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REGULAR PAPER

Characterization of chlorophyll–protein complexes isolated from a Siphonous green alga, *Bryopsis corticulans*

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Abstract Six chlorophyll–protein complexes are isolated from thylakoid membranes of *Bryopsis corticulans* by dodecyl-β-D-maltoside polyacrylamide gel electrophoresis. Unlike that of higher plants, the 77 K fluorescence emission spectrum of the CP1 band, the PSI core complexes of *B. corticulans*, presents two peaks, one at 675 nm and the other at 715–717 nm. The emission peak at 715–717 nm is slightly higher than that at 675 nm in the CP1 band when excited at 438 or 540 nm. However, the peak at 715 nm is obviously lower than that at 675 nm when excited at 480 nm. The excitation spectra of CP1 demonstrate that the peak at 675 nm is mainly attributed to energy from Chl *b* while it is the energy from Chl *a* that plays an important role in exciting the peak at 715–717 nm. Siphonaxanthin is found to contribute to both the 675 nm and 715–717 nm peaks. We propose from the above results that chlorophyll *a* and siphonaxanthin are mainly responsible for the transfer of energy to the far-red region of PSI while it is Chl *b* that contributes most of the transfer of energy to the red region of PSI. The analysis of chlorophyll composition and spectral characteristics of LHCP[1] and LHCP[3] also indicate that higher content of Chl *b* and siphonaxanthin, mainly presented in LHCP[1], the trimeric form of LHCII, are evolved by *B. corticulans* to absorb an appropriate amount of light energy so as to adapt to their natural habitats.

Keywords *Bryopsis corticulans* · Chlorophyll–protein complexes · Siphonaceous green algae · Siphonaxanthin and siphonein

Abbreviations

Chl	Chlorophyll
CP	Chlorophyll protein complex
Cpa	Chlorophyll protein complex of PSII core
β-DM	Dodecyl-β-D-maltoside
LHCI	Light-harvesting complex I
LHCII	Light-harvesting complex II
LHCP	Light-harvesting chlorophyll *a/b* protein complex
PAGE	Polyacrylamide gel electrophoresis
PSI	Photosystem I
PSII	Photosystem II

Introduction

Marine green algae possess pigment compositions that differ from those of higher plants for the adaptation to the light environments of their natural habitats in deeper water. With Siphonaxanthin and the associated esterified form, siphonein, a greater amount of Chl *b*, much more than those in freshwater algae and higher plants, is able to help absorbing the available green (500–550 nm) and blue-green light (Jeffrey 1965; Kageyama et al. 1977; Kleinig 1969; Ogawa et al. 1975; Yokohama et al. 1977; Yokohama 1981; Yokohama and Misonou 1980).

Measurements of fluorescence emission spectra of siphonaceous algae show that these marine green algae do not exhibit the 77 K fluorescence peak around 735 nm,

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which is the characteristic peak of PSI in higher plants and in some green algae as well (Anderson 1983, 1985). It therefore remains unclear how the siphonaceous algae modulate a balance in the light absorption between two photosystems to ensure an optimal utilization of the available light. Based on the results that the total antenna size of PSI exceeds that of PSII and on the pronounced 470 nm peak in the fluorescence excitation spectra of PSI complexes, recent studies lead to a hypothesis that LHCII is likely to serve as an antenna of PSI (Yamazaki et al. 2005, 2006). To prove this, further experimental evidence is needed. Indeed, siphonaxanthin, siphonein and larger amounts of chlorophyll *b* compared to chlorophyll *a*, in siphonaceous algae, are able to reinforce the light harvesting capacity of PSII and PSI, even to the blue-green and green regions (Anderson 1983, 1985; Nakayama et al. 1986, 1994; Nakayama and Okada 1990; Nakayama and Mimuro1994), but their roles in adjusting the energy transfer still need to be clarified. Furthermore, the role siphonaxanthin and siphonein play in adjusting the energy transfer to PSI seems to be neglected compared to that of Chl *b*, even though they are claimed to serve as antenna pigments of PSI (Anderson 1985). Hence, in the present research, a siphonaceous marine green alga, *Bryopsis corticulans*, inhabiting the intertidal zone, is used to prepare the chlorophyll–protein complexes by dodecyl-*β*-D-maltoside (*β*-DM) polyacrylamide gel electrophoresis (PAGE), with seven bands resolved and spectrally characterized. The characteristics of siphonaxanthin, siphonein and Chl *b* in each Chl–protein complexes, as well as their effects on modulating the excitation energy distribution, are investigated in detail.

Materials and methods

Plant materials and preparation of thylakoid membrane

B. corticulans was collected in the intertidal zone near Qingdao in China (36°04′N, 120°18′E). Intact chloroplasts were prepared from *B. corticulans* according to Yamazaki et al. (2005) with some modification. Fresh thalli were washed several times with cold double-distilled water and broken in a TSN buffer containing 50 mM Tris–HCl, pH 8.0, 0.3 M sucrose and 10 mM NaCl, followed by filtration through 8 layers of gauze and centrifugation at 8,000 *g* for 20 min. The pellets were collected and suspended in the TSN buffer and stored in liquid N_2 until use. All the procedures were performed in dim light at 4°C. Before use, the above chloroplasts were washed twice with 10 mM Hepes–NaOH (pH 7.4), 0.3 M sucrose, 2 mM NaCl, 2 mM $MgCl_2$, and 1 mM Ethylenediamine tetraacetic acid. Thylakoid membranes were prepared by osmotically disrupting chloroplasts in 10 mM Hepes–NaOH (pH 7.4) for 30 min at 0°C and collected by centrifugation at 38,000 *g* for 30 min. The hypotonic treatment was repeated three times, removing white starch grains at the bottom of the tubes after each centrifugation. The thylakoid membranes were quickly frozen in liquid nitrogen and stored at −80°C until use.

Chl concentrations and Chl *a/b* ratios were determined in 80% acetone (Porra et al. 1989).

Separation of pigment–protein complexes

The non-denaturing *β*-DM-PAGE was used for resolution of chlorophyll-protein according to the procedures in Anderson (1980) and Davis (1964) with some modifications (Yamazaki et al. 2006; Varsano et al. 2006). The separating gel contained 0.42 M Tris–HCl (pH 8.8), 0.05% *β*-DM with an acrylamide of 5% (the acrylamide to N, N′-methylenebisacrylamide weight ratio was 37.5:1), 0.07% ammonium persulphate, 0.03% N,N,N′,N′-tetramethylenediamine. The stacking gel contained 56 mM Tris–HCl (pH 6.8), 0.05% *β*-DM, 3% acrylamide, 0.07% ammonium persulphate, 0.1% N,N,N′,N′-tetramethylenediamine. *B. corticulans* thylakoids (0.9 mg Chl/ml) were solubilized in 0.15 M Tris (pH 6.8), 0.3 M sucrose, 10% glycerol and 0.8% *β*-DM with a *β*-DM/Chl weight ratio of 9:1 for 1h on ice, then centrifuged at 10,000 *g* for 5 min to remove insoluble materials. Then 150 µl of the supernatant was immediately applied to pre-cooled gel. Electrophoresis was carried out with 2.5 mA per tube gel for the stacking gel and 5 mA for the separating gel at 4°C. After the electrophoresis was finished, gels were photographed to record the chlorophyll bands and then immediately cut for spectrophotometric analysis.

Discontinuous Sodium dodecyl sulphate polyacrylamide gel electrphoresis (SDS-PAGE) was used to separate polypeptides of the chlorophyll-protein complexes according to the method of Laemmli (1970).

Determination of chlorophyll concentration and Chl a/b ratio of chlorophyll-protein complexes

The Chl-protein bands were extracted with 80% acetone for 2 h and their chlorophyll concentrations and Chl *a/b* ratios were determined according to the method of Porra et al. (1989).

Spectrophotometric measurements

Absorption spectra of thylakoids and gel slices were recorded with a Shimadzu UV-2550 spectrophotometer at room temperature.

77 K low temperature fluorescence spectra were recorded with a Hitachi F-4500 fluorometer. The fluorescence emission data were analyzed with a Gaussian deconvolution program of Origin 7.5.

Results and discussion

Absorption and fluorescence characteristics of *B. corticulans* thylakoids

We measure the room temperature absorption and 77 K fluorescence spectra for a thorough understanding of the basic characteristic of *B. corticulans* thylakoids. The results are given in Figs. 1 and 2, respectively. In the absorption spectrum, Chl *a* gives rise to two absorption peaks at 438 and 679 nm, and another two peaks at 476 and 652 nm attributed to Chl *b* (Fig. 1). The presence of siphonaxanthin and siphonein also efficiently makes a broad absorption in green region (500–540 nm).

Figure 2a clearly describes the 77 K fluorescence emission spectra of *B. corticulans* thylakoids excited at 438 nm (chlorophyll *a*). Its full display in this circumstance contains a prominent peak at 683 nm and three shoulders at 694, 702 and 713 nm (solid thick line, Fig. 2a), respectively, similar to the 685, 695 and 710 (718) nm peaks of other siphonaceous algae such as *B. maxim*a and *Codium* chloroplasts (Anderson 1983; Yamazaki et al. 2005). In order to reveal the origin of the fluorescence emission peaks, we fitted the emission spectrum by Gaussian components according to the assignments of emission bands to different components of the photosynthetic apparatus (Govindjee 1995; Krause and Weis 1991). The spectrum can be fitted well by 7 Gaussian sub-bands: 672.6, 680.6, 693, 705.8, 715.4, 724.2 and 744.8 nm (thin lines, Fig. 2a). The preceding data (Govindjee 1995; Krause and Weis 1991; Murata and Satoh 1986; Wen et al. 2005) provides feasibility for our assignment of emissions of 680.5 and 693 nm, originated from LHCII and the CP47 core antenna complexes of PSII, respectively. Correspondingly, the given data contribute to the following two possibilities: the 715.4 nm and 724.2 nm bands are produced by PSI, and the 672.6, 705.8 and 744.8 nm bands are suggested to be the vibrational satellites of PSII and PSI bands. PSI of *B. corticulans* notably exhibits two extraordinary emission bands at 715.4 nm and 724.2 nm, which are shorter than those of higher plants usually ranging at 714–725 nm and at 735–745 nm (Krause and Weis 1991). These results are

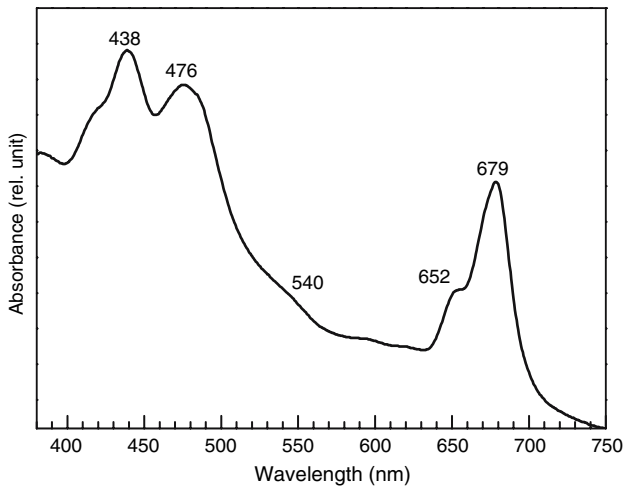

Fig. 1 The room temperature absorption spectrum of *Bryopsis corticulans* thylakoids at the chlorophyll concentration of 5 μg Chl/ml

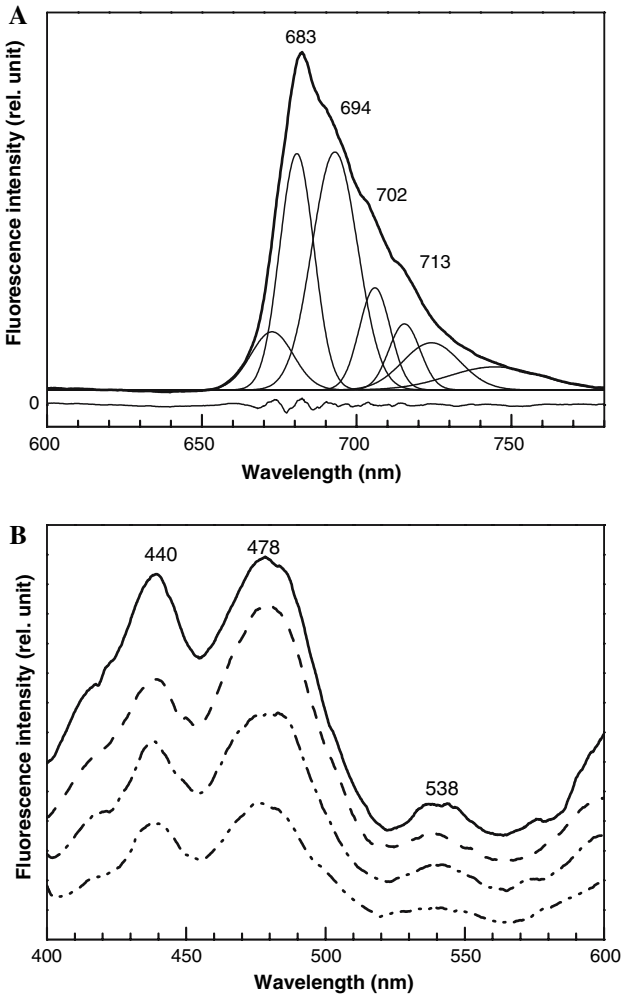

Fig. 2 The 77 K fluorescence spectra of *B. corticulans* thylakoids. (**a**) The Gaussian sub-band deconvolution for 77 K fluorescence spectra of *B. Corticulans* thylakoids with excitation at 438 nm. The *solid thick line* is the full 77 K fluorescence spectrum. The *thin lines* are the Gaussian sub-bands deconvoluted from the full spectrum. The *curve* below the spectrum is the residues of the fitting errors; (**b**) excitation spectra emitted with 683 nm (*solid*), 694 nm (*dash*), 702 nm (*dash dot*) and 713 nm (*dash dot dot*)

likely to prove that PSI of *B. corticulans* possesses a special structure and a distinct way in energy transfer.

The excitation spectra of four fluorescence emission bands shown in Fig. 2b display three peaks at 440, 478, 538 nm, reflecting that excitation energy absorbed by Chl *a*, Chl *b* and siphonaxanthin/shiphnein is efficiently transferred to two photosystems in the isolated thylakoids. Obviously, among these four wavelengths in the fluorescence emission (Fig. 2b), the Chl *b* band at 478 nm stands more pronounced than Chl *a* band at 440 nm. As a result, Chl *b* is found to be the factor that generally determines the energy transfer to both PSII and PSI. Moreover, the peaks at 538 nm in four excitation spectra also clarify that siphonaxanthin/siphonein is one of the determined factors that transfer energy to the far-red region and served as the antenna pigment of PSI as well as PSII.

Absorption characteristics of chlorophyll-protein complexes isolated from *B. corticulans* thylakoids

To further elucidate the characteristic of the *B. corticulans*, thylakoid membranes were solubilized at a ratio of *β*-DM: Chl = 9:1 and resolved by non-denaturing *β*-DM-PAGE, which minimizes the release of free pigments (Yamazaki et al. 2006). The resolved seven zones shown in Fig. 3a are designated as CP1a^1 and CP1a^2 (brown), CP1 (dark-green), LHCP^1 (dark-brown), CPa (light-green), LHCP^3 (yellow-brown) and free pigments in the order of increasing mobility. The nomenclatures we adopt correspond to those used by Anderson (1983), Nakayama et al. (1986), Wollman and Bennoun (1982), and Ish-Shalom and Ohad (1983). Without exception, the LHCP^2 band that is resolved from higher plant thylakoids is still in the absence in *B. corticulans*. However, this is consistent with that of *Codium* species (Anderson 1983). It is worthy of note that the amount of free carotenoid in the *β*-DM solubilized FP band is largely reduced in comparison with that resolved by SDS (data not shown).

Table 1 depicts the relative distribution of Chl and the Chl *a/b* ratios of these Chl-protein bands. It is evident that most of Chl are associated with LHCII (LHCP^1 and LHCP^3), which accounts for 60% of total Chl, while the CPa band, the PSII core complex with some light harvesting protein, 10.1%; CP1a$^{1-2}$ band, 12.8%. Judging from the Chl *a/b* ratio of 1.64, we assign the CP1a$^{1-2}$ as the partially dissociated PSI complex possessing PSI light-harvesting complexes. Compared with PSI of *Codium* (Chl *a/b* ratio of 5.0–7.0) (Anderson 1983) and *B. maxima* (Chl *a/b* ratio of 2.4) (Nakayama 1986), the CP1a$^{1-2}$ in *B. corticulans* is seen in a lower Chl *a/b* ratio. The CP1 band takes up 9.24% of Chl. It consists of two polypeptides belonging to PSI core proteins with apparent molecular mass of about 66 kDa and 64 kDa as resolved by SDS-PAGE (Fig. 3b). The fact that most of Chl in the CP1 band are Chl *a* is explained here from its high Chl *a/b* ratio of 9.55. Hence, unlike other siphonaceous algae, there are much more Chl *b* in LHCI of *B. corticulans* instead of the

Table 1 The relative distribution of chlorophyll and the Chl *a/b* ratios of chlorophyll-protein complexes of *Bryopsis corticulans* thylakoids resolved by non-denaturing *β*-DM-PAGE

Thylakoids	CP1a$^{1-2}$	CP1	LHCP^1	CPa	LHCP^3	Free pigment
100	12.8	9.2	42.3	10.1	17.5	8.1
1.27	1.64	9.55	0.71	3.08	0.83	1.65

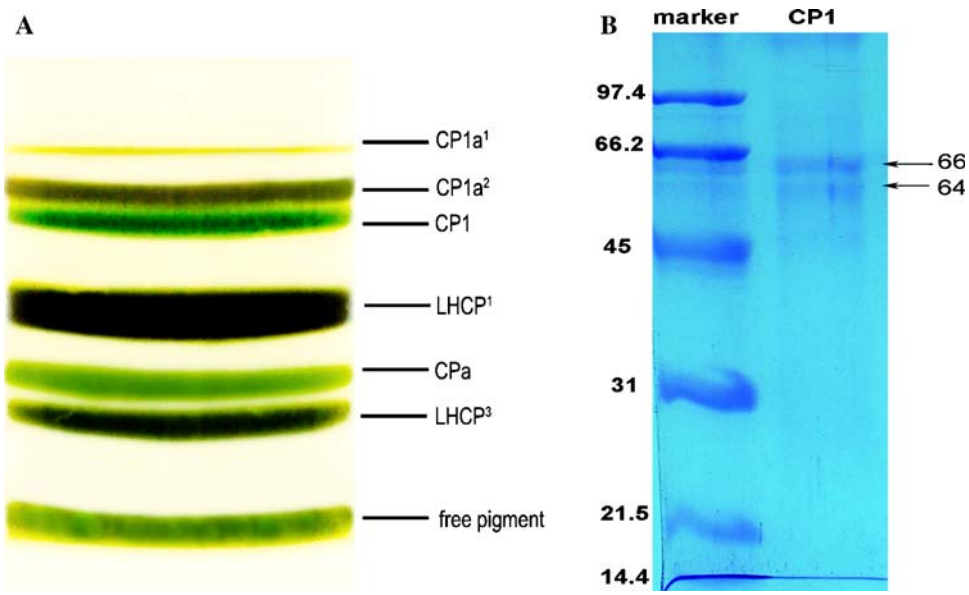

Fig. 3 (**a**) Chlorophyll-protein complexes bands of *B. corticulans* thylakoids (Chl *a/b* = 1.27) resolved by discontinuous *β*-DM-PAGE. CP1a^1 and CP1a^2, partially dissociated PSI complexes that possess LHCI; CP1, mainly composed of PSI core complex; LHCP^1, the trimeric LHCII; CPa, the PSII core complex with small amount of LHCII; LHCP^3, the LHCII monomer; (**b**) The protein compositions of CP1 band resolved by SDS-PAGE

PSI core complex. The free pigment band, however, occupies only 8.1% of the total Chl.

Figure 4a, b illustrates the absorption spectra of Chl–protein complexes. In Fig. 4a, the absorption spectrum of $CP1a^1$ exhibits significant peaks at 438 and 677 nm attributed to Chl *a* and a 467 nm peak attributed to Chl *b*. A similar absorption spectrum is also obtained for $CP1a^2$. The green CP1 band results mainly from Chl *a* absorption peak at 440 nm and 677 nm. And we can even observe the minor Chl *b* absorption in the blue-green region as is found by Nakayama (1986) in *Bryopsis maxima* in the present CP1 band. Thus, the above results further prove that Chl *b* in PSI is mainly located on its light-harvesting proteins.

CPa shows two predominant Chl *a* absorption peaks at 438, 671 nm and a Chl *b* absorption peak at 471 nm (in Fig. 4b). Absorption spectrum of $LHCP^1$, the trimeric Photosystem II light-harvesting complexes, enables us to see peaks at 437, 671 nm of Chl *a*, a broad peak containing Chl *b* and carotendoid absorption, as well as some absorption in the 500–550 nm region derived from siphonaxanthin/siphonein. Peaks at 437, 671 nm driven by Chl *a*, and 470 nm and a shoulder at 652 nm attributed to Chl *b* are also clearly presented in the absorption spectrum of the LHCII monomer $LHCP^3$.

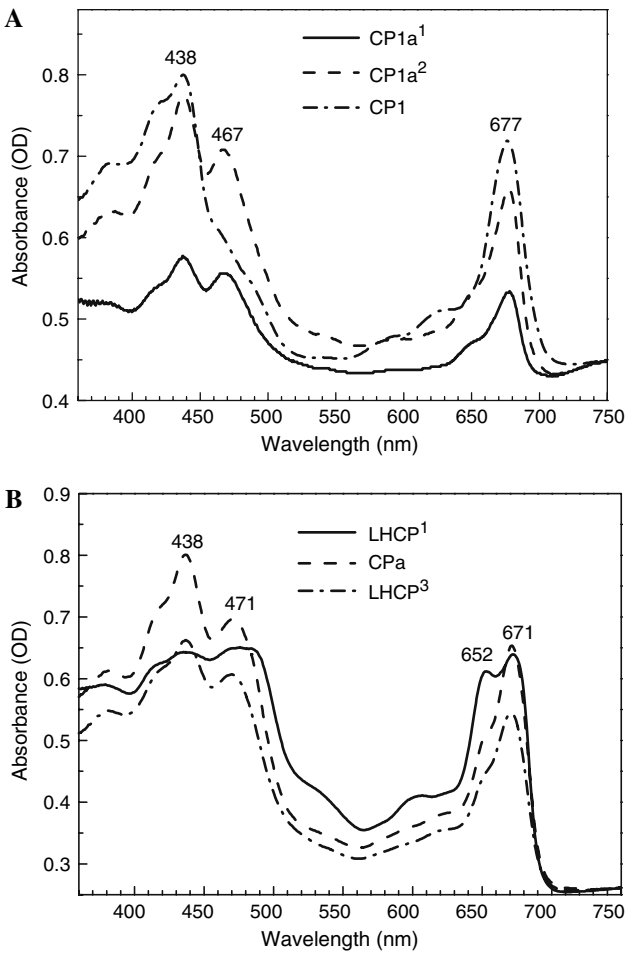

Fig. 4 Room temperature absorption spectra of chlorophyll-protein complexes resolved from *B. corticulans* thylakoids, normalized to the baseline, respectively. (**a**) $CP1a^1$, $CP1a^2$ and CP1; (**b**) $LHCP^1$, CPa and $LHCP^3$

Fluorescence characteristics of chlorophyll-protein complexes

We examine the low temperature fluorescence emission spectra of all Chl–protein complexes excited at 438, 480 and 540 nm in Fig. 5. There is only one unusual broad peak at 690 nm or 692 nm standing in fluorescence emission spectra of $CP1a^1$ and $CP1a^2$ bands, irrespective of the excitation wavelength. As once reported in the $CP1a^2$ band of SDS-solubilized *Codium* thylakoids (Anderson 1983), we also find it true that an emission can reach maximally to 698 nm. In Fig. 6, excitation spectrum of the $CP1a^1$ band shows peaks at 440, 475 and 544 nm while $CP1a^2$ has maxima at 440, 478 and 545 nm and a shoulder at 505 nm. Thus, it is clear that the emission band at about 690 nm of PSI is a result from Chl *a*, Chl *b* and siphonaxanthin. Their excitation spectra also allow us to see that both $CP1a^1$ and $CP1a^2$ contained high contents of Chl *b* and siphonaxanthin/siphonein.

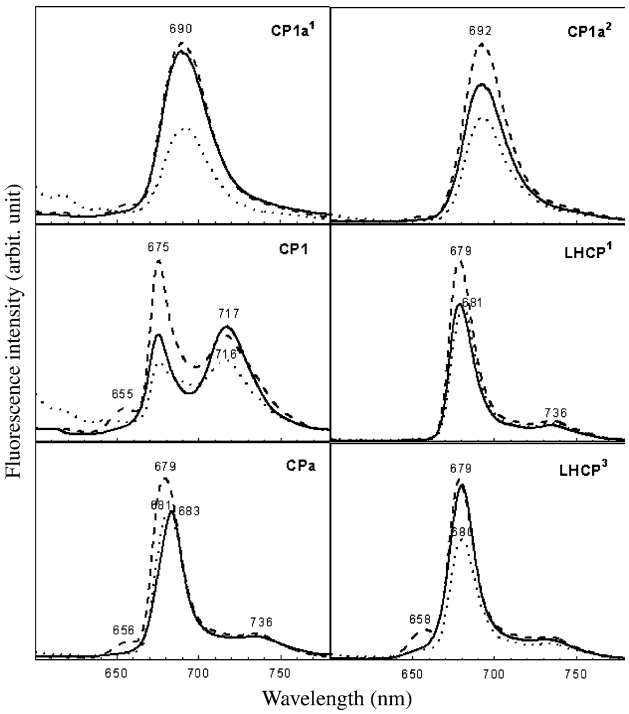

Fig. 5 The 77 K fluorescence emission spectra of Chlorophyll–protein complexes resolved from *B. corticulans* thylakoids excited with 438 nm (*solid*), 480 nm (*dash*), and 540 nm (*dot*), respectively

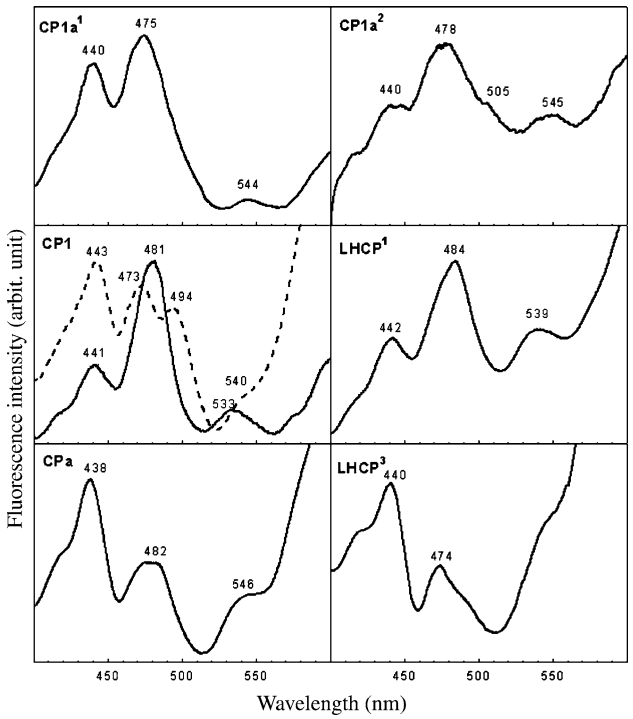

Fig. 6 The 77 K fluorescence excitation spectra of Chlorophyll–protein complexes resolved from *B. corticulans* thylakoid. $CP1a^1$ and $CP1a^2$, emission peak at 690 nm and 692 nm, respectively; CP1, emission peak at 675 nm (*solid*) and 715 nm (*dash*); $LHCP^1$, emission peak at 679 nm; CPa, emission peak at 683 nm; $LHCP^3$, emission peak at 680 nm

For CP1 band, the emission spectrum unfolds two prominent peaks, one unexpected at 675 nm and another around 715–717 nm, irrespective of the excitation wavelength. This means that energy absorbed by Chl *a*, Chl *b*, and siphonaxanthin/siphonein is efficiently transferred to both red and far-red region in PSI. The peak at 715–717 nm is slightly higher than the peak at 675 nm in CP1 when excited at 438 or 540 nm in comparison with the peak at 715–717 nm that is obviously lower than that at 675 nm in CP1 when excited at 480 nm. As a consequence, the energy is transferred to far-red region of PSI by Chl *a* and siphonaxanthin with higher efficiency than Chl *b*. The emission of PSI or CP1 band turns out to be similar at 671 and 715 nm for marine algae *Ulva pertusa* (Yamazaki et al. 2006) and *Codium* (Anderson 1983), while the PSI band of the closest species *B. maxima* (Yamazaki et al. 2006) has only one broad peak at 714 nm. It was once proposed that the peak at 675 nm might be an artifact (Anderson 1983; Bassi and Simpson 1987). However, the excitation spectrum in Fig. 6 proves that 675 nm emission spectrum is composed of Chl *a*, Chl *b* and siphonaxanthin/siphonein, clearly showing that 481 nm was extremely pronounced compared to Chl *a* at 441 nm and siphonaxanthin at 533 nm for excitation of CP1. Accordingly, we find that in CP1 the peak at 675 nm is mainly attributed to the energy from Chl *b* while it is the energy from Chl *a* which plays an important role in exciting the peak at 715–717 nm. Then, there can be little question that siphonaxanthin affect both 675 nm and 715 nm peaks. The experimental evidence is the extremely pronounced 481 nm peak in the excitation spectrum of CP1 at 675 nm that reveals a probability that LHCII works as an antenna of PSI (Yamazaki et al. 2005, 2006).

A strong peak at about 679–683 nm and a weak peak at 736 nm emerge in the fluorescence emission spectrum of CPa (Fig. 5). Spectra of light-harvesting complexes $LHCP^1$ and $LHCP^3$ draw similar modes with strong peaks at about 679 nm and minor peaks around 736 nm. An additional emission peak at 658 nm due to Chl *b* is clearly seen in $LHCP^3$ when excited at 480 nm wavelength, revealing the energy absorbed by Chl *b* is not completely transferred to Chl *a* while there is only one fluorescence peak at around 680 nm in $LHCP^1$ irrespective of the excitation wavelengths. These results suggest that $LHCP^1$ keeps its in vivo state and has higher energy transfer efficiency than that of $LHCP^3$. The 679 nm peak in $LHCP^3$, according to the excitation spectrum, is mainly attributed to Chl *a* at 440 nm, but Chl *b* at 474 nm and carotenoid at 494 nm shoulder exert minor effects. In the meantime, the 679 nm peaks in $LHCP^1$ yield to the contribution of 484 nm wavelength (combination of Chl *b* and carotenoid) and also of siphonaxanthin at 539 nm. These points taken together demonstrate that higher content of Chl *b*, carotenoid and siphonaxanthin exist mainly in the trimeric form of LHCII instead of in the monomer, although their *a*/*b* ratios stay almost the same as shown in Table 1.

Conclusion

The present work reports the detailed spectral characteristics of Chl-protein complexes separated from *B. corticulans* thylakoids by non-denaturing *β*-DM-PAGE.

There is a significant distinction in Chl compositions and fluorescence spectra between *B. corticulans* thylakoids and higher plants. Emission spectrum of CP1 band, which consists of PSI core proteins, demonstrates two peaks at 675 and 715–717 nm. Their excitation spectra clarify that it is the Chl *a* and siphonaxanthin that make the main contribution to the transfer of energy to the far-red band of PSI around 715–717 nm, while Chl *b* is responsible for 675 nm band of PSI. Although most of the Chl are located on LHCII, higher content of Chl *b* and siphonaxanthin in PSI might play a key role in balancing the energy distribution between PSII and PSI by extending the transfer of energy of PSI to the far-red region. However, further study is needed to reveal the arrangement of the siphonaxanthin-Chl *a*/*b*–protein complex and the energy transfer pattern.

In addition, it is indicated from Chl composition and spectral characteristics of HLCP[1], LHCP[3] and CP1a[1–2] bands that higher content of Chl *b* and siphonaxanthin are evolved by *B. corticulans* to absorb sufficient light for the marine green alga so as to adapt to the changing environment. Chl *b* has once been suggested to have a vital impact on photoprotection in that Chl *b* is inclined to dissociate from LHCII in the oligomerization state and thus reduced its light absorption capacities when the tide retreats (Chen et al. 2005). Combined with the fact that *B. corticulans* is a green alga inhabited in the intertidal zones with 1.5–3.0 m tide levels, we come to a conclusion that high content of Chl *b* and siphonaxanthin are able to help absorbing the blue-green and green light available and thus to obtain sufficient light energy for the alga in the water when the tide comes.

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