fluorescence rise in FTC. This interpretation corresponds to the finding that the higher second fluorescence rise was observed in samples with reduced LHC contents, e.g. in stromal thylakoids [21], leaves grown in stronger light conditions [44], greening or intermittent light grown leaves [23,24]. Detached LHCs, probably not contributing to this rise, could play the role of a non-fluorescent inner filter in the M2 FTC region.

No second fluorescence rise was detected with isolated PSII cores [34] (also our unpublished data). This leads us to suggest that some thylakoid membrane components or processes around the membranes are also involved in the fluorescence rise.

4.3. Fluorescence changes above the second rise in FTC

The fluorescence decrease appearing above the second fluorescence rise in FTC is pronounced in leaves with higher LHC contents (Fig. 1) [24]. The changes in the emission spectra show that the fluorescence decrease corresponds to a gradual disappearance of the emission form peaking at about 685 nm, which looks very similar to the emission spectrum detected at 25–55°C (Figs. 2 and 3). This suggests that, in the M2 FTC region, at least two Chl *a* spectral forms emit fluorescence: the first, peaking at 675 nm, is responsible for the second fluorescence rise in FTC; and the second originates from unchanged emission forms of Chl *a*. The larger the content of LHCs in the leaves, the more pronounced is the latter form in the fluorescence emission above 60°C (Figs. 1–5).

The most simple explanation of the observed fluorescence decrease in the M2 FTC region is a gradual denaturation of PSII leading to the formation of quenching centers or non-fluorescing aggregates.

5. Conclusion

The fluorescence changes during FTC up to about 55°C probably reflect an increase of fluorescence yield of antennae due to progressive impairment of the PSII function. The second fluorescence rise comes from the short-wavelength Chl *a* spectral form with an emission maximum at 675 nm. The absorption and emission spectral changes during this fluorescence rise imply that the rise originates in Chl *a* molecules with weakened interactions with proteins. FTCs measured with barley leaves with different LHC contents support the idea that the fluorescing Chl *a* molecules come from photosystem cores. The fluorescence decrease at 61–75°C, pronounced in fully developed green leaves with a large amount of LHCs, corresponds to the gradual disappearance of the spectral form peaking at about 685 nm.

6. Abbreviations

F_M	maximal fluorescence
FTC	fluorescence temperature curve
Chl *a*(*b*)	chlorophyll *a*(*b*)
IML	intermittent light
LHC	light-harvesting complex
M1	first maximum in FTC
M2	second maximum (region) in FTC
PAR	photosynthetically active radiation
PPC	pigment–protein complex
PSI(II)	photosystem I(II)
Q_A	primary chinone acceptor of PSII
RCII	reaction center of PSII

Acknowledgements

We would like to thank P. Kamínková and Dr. P. Krchňák for their technical assistance, Prof. D. Simpson for providing us with seeds of *chlorina f2* barley, and K. Klem for reproduction of the barley mutant. We are grateful to Dr. P. Šiffel for helpful discussions. This research was supported by grants 204/98/P205 from the Grant Agency of the Czech Republic, No. 3150 3010 from Palacký University and CEZ:J14/98:N70000010 from the Ministry of Education of the Czech Republic. F.V. thanks the Ministry of Education of the Czech Republic for its support (grant VS96085).

References

[1] J. Levitt, Responses of Plants to Environmental Stresses, Academic Press, New York, 1980.

[2] G. Papageorgiou, Chlorophyll fluorescence: an intrinsic probe of photosynthesis, in: Govindjee (Ed.), Bioenergetics of Photosynthesis, Academic Press, New York, 1975, pp. 316–371.

[3] J. Lavorel, A.L. Etienne, In vivo chlorophyll fluorescence, in: J. Barber (Ed.), Primary Progress in Photosynthesis, Elsevier, North-Holland Biomedical Press, Amsterdam, 1977, pp. 203–268.

[4] U. Schreiber, J.A. Berry, Heat-induced changes of chlorophyll fluorescence in intact leaves correlated with damage of the photosynthetic apparatus, Planta 136 (1977) 233–238.

[5] U. Schreiber, P.A. Armond, Heat-induced changes of chlorophyll fluorescence in isolated chloroplasts and related heat-damage at the pigment level, Biochim. Biophys. Acta 502 (1978) 138–151.

[6] R. Kuropatwa, J. Nauš, M. Mašláň, L. Dvořák, Basic properties of chlorophyll fluorescence temperature curve in barley leaves, Photosynthetica 27 (1992) 129–138.

[7] J. Fiala, P. Ilík, J. Skotnica, J. Nauš, Spectral changes of chlorophyll fluorescence during linear heating of barley leaves, in: G. Garab (Ed.), Photosynthesis: Mechanisms and Effects, Vol. 1, Kluwer Academic, Dordrecht, 1998, pp. 433–436.

[8] U. Schreiber, K. Colbow, W. Vidaver, Analysis of temperature-jump chlorophyll fluorescence induction in plants, Biochim. Biophys. Acta 423 (1976) 249–263.

[9] P.A. Armond, U. Schreiber, O. Björkman, Photosynthetic acclimation to temperature in the desert shrub, *Larrea divaricata*. II. Light-harvesting efficiency and electron transport, Plant Physiol. 61 (1978) 411–415.

[10] J.-M. Ducruet, Y. Lemoine, Increased heat sensitivity of the photosynthetic apparatus in triazine-resistant biotypes from different plant species, Plant Cell Physiol. 26 (1985) 419–429.

[11] N.G. Bukhov, S.C. Sabat, P. Mohanty, Analysis of chlorophyll *a* fluorescence changes in weak light in heat treated *Amaranthus* chloroplasts, Photosynth. Res. 23 (1990) 81–87.

[12] J.M. Briantais, J. Dacosta, Y. Goulas, J.M. Ducruet, I. Moya, Heat stress induces in leaves an increase of the minimum level of chlorophyll fluorescence, F_0: a time-resolved analysis, Photosynth. Res. 48 (1996) 189–196.

[13] Y. Yamane, Y. Kashino, H. Koike, K. Satoh, Increases in the fluorescence F_0 level and reversible inhibition of photosystem II reaction center by high temperature treatments in higher plants, Photosynth. Res. 52 (1997) 57–64.

[14] P. Pospíšil, E. Tyystjärvi, Molecular mechanism of high-temperature-induced inhibition of acceptor side of photosystem II, Photosynth. Res. 62 (1999) 55–66.

[15] P. Pospíšil, J. Nauš, Theoretical simulation of temperature induced increase of quantum yield of minimum chlorophyll fluorescence $\Phi_{F(0)}$, J. Theor. Biol. 193 (1998) 125–130.

[16] M. Havaux, Characterization of thermal damage to the photosynthetic electron transport in potato leaves, Plant Sci. 94 (1993) 19–33.

[17] J. Nauš, R. Kuropatwa, T. Klinkovský, P. Ilík, J. Lattová, Z. Pavlová, Heat injury of barley leaves detected by the chlorophyll fluorescence temperature curve, Biochim. Biophys. Acta 1101 (1992) 359–362.

[18] M. Havaux, Rapid photosynthetic adaptation to heat stress triggered in potato leaves by moderately elevated temperature, Plant Cell Environ. 16 (1993) 461–467.

[19] H.W. Bilger, U. Schreiber, O.L. Lange, Determination of leaf heat resistance: comparative investigation of chlorophyll fluorescence changes and tissue necrosis methods, Oecologia 63 (1984) 256–262.

[20] P. Pospíšil, J. Skotnica, J. Nauš, Low and high temperature dependence of minimum F_0 and maximum F_m chlorophyll fluorescence in vivo, Biochim. Biophys. Acta 1363 (1998) 95–99.

[21] W.J.S. Downton, J.A. Berry, Chlorophyll fluorescence at high temperatures, Biochim. Biophys. Acta 679 (1982) 474–478.

[22] N.A. Gaevsky, V.G. Ladygin, V.M. Gold, New data on the nature of high-temperature rise of chlorophyll fluorescence, Fiziol. Rast. 36 (1989) 274–277, in Russian.

[23] P. Ilík, J. Nauš, V. Špunda, M. Čajánek, D. Cikánek, P. Pospíšil, High-temperature chlorophyll fluorescence rise within 61–67°C. Spectroscopic study with intermittent light grown leaves, J. Photochem. Photobiol. B: Biol. 39 (1997) 243–248.

[24] P. Ilík, J. Nauš, D. Cikánek, R. Novotný, Chlorophyll fluorescence changes at high temperatures induced by linear heating of greening barley leaves, Photosynth. Res. 44 (1995) 271–275.

[25] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Methods Enzymol. 48 (1987) 350–382.

[26] B. Brossmann, J. Knoetzel, S. Jansson, Screening of chlorina mutants (*Hordeum vulgare* L.) with antibodies against light-harvesting proteins of PSI and PSII: absence of specific antenna proteins, Photosynth. Res. 52 (1997) 127–136.

[27] K. Ohashi, A. Murakami, A. Tanaka, H. Tsuji, F. Yoshihiko, Developmental changes in amount of thylakoid components in plastids of barley leaves, Plant Cell Physiol. 33 (1992) 371–377.

[28] T.C. Vogelmann, Penetration of light into plants, Photochem. Photobiol. 50 (1989) 895–902.

[29] D. Siefermann-Harms, H. Ninnemann, Pigments organization in the light-harvesting-chlorophyll-a/b protein complex of lettuce chloroplasts. Evidence obtained from protection of the chlorophylls against proton attack and from excitation energy transfer, Photochem. Photobiol. 35 (1982) 719–731.

[30] Govindjee, Sixty-three years since Kautsky: chlorophyll *a* fluorescence, Austr. J. Plant Physiol. 22 (1995) 131–160.

[31] R.C. Jennings, F.M. Garlaschi, R. Bassi, G. Zucchelli, A. Vianelli, P. Dainese, A study of photosystem II emission in terms of the antenna chlorophyll–protein complexes, Biochim. Biophys. Acta 1183 (1993) 194–200.

[32] H. Dau, K. Sauer, Exciton equilibration and photosystem II exciton dynamics — A fluorescence study in photosystem II membrane particles of spinach, Biochim. Biophys. Acta 1273 (1996) 175–190.

[33] G.H. Schatz, H. Brock, A.R. Holzwarth, Kinetic and energetic model of the primary processes in photosystem II, Biophys. J. 54 (1988) 397–405.

[34] W.P. Williams, K. Gounaris, Stabilization of PS-II-mediated electron transport in oxygen-evolving PS-II core preparations by the addition of compatible co-solutes, Biochim. Biophys. Acta 1100 (1992) 92–97.

[35] M. Havaux, F. Tardy, Thermostability and photostability of photosystem II in leaves of the Chlorina-f2 barley mutant deficient in light harvesting chlorophyll a/b protein complexes, Plant Physiol. 113 (1997) 913–923.

[36] J. Nauš, M. Šnáblová, L. Dvořák, Z. Kupka, Temperature dependence of in vivo chlorophyll fluorescence, Acta UPOL, Fac. Rer. Nat., Physica XXV 85 (1986) 47–63.

[37] K.A. Santarius, M. Exner, R. Thebud-Lassak, Effects of high temperature on photosynthetic apparatus in isolated mesophyll protoplasts of *Valerianella locusta* (L.) Betche, Photosynthetica 25 (1991) 17–26.

[38] D.C. McCain, J. Croxdale, J.L. Markley, Thermal damage to chloroplast envelope membrane, Plant Physiol. 90 (1989) 606–609.

[39] K. Gounaris, A.P. Brain, P.J. Quinn, W.P. Williams, Structural reorganization of chloroplast thylakoid membranes in response to heat stress, Biochim. Biophys. Acta 766 (1984) 198–208.

[40] P. Ilík, J. Nauš, D. Cikánek, V. Špunda, M. Čajánek, R. Novotný, D. Žák, An investigation of high-temperature region of chlorophyll fluorescence temperature curve, in: P. Mathis (Ed.), Photosynthesis: From Light to Biosphere, Vol. IV, Kluwer Academic, Dordrecht, 1995, pp. 885–888.

[41] E.A. Kuznetsova, Study of temperature dependence of plant leaf

fluorescence, Dokl. Acad. Nauk SSSR 256 (1981) 893–894, in Russian.
[42] K.A. Smith, P.S. Low, Identification and partial characterization of the denaturation transition of the photosystem II reaction center of spinach chloroplast membranes, Plant Physiol. 90 (1989) 575–581.
[43] K.A. Smith, B.K. Ardelt, N.P.A. Huner, M. Król, E. Myscich, P.S. Low, Identification and partial characterization of the denaturation transition of the light harvesting complex II of spinach chloroplast membranes, Plant Physiol. 90 (1989) 492–499.
[44] V. Špunda, J. Kalina, J. Nauš, R. Kuropatwa, M. Mašláň, M. Marek, Responses of photosystem 2 photochemistry and pigment composition in needles of Norway spruce saplings to increased radiation level, Photosynthetica 28 (1993) 401–413.