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Energy transfer processes in chlorophyll *f*-containing cyanobacteria using time-resolved fluorescence spectroscopy on intact cells ☆

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ABSTRACT

We examined energy transfer dynamics in the unique chlorophyll (Chl) *f*-containing cyanobacterium *Halomicronema hongdechloris*. The absorption band of Chl *f* appeared during cultivation of this organism under far-red light. The absorption maximum of Chl *f* in organic solvents occurs at a wavelength of approximately 40 nm longer than that of Chl *a*. *In vivo*, the cells display a new absorption band at approximately 730 nm at 298 K, which is at a significantly longer wavelength than that of Chl *a*. We primarily assigned this band to a long wavelength form of Chl *a*. The function of Chl *f* is currently unknown. We measured the fluorescence of cells using time-resolved fluorescence spectroscopy in the picosecond-to-nanosecond time range and found clear differences in fluorescence properties between the cells that contained Chl *f* and the cells that did not. After excitation, the fluorescence peaks of photosystem I and photosystem II appeared quickly but diminished immediately. A unique fluorescence peak located at 748 nm subsequently appeared in cells containing Chl *f*. This finding strongly suggests that the Chl *f* in this alga exists in photosystem I and II complexes and is located close to each molecule of Chl *a*. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

1. Introduction

Chlorophylls (Chls) play important roles in light harvesting, energy transfer, and electron transfer during photosynthesis. Various Chl species exist among photosynthetic organisms. General oxygenic phototrophs, such as higher plants, algae, and cyanobacteria, contain Chls *a*, *b*, and *c* as well as 3, 8-divinyl Chls *a* and *b*. In 1997, Miyashita et al. [1] found a red-absorbing Chl, Chl *d*, within the unicellular cyanobacteria *Acaryochloris marina*. Subsequently, the existence of Chl *d* was recognized in organisms worldwide [2–6]. Chl *d* serves as both an antenna pigment and as a primary electron donor [7–13]. Recently, Chen et al. [14] found a more red-shifted Chl, Chl *f*, within the filamentous cyanobacteria *Halomicronema hongdechloris*, which inhabits stromatolites at Hamelin Pool, Western Australia. The Chl content of *H. hongdechloris* varied under different light conditions. When under far-red light (>700 nm), the Chl *f* content increased to 10%–12.5% of the total Chl. When under white fluorescent light, the Chl *f* content decreased negligibly and the phycobilisome content increased [15]. In addition, the content of β-carotene (β-Car) increased during growth under far-red light. The absorption maximum of Chl *f* in organic solvents occurs at a wavelength approximately 40 nm longer than that of Chl *a*. The structure of Chl *f* was determined to be [2-formyl]-Chl *a* using mass spectroscopy and nuclear magnetic resonance (NMR) analysis [14,16].

The photochemical and photophysical functions of Chl *f* are not known. Therefore, we decided to use time-resolved fluorescence spectroscopy for an investigation of intact cells. Time-resolved fluorescence spectroscopy, with picosecond-to-nanosecond time resolution, has been used to monitor changes in Chl fluorescence as well as energy transfer from Chl *a* to Chl *f* in photosynthetic complexes. This approach will provide some initial information concerning the role of Chl *f* as a photosynthetic pigment in oxygenic photosynthesis.

Abbreviations: Chl, chlorophyll; PS, photosystem; TRFS, time-resolved fluorescence spectrum (spectra)

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2. Materials and methods

2.1. Culture conditions

H. hongdechloris was cultured in an 8-liter culture bottle containing IMK medium at 298 K, with stirring. Air was continuously supplied through a filter (Millipore Millex FG-50). The cells lacking Chl *f* were cultured under fluorescent light. The cells containing Chl *f* were cultured using a far-red, 740-nm LED lamp (Edison OPTO Corporation, Cherry Red). Chl concentrations were determined in 100% methanol using the extinction coefficients of $\varepsilon_{665.5} = 70.02$ and $\varepsilon_{707.0} = 71.11\ mM^{-1}\ cm^{-1}$ for Chl *a* and Chl *f*, respectively [17].

The absorption spectra were recorded using a spectrophotometer (JASCO V-660, Japan) equipped with an integrating sphere unit at 298 K and 77 K. A custom-made Dewar system was employed for measurements at 77 K. Steady-state fluorescence spectra were recorded using a spectrofluorometer (Shimadzu RF-5300PC) with a commercial Dewar system at 77 K. The spectral sensitivity of the fluorometer was corrected using the radiation profile of the standard lamp. The absorbance of the steady-state absorption were adjusted by 0.1–0.3 at Q_y band (pathlength = 10 mm). For steady-state fluorescence measurements (pathlength = 1 mm), fluorescence from the surface of sample frozen in liquid nitrogen was monitored a right angle with respect to the incident light. This method does not cause fluorescence self-absorption during measurements.

Time-resolved fluorescence spectra were measured at 77 K using the time-correlated single-photon counting method, as previously described [10,18,19]. All pigments were simultaneously excited using an excitation wavelength of 425 nm. The excitation laser intensity was adjusted to obtain fluorescence signals of less than 20,000 counts per second around the fluorescence peak wavelengths. Using this intensity, laser excitation with a repetition rate of 2.9 MHz did not damage the samples. Using these experimental conditions, annihilation effects can be ruled out [20]. For time-resolved measurements, fluorescence from the surface of sample frozen in liquid nitrogen was monitored at a right angle with respect to the incident light [21]. Fluorescence lifetimes were estimated by a convolution calculation [22].

3. Results

3.1. Steady-state absorption and fluorescence spectra

Fig. 1 shows the absorption spectra of intact cells cultured under a white fluorescent lamp (hereafter referred to as white light) or a far-red light at 298 K and 77 K. Under white light cultivation at 298 K (Fig. 1, dotted line), the Q_y maximum of Chl *a* was located at 676 nm and the phycobilisome peak maximum was located at 626.6 nm (Fig. 1, solid line). This absorption spectrum is similar to that of common cyanobacteria, such as *Synechocystis* sp. PCC6803 [23]. The methanol extract from the cells cultured under white light did not show the presence of Chl *f* (Fig. 1C), and an HPLC-based pigment analysis barely detected Chl *f* (data not shown). Cells cultured under far-red light displayed a Q_y maximum at 676 nm that is characteristic of Chl *a*, which is the same as that in cells cultured under white light. However, a broader shoulder at approximately 730 nm was also present, which indicated the presence of Chl *f* (Fig. 1, dotted line). The intensity of the phycobilisome band was much lower than that in cells cultured under white light. The content of carotenoids was greater in cells cultured under far-red light conditions. This tendency was consistent with a previous report [15]. It is possible that cells cultured under far-red light require a photo-protection system. The absorption spectra of the cells cultured under far-red light were similar to those presented in a previous report [15]. The spectrum of a methanol extract obtained from the cells cultured under far-red light revealed the presence of Chl *f* (Fig. 1D) and an HPLC-based pigment analysis also revealed the presence of approximately 10% Chl *f* (data not shown).

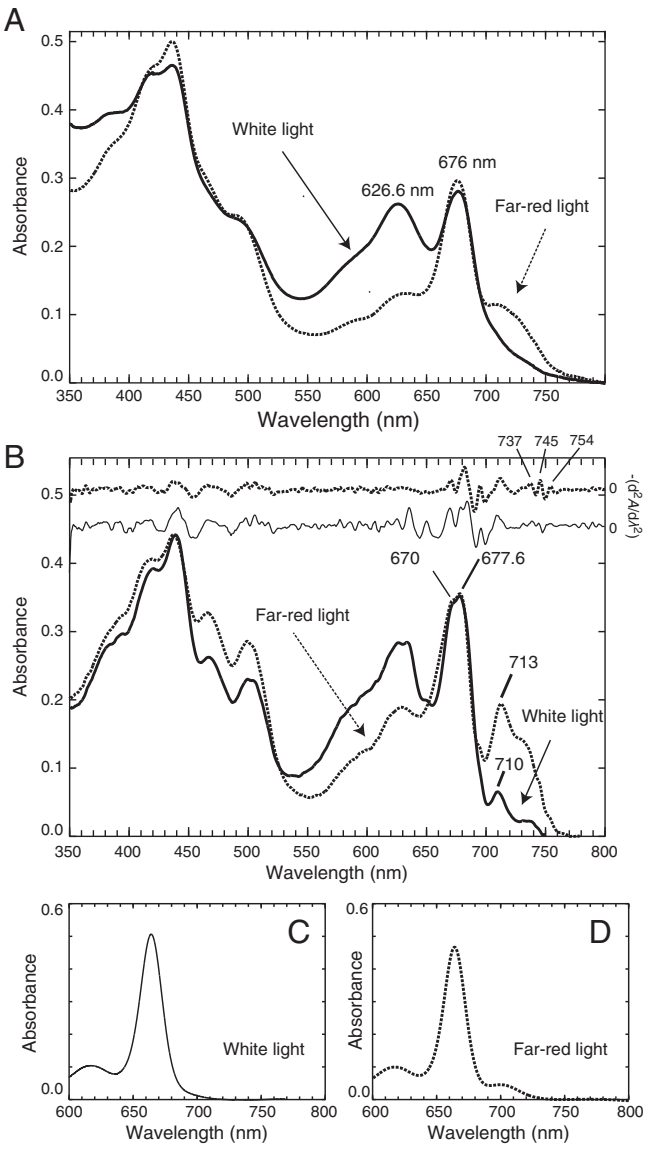

Fig. 1. Absorption spectra of the cells under white or far-red cultivated light. A: Absorption spectrum at 298 K of the cells under white light (solid) and far-red light (dotted). B: Absorption spectra at 77 K, and the second derivative spectra of the cells under white light (solid) and far-red light (dotted). C: Absorption spectrum at 298 K of a methanol extract from the cells cultured under white light. D: Absorption spectrum at 298 K of a methanol extract from the cells cultured under white light. The chlorophyll concentrations were adjusted to approximately 4 μg Chl mL^{-1}.

At 77 K, several absorption bands derived from Chls were resolved in the low-temperature spectrum (Fig. 1B). Chl *a*, peakings at 677.6 and 670 nm in the Q_y band, was present in cells cultured under both light conditions. Multiple Chl forms are common in the *in vivo* and *in vitro* absorption spectra of photosynthetic oxygenic organisms [24–26]. A unique peak at approximately 710 nm was resolved in the cells cultured under white light conditions.

An absorption band at 713 nm was also present at 77 K in cells cultured under far-red light, but its intensity was approximately 2.5 times greater than that in cells cultured under white light (Fig. 1B, dotted line). The peak maximum was red-shifted by 3 nm compared with the cells cultured under white light. The peak height of the 713-nm band was approximately half of that of the 677-nm main Chl *a* band. Because the amount of Chl *f* in cells cultured under far-red light was 10% of the total Chl, we also primarily assigned this band to Chl *a*.

Changes in the photosystem (PS) I/PS II ratio have also been reported in cyanobacteria under different light conditions [27–29]. We performed

an antibody analysis of the thylakoids of cells cultured under both light conditions using anti-PsaA/B [30] and anti-PsbA [25,31] antibodies. The amount of PS I was similar between cells cultured under white and far-red lights, whereas the amount of PS II in the cells cultured under far-red light was slightly greater than that in the cells cultured under white light (data not shown).

3.2. Fluorescence spectra

The steady-state fluorescence spectrum of the cells cultured under white light, obtained at 77 K with excitation at 425 nm (Fig. 2, solid line), was dominated by three bands at 646 nm, 684 nm, and approximately 730 nm, arising from phycobilisome, PS II and PS I, respectively [32]. The band at 730 nm was broad, which is similar to a previous report [15]. The unique absorption band of Chl *a* at 710 nm was probably responsible for some of the broadening. On the other hand, a fluorescence band at 745 nm dominated the spectrum of the cells cultured under far-red light. The other two bands, which originate from phycobilisome and PS II, were minor contributors located at 646 nm and 684 nm, respectively (Fig. 2, dotted line). This clear difference arises from the existence of Chl *f* in the cells cultured under far-red light.

3.3. Time-resolved fluorescence spectra

Time-resolved fluorescence spectroscopy (TRSF) is one of the most useful techniques to investigate energy transfer processed in photosynthetic systems because it only detects signals from excited states. There were clear differences between the TRFS at 77 K of the *H. hongdechloris* cells cultured under white light and *H. hongdechloris* cells cultured under far-red light. Spectra normalized to their maximum intensities are shown as individual traces in Fig. 3. In cells cultured under white light, the PS II fluorescence bands at approximately 685 nm (F685) and a PS I band at approximately 730 nm (F730) were clearly detected immediately following excitation. With time, F685 shifted to 695 nm (F695). These properties were very similar to those of common cyanobacteria [23]. Approximately 1 ns after excitation, a 742-nm fluorescence band (F742) appeared, and its relative intensity decreased with time. The lifetime of this band was analyzed to be approximately 1 ns (Fig. 3 and Table 1). At a later stage, F742 was diminished, but F730 remained. This shows that a partial energy transfer occurred from F730 to F742. The general fluorescence band of PS I appears at 720–730 nm. Therefore, F742 is a unique band of this alga that may originate from the Chl *a* absorption band at 710 nm. Judging from the TRFS of the cells cultured under white light, delayed fluorescence were observed in the Chl fluorescence region after 10 ns, showing three peaks at 695, 730 and 742 nm. This suggests that the excitation energy is shared between PS I and PS II in this alga under white light conditions [19,33].

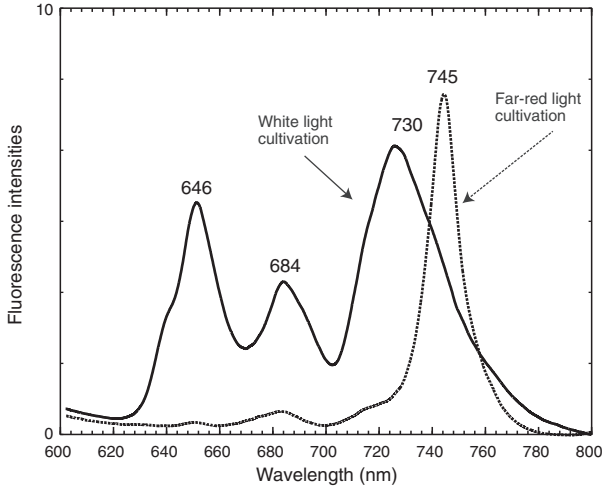

Fig. 2. Fluorescence spectra upon the excitation of cells cultured under white or far-red light at 425 nm at 77 K. Cells cultured under white light (solid line), and far-red light cells (dotted line). The chlorophyll concentrations were adjusted to approximately 3 μg Chl mL^{-1}.

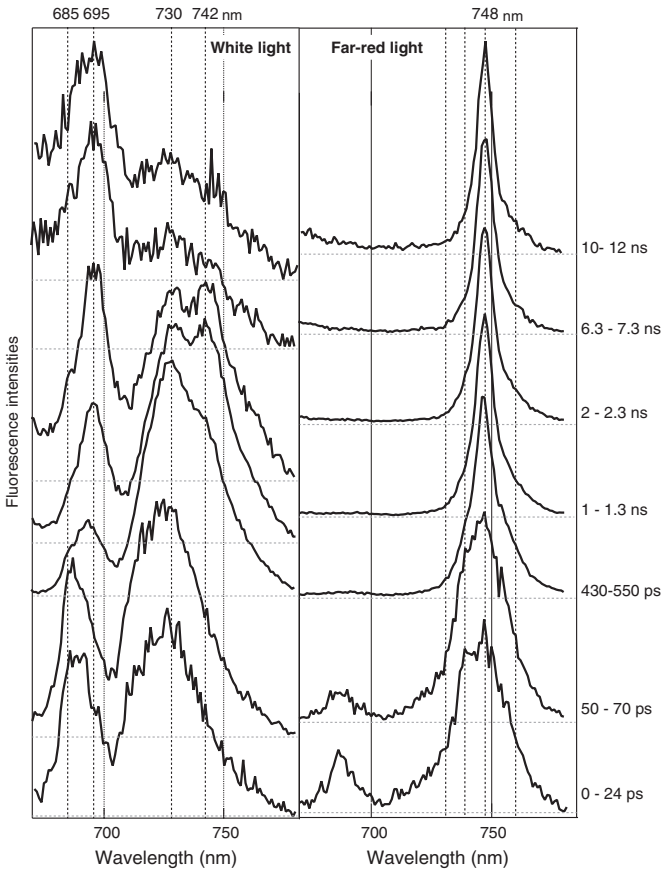

Fig. 3. Time resolved fluorescence spectra (TRFS). Left side: normalized TRFS, (670–800 nm) of the cells cultured under white light. Right hand: normalized TRFS (670–800 nm) of the cells under far-red light. The chlorophyll concentrations were adjusted to approximately 2 μg Chl mL^{-1}. The individual traces are normalized to their maximum intensities.

In the cells cultured under far-red light, F685 and a broad 748 nm florescence band (F748) appeared initially, and then F685 diminished immediately. It was shown that a rapid energy transfer occurred from F685 to F748. The bandwidth of the 748 nm band was initially broad. The band shape suggested that at least three components were present. The main band at 77 K (F748 which was assigned to Chl *f*) most likely arises from the absorption peak in the wavelength region longer than 720 nm that is characteristic of cells cultured under far-red light. Another of the three bands is most likely F730, which is assigned to the red (low energy) Chl *a* of PS I. However, F730 diminished within 2 ns after excitation. The remaining component, which is located at approximately 760 nm, may arise from an absorption peak at approximately 740–750 nm. This component also decreased in intensity soon after 1 ns. This time-dependent decrease in relative intensity at approximately 760 nm did not correspond to the rise lifetime at 745 nm (40 ps, Table 1). Judging from the time-resolved fluorescence spectra and its related table, it is difficult to transfer energy from 754 nm to 745 nm at 77 K.

4. Discussion

4.1. Absorption spectrum

In this study, we revealed the absorption and fluorescence properties from Chl *f*-containing cyanobacteria *H. hongdechloris*. At 77 K, a

Table 1
Fluorescence lifetime and its amplitude after excitation at 425 nm.

Cell	Wavelength (nm)	Lifetime (Amplitude)			
White light	685	150 ps (0.702)	520 ps (0.191)	1.8 ns (0.096)	5.1 ns (0.011)
	745	70 ps (−0.959)	400 ps (0.731)	1.0 ns (0.255)	3.0 ns (0.014)
Far-red light	685	<10 ps (0.857)	130 ps (0.111)	1.1 ns (0.023)	4.4 ns (0.009)
	745	40 ps (−0.885)	360 ps (0.379)	1.5 ns (0.415)	3.2 ns (0.206)

distinct absorption peak at approximately 710–713 nm was resolved in the cells cultured under both light conditions. This band was absent in *Synechocystis* sp. PCC6803. We assigned this band to a long wavelength form of Chl *a* because Chl *f* is not present under white light cultivation. Shubin et al. already reported this type of Chl *a* band in the filamentous cyanobacteria *Arthrospira* (*Spirulina*) *platensis* [34,35]. Cells cultured under white light showed an additional chlorophyll band at 730–740 nm (Fig. 1B, solid line). This band was also absent in *Synechocystis* sp. PCC6803 and Shubin et al. also reported this type of Chl *a* band in the filamentous cyanobacteria *A. platensis* [34,35]. The presence of Chl *f* was barely detectable in the methanol extract of cells cultured under white light (Fig. 1C). Therefore, we also assigned this long wavelength form Chl to Chl *a*. In photosystem I, the existence of red Chls *a* were reported by many groups, and those localizations were discussed in crystallographic structural analysis [36–40]. These red Chls *a* were located at peripheral position of PsaA and PsaB [37] and the fluorescence maximum of these red Chls *a* were approximately 720–730 nm. However, in *A. platensis*, apparent absorption peaks of the long wavelength form of Chls *a* were observed at 710–740 nm and these bands have not thus far been observed in other cyanobacteria (except for in this study). These long wavelength forms of Chls *a* of *A. platensis* were most likely located at an adjacent area between the PS I monomers [33]; the fluorescence maxima were located at 760 nm [41]. Thus, there was an apparent difference between red Chls *a* and the long wavelength form of Chl *a*. However, in *H. hongdechloris*, the long wavelength form of Chl *a* of the fluorescence maximum was not apparent at 760 nm. Thus, the long wavelength forms of Chl *a* of *H. hongdechloris* and *A. platensis* are different. The structure of the entire genome of *A. platensis* was recently determined [42]. However, the sequence of the *H. hongdechloris* genome has not been determined. Therefore, the location of the long wavelength form of Chl *a* in *H. hongdechloris* is currently unclear.

At 77 K in the cells cultured under far-red light, at least 3 peaks above 730 nm were recognized in the second derivative spectrum of the cells cultured under far-red light (737, 745, and 754 nm). Some of these peaks overlapped with Chl *a* in the absorption spectrum of the cells cultured under white light. However, the peak maxima in the second derivative absorption spectra differed slightly between the cells cultured under white and far-red lights. Therefore, these bands at 745 and 754 nm contained some contribution from Chl *f*.

4.2. Time-resolved fluorescence spectra

In the steady-state fluorescence spectrum at 77 K, a fluorescence band at 745 nm dominated the spectrum of the cells cultured under far-red light (Fig. 3). This unique fluorescence band arises from the existence of Chl *f*. In the time-resolved fluorescence spectra, the fluorescence band that arises from the red Chl *a* of PS II, which is F685 or F695, and the fluorescence band that arises from the red Chl *a* of PS I, which is F730, remained after a later period in the cells cultured under white light. However, these bands diminished immediately in cells cultured under far-red light. This implies that rapid energy transfer occurred from Chl *a* to Chl *f* in both photosystems. Therefore, the localization of Chl *f* must be very close to the red Chl *a* of PS I and of PS II, respectively. Moreover, the fluorescence rise lifetime component (40 ps) in the cells cultured under far-red light is faster than that of white light (70 ps) at 745 nm (Table 1). There could be a larger amount of Chl *f* in cells cultured under far-red light than red Chl *a* in cells cultured under white light. In the Chl *f* fluorescence, the amplitudes of longer lifetimes are higher, compared with those in the red Chl *a* fluorescence (Table 1), suggesting that the functions of Chl *f* and the Chl *a* are different. Therefore, Chl *f* seem to work as energy trap at 77 K. By combining the close proximity to Chl *a* and the longer lifetime of Chl *f*, Chl *f* may work as an energy donor of Chl *a* at a physiological temperature. Mimuro et al. have reported such an uphill energy transfer in a Chl *d*-dominated cyanobacterium [43].

Table 1 summarizes the lifetime values and their amplitudes at 685 nm and 745 nm. The former wavelength is assigned due to the Chl *a* fluorescence band, whereas the latter is due to the tail of Chl *a* fluorescence for the cells under white light and the Chl *f* band for the cells under far-red light. The amplitudes of the first and second components (<10 ps and 130 ps) of F685 contributed approximately 97% of the total amplitude to the fluorescence decay of the cells cultured under far-red light (Table 1). In the cells cultured under white light, the 150-ps component constituted approximately 70% of the total amplitude. These results indicate that a new energy transfer pathway from F685 was established in the cells under far-red light. In the fluorescence kinetics at 745 nm, a shorter rise time was observed for the cells under far-red light. It is likely that Chl *f* is located in the proximity of Chl *a*. Therefore, the rapid transfer of almost all the energy in Chl *a* to Chl *f* occurred in the cells cultured under far-red light. As a result, surprisingly, only F748 remained after 2 ns in cells cultured under far-red light.

A delayed (in the nanosecond time region) fluorescence component at approximately 683 nm is known to originate from the excited state by direct charge recombination between the special pair and the primary electron donor of PS II [25,44]. We further analyzed the delayed fluorescence in the nanosecond time region using decay curves at 77 K (Fig. 4). Basically, four lifetime components were necessary to fit the fluorescence rise and decay profiles in each of the cultivation conditions. However, to improve the fitting condition, a long-lived component with a time constant longer than 10 ns was required for the fluorescence kinetics at 685 nm. The lifetime of uncoupled Chl has been reported to be approximately 6 ns [45–47]. In a decay curve analysis, no lifetime component of 6 ns was observed at either 685 or 745 nm. Therefore, it was confirmed that the Chl *f* in this cyanobacterium is functional and is not released.

The relative amplitude of the delayed fluorescence was small in cells due to the large antenna size. However, it is possible to detect the decay curve of this fluorescence. Judging from the decay curve of fluorescence monitored at 685 nm (Fig. 4), the amplitude of the 10–20-ns components in cells cultured under far-red light is smaller than that of similar components from cells cultured under white light. Therefore, Chl *f* appears to exert a negligible effect on the primary electron transfer process in PS II. However, the amplitude was very small; therefore, further experiments are necessary at clarify this point using isolated PS complexes.

5. Summary

In this study, the spectroscopic features of the *H. hongdechloris* were demonstrated to be significantly different from those of common cyanobacteria because unique absorption and fluorescence bands were observed. We succeeded for the first time to measure the time-resolved fluorescence properties of the Chl *f*-containing

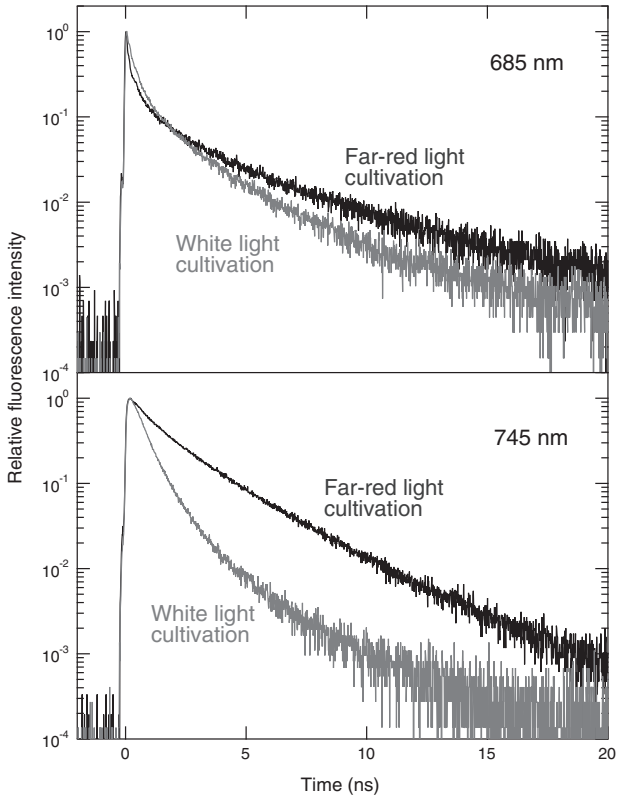

Fig. 4. Chlorophyll fluorescence decay curves at 77 K for *H. hongdechloris* under white light (black) and far-red light (gray) monitored at 685 nm (upper trace) and 745 nm (lower trace). The excitation wavelength was 425 nm. The chlorophyll concentration was approximately 2 μg mL^{-1}.

cyanobacterium *H. hongdechloris*. In particular, our analysis revealed that a rapid energy transfer occurred from red Chl *a* to Chl *f* in the PS I and PS II complexes. The Chl *f* works functionally in the antenna system of this cyanobacterium.

These findings naturally lead to future work that will be designed to study the transfer of excitation energy on the femtosecond time scale and to elucidate the localization of Chl *f* in isolated PS I and PS II complexes from the Chl *f*-containing cyanobacterium *H. hongdechloris*.

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